



**University of
Nottingham**
UK | CHINA | MALAYSIA

Hydrogen Sulfide Generation and Signalling in the Heart and Coronary Artery of the Pig

Yasir Yaseen Al-Taie,

B. Pharm., MSc. Pharmacology

School of Life Sciences

Faculty of Medicine and Health Sciences

University of Nottingham

**Thesis submitted to the University of Nottingham for
the degree of Doctor of Philosophy**

February 2021

Abstract

Cardiovascular diseases (CVD), such as hypertension, angina, myocardial infarction, arrhythmia, heart failure, and stroke, are some of the most common causes of death around the world. Hydrogen sulfide (H₂S) is considered as a bio-modulator gasotransmitter molecule. In recent years, evidence that H₂S may have an influential role in several important biological processes, especially in the cardiovascular system (CVS) has increased. Many studies have reported the production of H₂S within the body. H₂S appears to play a role in the regulation of vascular tone. Therefore, there is accumulated evidence regarding the role of H₂S in the vasculature, while there is little information about the role of H₂S in the heart. Many cardiovascular pathologies have been associated with low H₂S production, such as hypertension, ischaemic heart diseases (IHD), and atherosclerosis.

The present study measured H₂S generation in the heart by investigating the expression level of H₂S synthesising enzymes cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and mercaptopyruvate sulfurtransferase (MST) using immunoblotting and investigating the activity of these enzymes via the development of assays using the methylene blue and SF7-AM (sulfidefluor-7 acetoxymethyl ester) methods. We found that CBS and MST were expressed in the heart, but CSE was not detected. CBS detected in the heart was consistently present at a higher MW band, suggesting the less active form, in comparison with the liver that had higher expression of the lower MW form which is thought to be the active form. This may reflect the difference in biological effect of H₂S and post-translational modifications (PTMs) in the heart compared to the liver. We found that there was a low amount of H₂S produced in the heart compared to the liver using both the methylene blue and SF7-AM methods. Myocardium added to isolated porcine coronary arteries (PCA) in tissue baths produced a relaxation response, which was inhibited by H₂S synthesising enzyme/s inhibitors, suggesting that H₂S synthesised in the myocardium might regulate coronary artery tone in a paracrine manner.

There were no significant effects of SAM (S-adenosyl methionine, a positive CBS modulator) and gender on synthesis of H₂S, and degassing on detection of H₂S. In contrast, alkaline pH led to an increase of H₂S measurement and dialysis of the heart cytosol led to an increase of detection of H₂S. Briefly, there was detected enzyme activity in the heart, but it was lower than the liver by both the methylene blue and SF7-AM methods. The detected H₂S production in the heart causes relaxation of the coronary artery, which suggests that H₂S synthesised in the myocardium might regulate coronary artery tone in a paracrine manner.

Measurement of enzyme activity only indicates the potential to produce H₂S, not the amount that the tissue actually produces. Measurement of sulfhydrylation levels may be a way of determining the level of H₂S produced in the tissue. In the biotin switch assay (BSA), there was an increase of sulfhydrylation level of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and MEK1 (mitogen-activated extracellular signal-regulated kinase 1), but the results were variable between different tissues. In the results of the red maleimide assay, there was a decrease in the fluorescence detected in the presence of Na₂S and the presence of DTT (dithiothreitol) of GAPDH and MEK1 proteins. There was a difference in the fluorescence of bands which ran at the same place as MEK1 and GAPDH.

As a result of running the samples on polyacrylamide gels in non-reducing conditions to detect proteins in the red maleimide assay, we observed the shifting of GAPDH and MEK1 bands to a lower MW in the presence of Na₂S and absence of DTT. The same observation was also detected after immunoprecipitation of the heart homogenate and Western blotting. This shift to the lower MW band could be a simpler method for detecting changes in sulfhydrylation. Therefore, depending on our results of sulfhydrylation, there is detected sulfhydrylation in the heart by our assays and sulfhydrylation is a potential signalling pathway of H₂S in the heart and could be used as an index for H₂S production in the heart.

Next, the present study investigated the effects of three different types of H₂S donors (sources, drugs), Na₂S fast-releasing H₂S salt, GYY4137 slow-releasing H₂S donor, and AP39 mitochondria-targeted H₂S donor on vascular tone in PCA using organ-bath. Then, this study compared the effects of different types of H₂S sources on vascular tone in PCA under different conditions in order to determine whether there is a difference in the signalling pathways activated by these three different H₂S sources. The results of this study demonstrated there was no difference in the relaxation responses and mechanisms of relaxation responses between three different H₂S donors, Na₂S, GYY4137 and AP39. Thus, the studies looking at the vascular responses to H₂S using the salts might be useful in determining the response to H₂S in PCA.

The results of this study demonstrated that there was a significant enhancement of the relaxation responses of all three donors in the presence of L-NAME. These results suggest chemical interaction of H₂S with NO might lead to formation of inactive nitrosothiol, which may not be able to contract or relax blood vessels. In contrast to previous studies, the results of this study demonstrated that there was a significant enhancement of the relaxation responses of all three donors in the presence of TEA (non-selective K⁺ channels blocker) and glibenclamide (K_{ATP} channels blocker) and these responses might be due to potassium channels blockade increasing sensitivity to other mechanisms of H₂S, such as calcium channels blockade.

Interestingly, the blocking of potassium channels by TEA and K_{ATP} channels by glibenclamide led to an enhancement of the H₂S-mediated relaxation, and these results are in contrast with previous studies that have reported that H₂S relaxation was mediated via potassium channels. Furthermore, there was significant inhibition of calcium-induced contractions by all three H₂S donors, and this inhibition was maintained after incubation of PCA with all the three different H₂S sources for 15, 30 and 60 min. Furthermore, there was a significant inhibition of contraction of PCA induced by BayK8644, L-type voltage-gated calcium channel opener by all three different H₂S sources, and thus, these results suggest that H₂S acts by blocking calcium channels and also inhibiting a calcium-induced contraction pathway.

Hypoxia plays critical roles in IHD. Therefore, studying the role of H₂S as an oxygen sensor using three different H₂S donors would be useful and interesting. Na₂S salt and AP39 caused no significant effect in hypoxia response, hypoxia-induced relaxation, but they caused a significant decrease in the recovery response, reoxygenation-induced contraction. In contrast, GYY4137 caused both a significant enhancement of the hypoxia response and a significant decrease in the recovery response to reoxygenation. Therefore, these results of H₂S donors, such as GYY4137 in PCA may be beneficial to decrease ischaemia-reperfusion injury (IRI). Thus, H₂S could be important for the CVS and many cardiovascular pathologies, which have been associated with low H₂S production. Therefore, H₂S donors may be used as a cardiovascular protective drug against IRI.

In conclusion, the present study demonstrated that H₂S is produced in the heart and causes relaxation of the coronary artery. Sulfhydrylation measured by our assays in the heart could be used as an index of H₂S production and potential signalling pathway for H₂S. There was no difference in the relaxation responses and mechanisms of relaxations, such as NO, potassium channels, calcium channels among the three different H₂S donors, Na₂S, GYY4137 and AP39. There was a difference between the effects of Na₂S and AP39 compared to GYY4137 in response to hypoxia and reoxygenation. Therefore, measurement of H₂S generation and sulfhydrylation levels could be used as an index for changes in cardiac dysfunction. Thus, H₂S sources could be beneficial in IHD, which are associated with low H₂S generation and sulfhydrylation levels.

Keywords: hydrogen sulfide, SF7-AM, CBS, sulfhydrylation, red maleimide, U46619, calcium, Na₂S, GYY4137, AP39, and hypoxia.

Abstracts for Conferences

1. Y. Y. Al-Taie, R. E. Roberts, M. J. Garle, S. P. Alexander (2016). The hydrogen Sulphide Enzymatic Generation and Regulation in the Porcine Heart. British Pharmacological Society (BPS) Winter Meeting, London, Queen Elizabeth II Conference Centre. Poster presentation. www.pA2online.org, 153P.
2. Y. Y. Al-Taie, R. E. Roberts, M. J. Garle, S. P. Alexander (2017). Development of a SF-7 AM based fluorescence assay for detection of Hydrogen Sulphide in the Porcine Heart. BPS Winter Meeting, London, Queen Elizabeth II Conference Centre. Poster presentation. www.pA2online.org, 132P.
3. Y. Y. Al-Taie, R. E. Roberts, M. J. Garle, S. P. Alexander (2018). Potential Signalling Pathways of Distinct Hydrogen Sulphide Donors (Na_2S , GYY4137, and AP39) in the Relaxation Response of Porcine Coronary Artery. BPS Winter Meeting, London, Queen Elizabeth II Conference Centre. Poster presentation.
4. Y. Y. Al-Taie, R. E. Roberts, M. J. Garle, S. P. Alexander (2019). Assay Development for Measurement of the Sulfhydration in the Pig Heart as a Potential Signalling Pathway of Hydrogen Sulfide. BPS Winter Meeting, Edinburgh, Edinburgh International Conference Centre. Poster presentation.
5. Y. Y. Al-Taie, R. E. Roberts, M. J. Garle, S. P. Alexander (2019). The Potential Role of Myocardium-Mediated Hydrogen Sulfide in the Relaxation Response of Porcine Coronary Artery. BPS Winter Meeting, Edinburgh, Edinburgh International Conference Centre. Poster presentation.

Acknowledgements

"O My Lord, increase me in knowledge" (the Holy Qur'an: Surah Ta-Ha, verse 114).

The Prophet Muhammad, may Allah's peace and blessing be upon him, said "Allah makes the way to Jannah easy for him who treads the path in search of knowledge."

Praise be to ALLAH, Lord of the Universe for helping me fulfil this work and providing me with willingness and capability to complete this doctoral thesis, and being with me during the hard moments that I faced throughout my study.

I would like to express my sincerest and greatest thanks and gratitude to my supervisors Dr Stephen Alexander and Dr Richard Roberts, for their valuable supervision, support, enthusiasm, patience and help during my PhD study. It has been an honour to be their PhD student and member of their research group. Their guidance helped me all the time of research and developed me academically and personally. I could not have imagined having better supervisors for my PhD study and making my dream real.

Special thanks to Dr Michael Garle for his general support and advice in E34 laboratory.

Sincere appreciation towards the Higher Committee for Education Development in Iraq (HCED) for their sponsorship of my PhD study. Many thanks to all staff and members in the Medical School, Life Sciences, University of Nottingham.

Eternal appreciations and thanks go to my beloved mother and father (mercy of Allah may be upon them). From the bottom of my heart I would like to express my deepest and sincerest thank and gratitude to my wife Dr Asmaa Khaleel, and my kids Ammar, Lana and the newcomer and little angel "Bana" for their tolerance, patience, encouragement and understanding. I will never be able to pay my debt of gratitude in full. Thanks for being in my life, I love you. Without your support this work would have been impossible. Thanks extend to all my family and my friends for their encouragement and support, and to everyone who helped me through my PhD journey, especially at difficult times. I hope I continue to make you all proud.

Dedication

To my beloved mother, Nahidah Abdulrazak and father, Yaseen Al-Taie, who sadly lost their lives during the dramatic events in Iraq.

Their love had inspired me to pursue my dreams. Unfortunately, you will not see your dream comes true. I hope you're proud of your little boy. I can see your smile from Heaven.

May their souls rest in peace.

Table of Contents

Abstract	I
Abstracts for Conferences	V
Acknowledgements	VI
Dedication	VII
List of abbreviations	XVI
Declaration	XIX
Chapter one: General Introduction.....	1
• 1.1. Background	2
• 1.2. H₂S chemistry	4
• 1.3. Biosynthesis and regulation of H₂S	6
• 1.3.1. H₂S Biosynthesis and regulation via CBS	7
• 1.3.1.1. Regulation and post-translational modifications (PTMs) of CBS	7
• 1.3.1.2. Regulation of protein levels	8
• 1.3.1.3. Post-translational modifications	8
• 1.3.2. H₂S Biosynthesis and regulation via CSE	12
• 1.3.3. H₂S Biosynthesis and regulation via MST	14
• 1.3.4. Comparison of variable tissue distributions of CBS, CSE and MST	16
• 1.4. Metabolism of H₂S	19
• 1.4.1. Oxidation	19
• 1.4.2. Methylation	20
• 1.4.3. Miscellaneous	20
• 1.5. Endogenous H₂S concentrations	21
• 1.6. Measurement of H₂S	23
• 1.6.1. Colorimetric assay: The methylene blue assay	24
• 1.6.2. Sulfide ion-selective electrode (SISE)	25
• 1.6.3. Polarographic H₂S sensor (PHSS)	26
• 1.6.4. Fluorescent probes	27
• 1.6.4.1. Reduction-dependent fluorescent probes	28
• 1.6.4.2. Nucleophilic-dependent fluorescent probes	29
• 1.6.4.3. Metal-sulfide based fluorescent probes	30

- **1.6.5. Carbon Nanotubes (CNT) 30**
- **1.6.6. Gas chromatography (GC) 30**
- **1.7. Mechanism of action (signalling pathways) of H₂S 31**
- **1.7.1. H₂S effects on ion channels 32**
- **1.7.1.1. Potassium channels 32**
- **1.7.1.2. Transient receptor potential channels 33**
- **1.7.1.3. Chloride channels 33**
- **1.7.1.4. Calcium channels 34**
- **1.7.2. Antioxidant effects of H₂S 36**
- **1.7.3. Effects of H₂S on mitochondrial function 39**
- **1.7.4. Sulfhydration 42**
- **1.7.5. H₂S as an oxygen sensor/transducer 44**
- **1.7.6. H₂S as an inflammatory modulator 46**
- **1.7.7. Effects of H₂S on angiogenesis and kinases 47**
- **1.7.8. Miscellaneous effects of H₂S 49**
- **1.7.9. Interaction of H₂S with NO 49**
- **1.8. The biological significance of H₂S in heart and vascular diseases 50**
- **1.9. Approaches to studying H₂S biology 55**
- **1.10. Therapeutic implications of H₂S releasing agents 57**
- **1.11. Aims of the study 63**

Chapter two: Measurement of Hydrogen Sulfide Generation in the Heart 64

2.1. Introduction 65

- **2.2. Materials 66**
- **2.3. Methodology 67**
- **2.3.1. Western blotting 67**
- **2.3.1.1. Sample preparation 67**
- **2.3.1.2. Lowry protein assay 67**
- **2.3.1.3. Polyacrylamide gel electrophoresis: sample loading and running phase 68**
- **2.3.1.3.1. Western blotting 68**
- **2.3.1.3.2. Antibody incubation 69**
- **2.3.2. Enzyme activity assays 70**
- **2.3.2.1. Determination of Sulfide (as hydrogen sulfide) by the methylene blue assay 70**

- **2.3.2.1.1. Sample preparation 70**
- **2.3.2.1.2. Measuring CBS activity 70**
- **2.3.2.1.2.1. Effects of dithiothreitol 71**
- **2.3.2.1.2.2. Effects of aspartic acid on H₂S production 71**
- **2.3.2.1.2.3. Effects of degassing on H₂S production 71**
- **2.3.2.1.3. Measuring MST activity 72**
- **2.3.2.2. SF7-AM Fluorescence 72**
- **2.3.2.2.1. Quantification of H₂S using SF7-AM 72**
- **2.3.2.2.2. Sample preparation 72**
- **2.3.2.2.2.1. The effect of tissue dialysis 73**
- **2.4. Statistical analysis 73**
- **2.5. Results 74**
- **2.5.1. Western blotting 74**
- **2.5.1.1. Cystathionine β-synthase (CBS) expression 74**
- **2.5.1.2. Cystathionine γ-lyase (CSE) expression 79**
- **2.5.1.3. Mercaptopyruvate sulfurtransferase (MST) expression 82**
- **2.5.2. Enzyme assays 86**
- **2.5.2.1. Measuring CBS activity using the methylene blue detection method 86**
- **2.5.2.1.1. Selection of blanks 86**
- **2.5.2.1.2. Effects of dithiothreitol 88**
- **2.5.2.1.3. Effect of aspartic acid on H₂S production 89**
- **2.5.2.1.4. The effects of DTT on L-cysteine-derived H₂S production 91**
- **2.5.2.1.5. H₂S production in different tissues 92**
- **2.5.2.1.6. Effect of pH on H₂S production 93**
- **2.5.2.1.7. Time profile of H₂S production at pH 9.0 96**
- **2.5.2.1.8. Effect of degassing buffers on H₂S production 97**
- **2.5.2.1.9. Effect of S-adenosyl-L-methionine on H₂S production 99**
- **2.5.2.2. Measuring MST activity using the methylene blue detection method 102**
- **2.5.2.2.1. MST and CBS activity in porcine ventricles 106**
- **2.5.2.2.2. Effect of PPA on MST activity 108**
- **2.5.2.3. Monitoring H₂S production using SF7-AM fluorescence 110**
- **2.5.2.3.1. Na₂S as a standard 111**
- **2.5.2.3.2. CBS activity in atria and ventricles 112**
- **2.5.2.3.3. The influence of gender on CBS activity 113**
- **2.5.2.4. Measurement of MST enzyme activity using SF7-AM 115**
- **2.5.2.4.1. MST activity in porcine atria and ventricles cytosols 115**
- **2.5.2.4.2. Particulate fractions from atria and ventricles 117**
- **2.5.2.4.2.1. Effect of PPA on MST activity 119**
- **2.5.2.4.3. pH sensitivity of MST activity 121**

- 2.5.2.4.4. The effects of dialysis 122
- 2.5.3. The effect of myocardium-derived H₂S on the relaxation responses in U46619-precontracted PCA 124
- 2.5.3.1. Tissue preparation 124
- 2.5.3.2. Isolated organ bath experiments 124
- 2.5.3.3. Effect of myocardial tissue on U46619-induced tone 125
- 2.5.3.4. The effects of H₂S synthesising enzymes inhibitors on myocardial-induced relaxation response 125
- 2.5.3.5. The effects of hypoxia on myocardial-induced relaxation response 125
- 2.5.3.6. Results 126
- 2.5.3.6.1. Effect of myocardial tissue on U46619-induced tone 126
- 2.5.3.6.2. The effect of H₂S synthesising enzyme inhibitors 128
- 2.5.3.6.3. The effect of H₂S synthesising enzyme candidate CDS on myocardium-derived relaxation responses in U46619-precontracted PCA 130
- 2.5.3.6.4. The effect of hypoxia on myocardial-induced relaxation response 133
- 2.6. Discussion 134
- 2.6.1. Pig heart tissues 135
- 2.6.1.1. The effects of DTT 136
- 2.6.1.2. The effects of L-cysteine 137
- 2.6.1.3. The effects of aspartic acid 138
- 2.6.1.4. The effects of change of pH 139
- 2.6.1.5. Time profile for detection of H₂S (incubation time) 139
- 2.6.1.6. The effects of H₂S synthesising enzymes inhibitors 140
- 2.6.1.7. The effects of degassing 141
- 2.6.1.8. The effects sample dialysis 141
- 2.6.2. Heart tissues of different species 142
- 2.6.3. Comparison of different tissues of the pig 144
- 2.6.4. Pig heart tissues compared to different tissues and species 147
- 2.6.4.1. Presence of low and high MW bands of CBS 147
- 2.6.4.2. CBS, CSE and MST expressions 147
- 2.6.4.3. Quantification of enzymatic production of H₂S 148
- 2.6.4.4. Possible explanations of low enzymatic production of H₂S 148
- 2.6.4.5. The effects of SAM 149
- 2.6.4.6. The effects of gender 150
- 2.6.4.7. Methylene blue assay compared to SF7-AM 151
- 2.6.5. Myocardial tissue effect on PCA vascular tone 153
- 2.6.6. Summary 154

Chapter Three: Development of an Assay to Measure Sulphydration in the Pig Heart..... 155

- **3.1. Introduction 156**
- **3.2. Materials and chemicals 158**
 - **3.2.1. Antibodies used in Western blotting 158**
- **3.3. Methodology 159**
 - **3.3.1. Biotin Switch Assay (BSA) 159**
 - **3.3.1.1. Methodology 161**
 - **3.3.1.2. Sample preparation 161**
 - **3.3.1.3. Antibody incubation in Western blotting 162**
- **3.4. Statistical analysis 163**
- **3.5. Results 164**
 - **3.5.1. Biotin switch assay (BSA)..... 164**
 - **3.5.1.1. Detection of the presence of sulphydrated proteins 164**
 - **3.5.1.2. GAPDH for detection of sulphydrated proteins 165**
 - **3.5.1.3. Effect of hydrogen peroxide (H₂O₂) on the detection of sulphydrated proteins 166**
 - **3.5.1.4. Effect of biotin concentrations effect on the detection of sulphydrated proteins 169**
 - **3.5.1.5. Effect of dithiothreitol (DTT) on the detection of sulphydrated proteins 172**
 - **3.5.1.6. Mitogen-activated protein kinase (MEK1) sulphydration 173**
 - **3.5.2. Red Maleimide assay (RMA) 175**
 - **3.5.2.1. Methodology 176**
 - **3.5.2.2. Materials 176**
 - **3.5.2.3. Results 177**
 - **3.5.2.3.1. GAPDH and Red Maleimide detection 177**
 - **3.5.2.3.2. MEK1 and Red Maleimide detection 179**
 - **3.5.2.3.3. Molecular weight shifts after non-exposure to DTT 182**
 - **3.5.3. Immunoprecipitation (IP) of GAPDH in PHH 185**
 - **3.5.3.1. Principle and aim 185**
 - **3.5.3.2. Methodology 185**
 - **3.5.3.3. Results of immunoprecipitation: GAPDH signal after exposure to DTT 185**
 - **3.5.4. GAPDH sulphydration in the PCA 188**
 - **3.5.4.1. Methodology 188**
 - **3.5.4.2. Results 188**
- **3.6. Discussion 190**
 - **3.6.1. The biotin switch assay results 191**

- 3.6.2. The red maleimide assay results 194
- 3.6.3. The immunoprecipitation and immunoblotting results196
- 3.6.4. Summary 197

Chapter Four: Comparison of Signalling Pathways of Hydrogen Sulfide Sources (Na₂S, GYY4137 and AP39) in the Relaxation Response of Porcine Coronary 198

- 4.1. Introduction 199
- 4.2. Materials and Methods 201
 - 4.2.1. Tissue Preparation 201
 - 4.2.2. Isolated organ bath experiments 201
 - 4.2.3. Effect of different H₂S sources on U46619-induced contractions 202
 - 4.2.4. The effect of different H₂S sources on calcium-induced contractions 202
 - 4.2.5. The effect of different H₂S sources on the BayK8644-induced contractions 203
 - 4.2.6. Investigating the mechanism/s of relaxation responses to different H₂S sources in the isolated pre-contracted PCA 203
 - 4.2.7. The effects of oxygen level on the relaxation responses of different sources of H₂S 204
 - 4.2.8. The effects of different sources of H₂S before or after U46619 on the hypoxia and recovery responses in PCA 204
 - 4.2.9. The effects of endogenous H₂S on responses to hypoxia and reoxygenation 205
 - 4.2.10. Defining time-dependent parameters of changes in tone 205
 - 4.2.11. Drugs and Chemicals 206
- 4.3. Statistical Analysis 207
- 4.4. Results 208
 - 4.4.1. The effects of Na₂S and NaHS on vascular tone 208
 - 4.4.2. The effects of GYY4137 on vascular tone 210
 - 4.4.3. The effects of AP39 on vascular tone 211
 - 4.4.4. The effects of the nitric oxide synthase inhibitor, L-NAME, on responses to three different sources of H₂S 213

- 4.4.5. The effects of the spasmogen on responses to different sources of H₂S 214
- 4.4.6. The effects of the potassium channel blocker, tetraethyl ammonium chloride (TEA) on relaxation responses to different sources of H₂S 215
- 4.4.7. The effects of potassium channel blocker, tetraethylammonium chloride (TEA) on the time dependence of responses to different sources of H₂S 216
- 4.4.8. The effect of K_{ATP} channel blockade on the relaxation evoked by three different sources of H₂S 218
- 4.4.9. The effect of tolbutamide on the relaxation responses to different sources of H₂S 220
- 4.4.10. The effect of the K_{ATP} channel blocker, glibenclamide on the relaxation responses of the K_{ATP} channel opener, pinacidil 222
- 4.4.11. The effect of glucose removal on relaxation responses to three different sources of H₂S 223
- 4.4.12. The effect of glucose on relaxation responses of submaximal concentrations of the mitochondrial-targeted H₂S donor AP39 224
- 4.4.13. The effect of the mitochondrial uncoupling agent FCCP on relaxation responses to submaximal concentration of the mitochondrial-targeted H₂S donor AP39 225
- 4.4.14. The effect of different sources of H₂S on calcium-induced contractions..... 226
- 4.4.15. The effect of three different sources of H₂S on BayK8644-precontracted PCA 228
- 4.4.16. The effect of capsazepine on relaxation responses to three different sources of H₂S in U46619-precontracted PCA 229
- 4.4.17. The comparison of 95 % oxygen and 21 % oxygen on relaxation responses in U46619-precontracted PCA..... 230
- 4.4.18. The effects of different sources of H₂S before or after U46619 on the hypoxia and recovery responses in PCA 232
- 4.4.18.1. The effects of 1 mM Na₂S added before U46619 addition 232
- 4.4.18.2. The effects of 100 μM GYY4137 added before U46619 addition 234
- 4.4.18.3. The effects of 30 μM AP39 addition before U46619 addition 236
- 4.4.18.4. The effects of 1 mM Na₂S addition after U46619 addition 238

• 4.4.18.5. The effects of 100 μ M GYY4137 addition after U46619 addition	240
• 4.4.18.6. The effects of 30 μ M AP39 addition after U46619 addition	242
• 4.4.19. The effects of inhibition of endogenous H ₂ S production on the recovery response after 15 min hypoxia in the U46619-precontracted PCA	244
• 4.5. Discussion	246
• 4.5.1. L-NAME effects: NO role	250
• 4.5.2. KCl, TEA and glibenclamide effects: K ⁺ channels role and K _{ATP} channels roles	252
• 4.5.3. The role of calcium channels	254
• 4.5.4. The role of TRPV1 channels	256
• 4.5.5. The role of mitochondria	257
• 4.5.6. The effects of hypoxia	258
• 4.5.7. Summary	264
Chapter Five: General Discussion.....	265
• 5.1. Measurement of Hydrogen Sulfide Generation in the Heart	268
• 5.2. Development of an Assay to Measure Sulfhydration in the Pig Heart	274
• 5.3. Comparison of Signalling Pathways of Hydrogen Sulfide Sources (Na ₂ S, GYY4137 and AP39) in the Relaxation Response of Porcine Coronary Artery	277
Conclusion	281
References	283
Appendices: The attended training courses and seminars ...	315

List of abbreviations

AA	Aspartic acid
ACE	Angiotensin converting enzyme
ACEI	Angiotensin converting enzyme inhibitor
AOAA	Aminooxyacetic acid
AP39	[10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl)phenoxy)decyl) triphenylphosphonium bromide]
B	Buffer
BCA	β -cyanoalanine
Biotin-HPDP	(N-[6-(biotinamido) hexyl]-3-(2-pyridyldithio) propionamide)
BP	Blood pressure
BSA	Biotin switch assay
cAMP	Cyclic adenosine monphosphate
cAMP/PKG	Cyclic AMP-dependent/protein kinase G
CAT	Cysteine aminotransferase
CBS	Cystathionine β -synthase
CDS	(1-(3,4-Dihydroxyphenyl)-2-[(4-hydroxy-6-methyl-2-pyrimidinyl) sulfanyl])
CGRP	Calcitonin-gene related peptide
CNS	Central nervous system
CNT	Carbon nanotubes
CO	Carbon monoxide
CSE	Cystathionine γ -lyase
CVD	Cardiovascular diseases
CVS	Cardiovascular system
DAO	D-amino acid oxidase
DHLA	Dihydrolipoic acid
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factors
EGTA	Ethylene glycol bis (β -aminoethyl ether)-N, N, N', N'- tetra acetic acid
FCCP	(carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gas chromatography
GY4137	[Morpholin-4-ium 4 methoxyphenyl (morpholino) phosphinodithioate]
H ₂ O ₂	Hydrogen peroxide
H ₂ S	Hydrogen sulfide
HEK 293	Huamn embrionic kidney 293 cells
HIF-1 α	Hypoxia-inducible factor-1 alpha
HPLC	High pressure liquid chromatography
HUVECs	Human umbilical vein endothelial cells
IHD	Ischaemic heart diseases
InsP ₃	Inositol-1, 4, 5 triphosphate
IP	Immunoprecipitation
IRI	Ischaemia-reperfusion injury

kDa	Kilodalton
Keap1	Kelch-like ECH-associated protein 1
KO	Knockout
L-NAME	N-nitro L-arginine methyl ester
LPS	Lipopolysaccharide
MBB	Monobromobimane
MDA	Malondialdehyde
MEK1	Mitogen-activated extracellular signal-regulated kinase 1
MMTS	S-methyl methanethiosulfonate
MST	Mercaptopyruvate sulfurtransferase
MW	Molecular weight
MWM	Molecular weight marker
Na ₂ S	Sodium sulfide
NaHS	Sodium hydrosulfide
NF-κB	Nuclear factor kappa enhancer of β-cells
NO	Nitric oxide
Nrf2	Nuclear-factor-E2-related factor transcription factor
NS	Non-significant
NSAIDs	Non-steroidal anti-inflammatory drugs
OD	Optical density
One-way ANOVA	One-way analysis of the variance
PCA	Porcine coronary artery
PHC	Porcine heart cytosol
PHH	Porcine heart homogenate
PHSS	Polarographic hydrogen sulfide sensor
PLN	Phospholamban
PLP	Pyridoxal 5-phosphate
PPA	Phenylpyruvic acid
PPG	Propargyl glycine
PTMs	Post-translational modifications
PVAT	Perivascular adipose tissue
Ratio of the low MW/ high MW	Ratio of the low molecular weight/ high molecular weight
RBC	Rat brain cytosol
RBCs	Red blood cells
RFU	Relative fluorescence unit
RHC	Rat heart cytosol
RLC	Rat liver cytosol
RLH	Rat liver homogenate
RM	Red maleimide
RMA	Red maleimide assay
ROS	Reactive oxygen species
RT	Room temperature
SAM	S-adenosyl methionine
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SF7-AM	Sulfidefluor7-acetoxymethyl ester
SHR	Spontaneously hypertensive rats
SISE	Sulfide ion selective electrode
SMCs	Smooth muscle cells
SP1	Specific protein 1

SP3	Specific protein 3
SQOR	Sulfide quinone oxidoreductase
STS	Sodium thiosulfate
TBST	Tris-buffered saline-Tween 20
TCEP	Tris (2-carboxyethyl)phosphine
TEA	Tetraethylammonium chloride
TGF- α	Transforming growth factor-alpha
TRP	Transient receptor potential
TRPA	Transient receptor potential-ankyrin
TRPV	Transient receptor potential-vanilloid
VEGF	Vascular endothelial growth factor

Declaration

I declare that the work carried out in the PhD thesis was all a result of my own work.

Chapter One

General Introduction

1.1. Background

Hydrogen sulfide (H₂S) is a small, flammable, colourless, gaseous molecule endogenously produced in mammalian tissues (Wang, 2012; Wen et al., 2018). H₂S has a characteristic offensive odour of rotten eggs (Wang, 2012; Beltowski, 2015). For a long time, H₂S was only known as a pollutant chemical, which had toxic effects (Reiffenstein et al., 1992; Wang, 2012; Beltowski, 2015). However, it is now clear that H₂S can be synthesised endogenously, and it is recognised as the third member of the gas transmitter family, which includes nitric oxide (NO) and carbon monoxide (CO). H₂S was firstly described by the Kimura group in 1996 as an important physiological mediator (Abe and Kimura, 1996). Therefore, increasing research has been focused on this interesting signalling molecule (Shen et al., 2015; Meng et al., 2017). The principal H₂S synthesising enzymes in mammals are cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and mercaptopyruvate sulfurtransferase (MST) combining with cysteine aminotransferase (CAT), by the metabolism of L-cysteine as the main substrate (figure 1.1: A) (Chan and Wallace, 2013; Kolluru et al., 2013; Shen et al., 2011; Polhemus and Lefer 2014; Beltowski, 2015).

Over the last two decades or so, the physiological significance of H₂S has been recognised because it is produced in low concentrations (basal levels) in mammals. Therefore, this biological relevance changed the image of H₂S from toxic chemical to a physiologically-active molecule (Szabo et al., 2018). H₂S has multiple important physiological and pathological effects; it is involved in various systems, such as the cardiovascular, nervous, endocrine, respiratory and gastrointestinal systems. The medical importance of H₂S remains a scientific challenge, and a hot topic among the research communities and studies are still at an early stage. Most of the detailed biological effects of H₂S are still unclear and need further research (Wang, 2012; Ahmad et al., 2015; Zhang et al., 2018).

Cardiovascular diseases (CVD), such as hypertension, angina, myocardial infarction, arrhythmia, heart failure, and stroke are some of the most common causes of death around the world (Guo et al., 2013; Polhemus et al., 2014). Additionally, hypertension is a common cause of IHD (ischaemic heart diseases). Most of the causes of hypertension are unknown (Carretero et al., 1998; Klabunde, 2012; Al-Taie, 2011; Al-Taie and Al-Youzbaki, 2013). Accumulated evidence has demonstrated that H₂S plays a crucial role in homeostasis and cardioprotection (Mancardi et al., 2009; Shen et al., 2015). Several cardiovascular diseases are linked with disturbances of H₂S and homocysteine levels, such as very low levels of H₂S and high levels of homocysteine and *vice versa* (Polhemus et al., 2014; Shen et al., 2015;

McCully, 2015), because CBS uses homocysteine to produce L-cysteine, which is metabolised to H₂S by CBS and therefore, disturbance of CBS would lead to elevation of homocysteine level and decrease of H₂S level. Some reports found that H₂S levels did not demonstrate a direct relationship with different stages of heart failure (Salloum, 2015). However, it has been reported that H₂S concentrations measured in plasma were found to be very low in patients with coronary artery disorders, myocardial infarction and heart failure in comparison with angiographically normal people (Kovačić et al., 2012; Polhemus et al., 2014; Salloum, 2015). Therefore, understanding the synthesis, regulation, level, mechanism and signalling pathways of H₂S in the heart may decrease the ambiguity that surrounds H₂S biology. This understanding may reveal a promising drug target, which may have several pharmacological implications for a plethora of diseases, in particular CVD (Polhemus et al., 2014; Yu et al., 2014; Susuki et al., 2016; Wen et al., 2018).

Thus, in recent years, evidence that H₂S may have an influential role in several important biological processes has increased. Many studies have highlighted the production of H₂S within the body, including a lot of data demonstrating biological effects of H₂S in the vasculature. However, the detailed mechanisms of H₂S generation, regulation, signalling and effects in the heart are still unclear and need to be clarified, especially in the myocardium of mammals.

1.2. H₂S chemistry

H₂S is a weakly acidic molecule. In aqueous solution, it dissociates into about two-thirds hydrosulfide anion (HS⁻) and an extremely small amount of sulfide anion (S²⁻) which presents only at high pH, while about one-third remains as undissociated volatile H₂S under physiological conditions (pH 7.4, 37 °C) (Hughes et al., 2009; Shen et al., 2011; Wen et al., 2018). Excess of H₂S can be stored within the body in two different bound forms (intracellular sulfur stores): sulfane sulfur (reductant/alkaline-labile) (persulfide, polysulfide, thiosulfate, polythionates and thiosulfonate) and acid-labile sulfur (iron-sulfur clusters), which may be important for cells and situations to perform the physiological effects of H₂S (Ubuka, 2002; Ishigami et al., 2009; Shibuya et al., 2009; Beltowski et al., 2015) (figure 1.1: B). Since sulfane sulfur releases stored H₂S under reducing or alkaline conditions, while acid-labile stores release stored H₂S under acidic condition (Li et al., 2011). Therefore, synthesised H₂S could be stored transiently as a sulfane sulfur and acid-labile sulfur forms. Whether the specific active form in biology is H₂S or HS⁻ or both is unknown, but HS⁻ may be the active form.

H₂S dissociates as the following: $\text{H}_2\text{S} \leftrightarrow \text{HS}^- \leftrightarrow \text{S}^{2-}$. The dissociation constants (pK_a) are for the first reaction (H₂S ↔ HS⁻) 7.04 and 11.96 for the second reaction (HS⁻ ↔ S²⁻) (Tangerman, 2009; Kolluru et al., 2013; Beltowski, 2015). Moreover, H₂S is five times more soluble in lipid than water. Thus, H₂S is able to diffuse through plasma membranes of all cells to exert autocrine/or paracrine effects without any transporter or channel. This property might be the cause of its short half-life because of its rapid multiple clearance pathways (about seconds-minutes) and multiple biological actions (Li et al., 2011; Whiteman et al., 2011; Wang, 2012; Shen et al., 2015). Therefore, H₂S biogenesis might follow a pulsatile pattern; in other words, an increase of H₂S biogenesis during a short period and then return to basal levels. Therefore, H₂S biogenesis may need a real-time (dynamic) technique for its measurement.

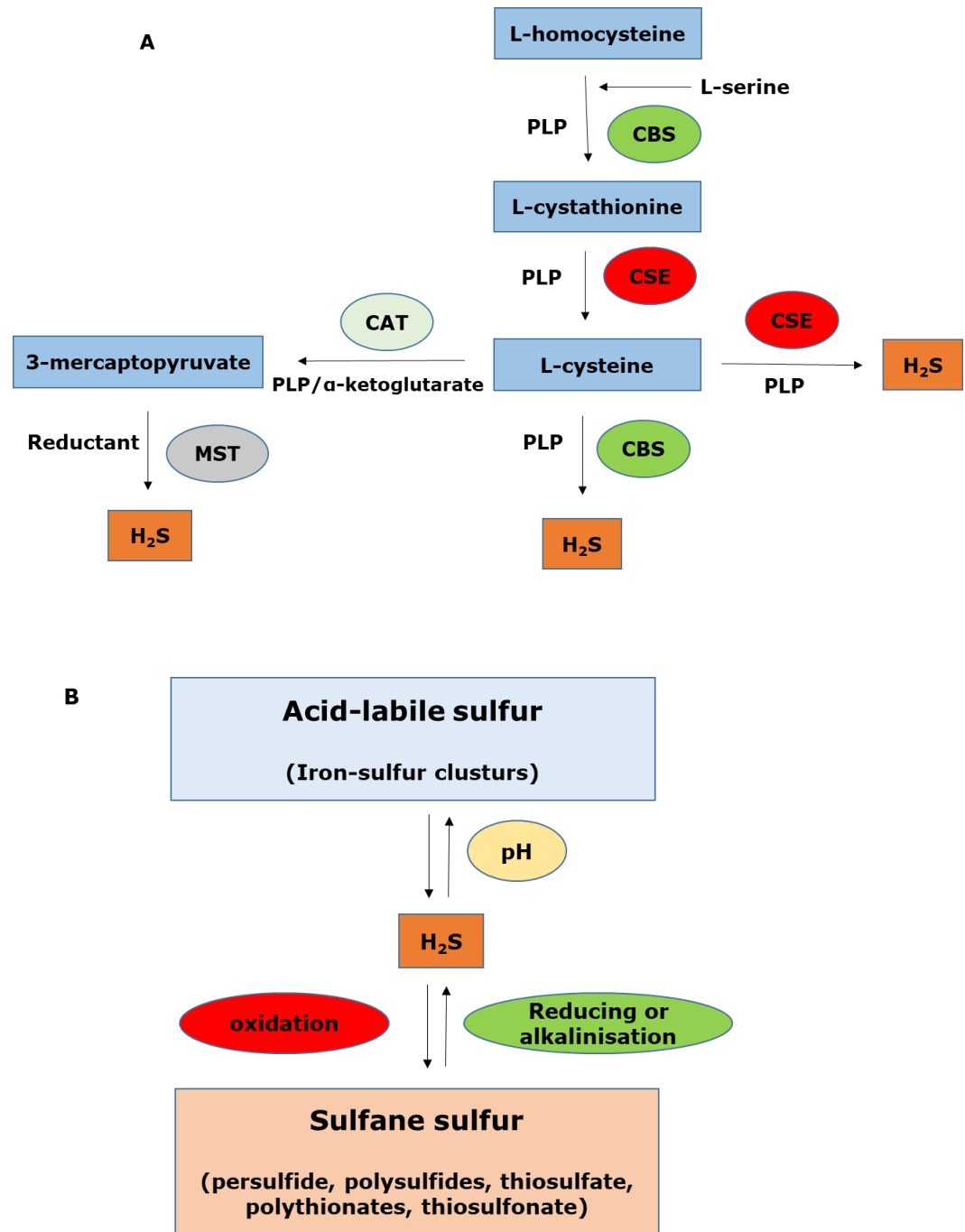


Figure 1.1: (A) Enzymatic biosynthesis of H₂S via CBS (cystathionine β-synthase), CSE (cystathionine γ-lyase), MST (3-mercaptopyruvate sulfurtransferase) with CAT (cysteine aminotransferase). PLP (pyridoxal 5-phosphate). (B) H₂S storage forms and release under different conditions, acid-labile sulfur form releases H₂S under acidic pH condition, sulfane sulfur form releases H₂S under reducing or alkalisation condition (Chan and Wallace et al., 2013; Kolluru et al., 2013).

1.3. Biosynthesis and regulation of H₂S

Generally, biogenesis of H₂S is controlled by CBS and CSE and MST. These enzymes were detected in different species, including mice, rats, pigs, guinea pigs, humans, rabbits, dogs, fish, frogs, cows and bacteria (Whiteman et al., 2011; Wang, 2012; Shen et al., 2015; Cao et al., 2019). These enzymes are expressed and distributed in most organs (heart, blood vessels, liver, kidney, brain, hippocampus, lung, placenta, eye, gastrointestinal tract and pancreas) with different tissue distribution across various studies (Levonen et al., 2000; Sidhu et al., 2001; Whiteman et al., 2011; Wang, 2012; Lin et al., 2013; Wen et al., 2018). Moreover, H₂S was detected in various samples, such as blood, plasma, serum, tissues and urine (Li et al., 2011; Wang, 2012). Therefore, this extensive presence of H₂S synthesising enzymes and H₂S production indicates important biological effects of H₂S in the body.

H₂S is mainly synthesised endogenously from L-cysteine or its derivative homocysteine by CBS and CSE through enzymatic desulfhydration reactions. Therefore, L-cysteine is the principal substrate for CBS and CSE, which metabolise L-cysteine to H₂S and thus, L-cysteine was used by many studies as a H₂S precursor (H₂S prodrug) to augment endogenous H₂S generation (Cheng et al., 2004; Al-Magableh and Hart, 2011; Bucci et al., 2012; Asimakopoulou et al., 2013; Chitnis et al., 2013; Rashid et al., 2013; Candela et al., 2016). CBS and CSE both require pyridoxal 5-phosphate (PLP) as a cofactor. PLP is a vitamin B6 (pyridoxine) derivative. Moreover, H₂S is synthesised via PLP-independent enzyme MST coupling with CAT (figure 1.1: A). CBS and CSE, as well as CAT are PLP-dependent enzymes. Therefore, in the absence of PLP, there would be no conversion of L-cysteine to H₂S via CBS/CSE and mercaptopyruvate via CAT. Therefore, in situations associated with low or restriction vitamin B6 and L-cysteine nutrition. This would lead to decrease of endogenous H₂S production rates via these enzymes in tissues and these situations were reported to be associated with increase of cardiovascular pathologies (DeRatt et al., 2014; Mys et al., 2017). Thus, application of PLP (vitamin B6) and L-cysteine leads to stimulation of the endogenous H₂S production via CBS and CSE as well as CAT, and cardiovascular protective effects.

H₂S is synthesised via non-enzymatic and enzymatic pathways. Non-enzymatic pathways play a minor role in endogenous H₂S production, such as via thiol or thiol-containing molecules reduction or metabolism (Li et al., 2011; Wen et al., 2018).

1.3.1. H₂S Biosynthesis and regulation via CBS

H₂S is mainly synthesised endogenously from L-cysteine, or its derivative homocysteine, by CBS. L-cysteine has been employed in several experiments as an enhancer of exogenous H₂S generation and H₂S precursor (Cheng et al., 2004; Rashid et al., 2013). CBS metabolises L-cysteine via hydrolysis and produces H₂S (Wang, 2012; Kolluru et al., 2013). CBS is a cytosolic and PLP dependent enzyme. In the absence of PLP, there would be no conversion of L-cysteine to H₂S via CBS (Meier et al., 2001; Banerjee and Zou, 2005; Wang et al., 2012; Cao et al., 2019).

CBS has different roles in addition to H₂S biosynthesis. For example, CBS metabolises (detoxifies) homocysteine via condensation with L-serine to produce cystathionine (Wang, 2012; Cao et al., 2019). Therefore, a mutation or abnormality in CBS results in hyperhomocysteinemia, which is a risk factor for CVD (McCully, 2015). CBS was reported to present predominantly in rat liver, kidney and brain (Abe and Kimura, 1996; Shibuya et al., 2009; Asimakopoulou et al., 2013; Wen et al., 2018; Cao et al., 2019). Furthermore, Abe and Kimura found that AOAA (aminooxyacetic acid, non-selective CBS inhibitor) inhibited endogenous H₂S generation in the rat brain (Abe and Kimura, 1996). Thus, CBS is thought to be the predominant enzyme for endogenous synthesis of H₂S in the rat and mouse brain (Abe and Kimura, 1996; Al-Magableh and Hart, 2011). CBS was also detected in placenta (Cindrova-Davies et al., 2013), pig bronchioles, trachea and pulmonary arteries (Rashid et al., 2013), mouse hearts (Kondo et al., 2013), human coronary artery smooth muscle cells and porcine coronary artery (PCA) (Donovan et al., 2017), porcine perivascular adipose tissue (PVAT) and myocardium (Donovan et al., 2018). Thus, H₂S plays important roles in the control of vascular tone and cardiac function.

1.3.1.1. Regulation and post-translational modifications (PTMs) of CBS

CBS is a homotetramer and consists of subunits, each one has a molecular weight (MW) about 63 kDa. CBS has two distinct terminal domains. The N-terminal has a catalytic site, which has heme (important redox sensor) and a PLP binding site (figure 1.2). C-terminal is a regulatory site responsible for allosteric activation and controls the sulfhydration of cysteine residues (Banerjee and Zou, 2005; Yang et al., 2008; Jiang et al., 2010). Moreover, S-adenosyl methionine (SAM) was reported as a positive allosteric modulator of CBS (Abe and Kimura, 1996) by binding to C-terminal part (domain) of CBS removing the auto-inhibitory effect of the C-terminal and transforming CBS to the truncated CBS, which is the active version of CBS that has a molecular weight of about 48 kDa (Skovby et al., 1984; Janosik et al., 2001; Zou and Banerjee, 2003). This process leads to an increase in cysteine entrance to the catalytic domain of CBS (Wang, 2012; Ereño-Orbea et al., 2014). Deletion of the C-

terminal leads to loss of modulation to allosteric activators, such as SAM (Janosik et al., 2001). The structure of the catalytic site may be pivotal in enzymatic reactions with substrates and intermediates (Taoka et al., 1998). Moreover, calmodulin could bind to the C-terminal and augment the catalytic domain of CBS in the brain (Kimura, 2002). Therefore, CBS could be stimulated by the Ca²⁺/calmodulin pathway (Banerjee et al., 2003; Wang, 2012).

1.3.1.2. Regulation of protein levels

It was reported that insulin and glucocorticoid caused an increase of CBS transcription (CBS mRNA) in the liver (Ratnam et al., 2002). Furthermore, the expression levels of CBS protein in rat astrocytes was increased by transforming growth factor- α (TGF- α), epidermal growth factors (EGF), dexamethasone and cAMP (cyclic adenosine monophosphate) (Enokido et al., 2005). Moreover, CBS and also CSE translation level could be regulated by Nrf2 (nuclear-factor-E2-related factor transcription factor), SP1 (specific protein 1), and SP3 (specific protein 3) (Sbodio et al., 2019). However, these studies have limitations. For example, Ratnam's study did not measure parallel CBS enzyme activity or H₂S level and changes in CBS mRNA may not result in changes in CBS protein expression and activity. Similarly to Ratnam's study, Enokido's study did not measure CBS enzyme activity or H₂S level. Therefore, it would be important to measure H₂S synthesising enzyme/s transcription, expression, H₂S production levels.

1.3.1.3. Post-translational modifications

Regulation of protein biosynthesis consists of transcription at the genetic level, which is the long-term stage and PTMs (post-translational modifications) as an acute stage. PTMs are groups of processing events that happen during the formation of mature protein as a central part of cell signalling. PTMs may involve proteolysis or the addition of functional groups, such as phosphate, acetate, methyl and amide or the linkage with lipid or carbohydrate in order to get the complete active version of the protein (Kabil et al., 2006; Beltrao et al., 2013). These events cause conversion of propeptide to mature protein form (Beltrao et al., 2013). PTMs have multiple effects on enzyme activity, function, stability (metabolism), localisation (residence) as well as interactions with other proteins (Paul and Snyder, 2012).

There are several PTMs of CBS. For example, proteolytic cleavage of peptide bonds to change the larger peptide protein that is about 63 kDa to mature protein of about 48 kDa (high MW CBS). In line with this idea, Skovby and his group reported that two polypeptides were found in the fresh cytosol of rat liver, one major protein with a high MW about 63 kDa and one minor protein with low MW about 48 kDa (Skovby et al., 1984). However, after one-week incubation of fresh rat liver cytosol at 4°C, this resulted in an increase of the CBS activity by about two or three-fold in parallel with a relative increase of the smaller polypeptide and disappearance of the larger molecular weight CBS. They demonstrated that the specific activity of the smaller polypeptide was equal to about sixty times more than the larger polypeptide.

This increase of the smaller polypeptide activity is associated with thirty times increased affinity toward homocysteine. Alteration of the high MW peptide to the lower MW peptide could be suppressed by protease inhibitors including antipain, leupeptin, and N-alpha-p-tosyl-L-lysine chloromethyl ketone, while it could be increased by proteases, such as trypsin, which triggers proteolysis and increase of the CBS activity. They inferred that PTMs of CBS via proteolysis from less active to more active forms might depend on physiological factors, such as the rate of proteolysis in cells and tissues (Skovby et al., 1984).

Additionally, Zou and Banerjee's study showed that the treatment of hepatocytes with tumour necrosis factor-alpha (TNF- α) leads to oxidative stress and cellular lesion; which in turn results in an increase of CBS activity via targeted proteolytic cleavage to the smaller peptide (a more active form of CBS) within 24 h (Zou and Banerjee, 2003).

Another example of PTMs of CBS; human CBS is reported to be phosphorylated at serine residue (27) on the heme-binding domain (Rigbolt et al., 2011) and serine residue (199) on the catalytic domain (Zhou et al., 2013), although these studies did not clarify by which kinase CBS was phosphorylated and also did not measure the phosphorylated CBS activity. In another study, di Villa Bianca's group reported that human CBS of urothelial cells might be phosphorylated, but on a different serine by cyclic AMP-dependent/protein kinase G (cAMP/PKG) pathway at serine 227 residue that augmented CBS activity (di Villa Bianca et al., 2015).

An additional example of PTMs of CBS is sumoylation. It was reported that CBS activity is modulated by sumoylation pathway (Ubiquitin-like modifier protein, SUMO-1) at lysine 211 (K211) in both *in vivo* and *in vitro* experiments (Kabil et al., 2006) by detection of two bands of CBS at about 63 and 80 kDa (Kabil et al., 2006). Sumoylation of CBS targeted the C-terminal, the regulatory domain for CBS. Also,

they found that the sumoylated CBS is identified within the platform (scaffold) of the nucleus. They suggested that it might be due to a genetic/sophisticated control of CBS activity due to unknown presence of sumoylated CBS in the nuclear compartment. These results suggested that sumoylation might regulate CBS activity. However, whether the sumoylated CBS is active or not is unclear because this study did not measure the enzyme activity of sumoylated CBS. In another study, CBS activity was attenuated *in vitro* by sumoylation (Agrawal and Banerjee, 2008).

An additional example of CBS PTMs is glutathionylation of human CBS at cysteine 346 (C346), which leads to an increase of CBS activity by about two-fold *in vitro* under oxidative stress conditions (Niu et al., 2015).

CBS contains unique heme in the N-terminal catalytic site; this group is imperative for redox reaction sensitivity (redox-sensor). CBS also has oxidoreductase components (motifs), which might participate in redox sensing (Meier et al., 2001; Taoka et al., 2002). Moreover, Taoka's study reported that heme was present in the crystallography of CBS, while oxidoreductase parts were absent (Taoka et al., 2002). Taoka's study also found that the replacement of any cysteine residue with a non-redox active amino acid did not result in a change of CBS activity to reducing agents; while the deletion of heme leads to a loss of redox sensitivity of CBS (Taoka et al., 2002). They suggested that the heme is the principal redox sensor in CBS. Thus, measurement of CBS activity might be a biomarker of oxidative stress. Thus, any mutation of this group could affect activity, regulation and stability of CBS (Meier et al., 2001; Taoka et al., 2002).

It has been reported that the activity of CBS was attenuated under reducing states and favoured ferric (Fe^{3+}) state of CBS (Taoka et al., 1998; Taoka et al., 2002). Moreover, human CBS activity is halved under reducing conditions, and re-oxidation leads to regain of CBS activity (Taoka et al., 1998). Therefore, human CBS activity is about doubled under oxidation circumstances (Taoka et al., 2002). The redox form of heme is affected by pH. For example, alkaline pH prefers ferrous (Fe^{2+}), while acidic pH produces ferric (Fe^{3+}) active version of human CBS (Pazicni et al., 2004). Conversely, in rat kidney, ischaemia leads to decrease of pH, and CBS activity and alkalinisation leads to recovery of CBS activity (Prathapasinghe et al., 2008).

Moreover, binding of NO and CO to the heme of CBS results in attenuated CBS activity (Taoka and Banerjee, 2001; Zhao et al., 2001). For instance, nitrosylation by NO led to a decrease of recombinant human CBS (Taoka and Banerjee, 2001), and rat CBS (Prathapasinghe et al., 2008) activity in vitro. CBS is PLP dependent enzyme, and in the absence of PLP, there is no conversion of L-cysteine to H₂S via CBS (Meier et al., 2001; Banerjee and Zou, 2005; Wang et al., 2012). In summary, these observations demonstrate that CBS activity depends on several factors, such as PLP, proteolysis, and heme group availability.

(A)

N-terminal	Phosphorylation at 27 (serine)	Heme binding site at 52 (cysteine) and 65 (histidine)	PLP binding site at 119 (lysine)	Phosphorylation at 199 (serine)	Sumoylation at 211 (lysine)	Phosphorylation at 227 (serine)	SAM binding site 414-551 (lysine)	C-terminal
------------	--------------------------------	---	----------------------------------	---------------------------------	-----------------------------	---------------------------------	-----------------------------------	------------

(B)

10	20	30	40	50
MPSETPQAEV	GPTGCPHRSG	PHSAKGSLEK	GSPEDKEAKE	PLWIRPDAPS
60	70	80	90	100
RCTWQLGRPA	SESPHHHTAP	AKSPKILPDI	LKKIGDTPMV	RINKIGKKFG
110	120	130	140	150
LKCELLAKCE	FFNAGGSVKD	RISLRMIEDA	ERDGTLPKPGD	TIIEPTSGNT
160	170	180	190	200
GIGLALAAAV	RGYRCIIVMP	EKMSSEKVDV	LRALGAEIVR	TPTNARFDSP
210	220	230	240	250
ESHVGVAVRL	KNEIPNSHIL	DQYRNASNPL	AHYDTTAEI	LQQCDGKLDM
260	270	280	290	300
LVASVGTGGT	ITGIARKLKE	KCPGCRIGV	DPEGSILAEP	EELNQTQTT
310	320	330	340	350
YEVEGIGYDF	IPTVLDRTVV	DKWFKSNDDEE	AFTFARMLIA	QEGLLCGSA
360	370	380	390	400
GSTVAVAVKA	AQELQEGQRC	VVILPDSVRN	YMTKFLSDRW	MLQKGFLEE
410	420	430	440	450
DLTEKKPWWW	HLRVQELGLS	APLTVLPTIT	CGHTIEILRE	KGFDQAPVVD
460	470	480	490	500
EAGVILGMVT	LGNMLSSLLA	GKVQPSDQVG	KVIYKQFKQI	RLTDTLGRLS
510	520	530	540	550
HILEMDHFAL	VVHEQIQYHS	TGKSSQRQMV	FGVVTAIDLL	NFVAAQERDQ K

S27, S199, and S227 are targets for phosphorylation (serine)

K119 is a target for PLP (lysine)

K211 for sumoylation (lysine)

V414 (valine) and K551 (lysine) are targets for interaction with SAM

Figure 1.2: (A and B) The active residues (reactive sites) of CBS (Janosik et al., 2001; Kabil et al., 2006; Rigbolt et al., 2011; Zhou et al., 2013).

1.3.2. H₂S Biosynthesis and regulation via CSE

CSE is a cytosolic and PLP dependent enzyme consisting of about 45 kDa subunits. CSE hydrolyses L-cysteine to produce H₂S (Kolluru et al., 2013; Zhao et al., 2014). Additionally, after the conversion of L-homocysteine to cystathionine via CBS, CSE metabolises cystathionine to L-cysteine as part of the transsulfuration pathway (Kolluru et al., 2013; Sbodio et al., 2019). In contrast to CBS, CSE has been reported to be abundant in the vasculature (Hosoki et al., 1997; Zhao et al., 2001).

In the vasculature, CSE has been shown to be expressed in rat portal vein and aorta (Hosoki et al., 1997), rat aorta, mesenteric, tail and pulmonary arteries (Zhao et al., 2001; Cheng et al., 2004), rat aorta (Geng et al., 2004; Brancaleone et al., 2015), rat mesenteric artery (Candela et al., 2016), human internal mammary artery (Webb et al., 2008), human aorta smooth muscle cells (yang et al., 2011), mouse aorta (Al-Magableh and Hart, 2011), mice mesenteric artery smooth muscle cells (Fu et al., 2012), mouse aorta and coronary arteries (Kuo et al., 2015), PCA (Donovan et al., 2017, Donovan et al., 2018), bovine aortic endothelial cells (BAECs), human umbilical vein endothelial cells (HUVECs) (Yang et al., 2008), human aortic endothelial cells (HAECs) (Kuo et al., 2015; Leucker et al., 2017). Asimakopoulou's study reported that relative abundancy of CSE was higher than CBS in rat aorta (Asimkopoulou et al., 2013). Endogenous H₂S production was inhibited by β -cyanoalanine (BCA, CSE inhibitor) and propargylglycine (PPG, CSE inhibitor) in the blood vessels, such as rat portal vein and aorta (Hosoki et al., 1997), and mouse aorta (Al-Magableh and Hart, 2011).

CSE is also present in human liver (Levonen et al., 2000; Webb et al., 2008), rat liver, kidney and brain (Abe and Kimura, 1996; Hosoki et al., 1997; Zhao et al., 2001; Ishii et al., 2004; Mok et al., 2004; Shibuya et al., 2009; Kuo et al., 2015), mouse liver and kidney (Li et al., 2005; Al-Magableh and Hart, 2011), mouse hepatocytes (Untereiner et al., 2016), pig liver, bronchioles, trachea and pulmonary arteries (Rashid et al., 2013), the carotid bodies of mice and rats (Peng et al., 2010), rat brain and heart (Geng et al., 2004) and porcine myocardium (Donovan et al., 2018). CSE is also reported to be expressed in other tissues, such as ileum (Hosoki et al., 1997) and placenta (Cindrova-Davies et al., 2013).

Swaroop's study reported that CSE expression in rat liver was about 100-fold higher than CSE expression in rat brain (Swaroop et al., 1992). In line with this idea, Ishii's study reported that CSE was about 50-fold more expressed in rat and mouse liver tissues than other tissues and also CSE activity was reported to be the highest in liver tissues (Ishii et al., 2004). In another study, endogenous H₂S production in CSE-KO mice liver was about one-third of wild-type mice (controls) (Untereiner et al., 2016). They suggested that CSE may contribute to about two-thirds of endogenous H₂S production in mouse liver. Similarly, CSE expression in rat liver was higher than rat aorta, tail, mesenteric, and pulmonary arteries (Zhao et al., 2001).

There are several factors that can modulate CSE activity (Zhao et al., 2014). CSE activity is modulated by calcium level. For example, low calcium level causes CSE activation, while high calcium level results in inhibition of purified rat liver CSE (Mikami et al., 2013). SP1 and TNF- α increased CSE expression in human aorta smooth muscle cells (Yang et al., 2011) and mice peritoneal macrophages (Sen et al., 2012). An elevated level of ROS (reactive oxygen species) leads to an increase in CSE expression and activity in rat kidney mesangial cells (Hassan et al., 2012). Dexamethasone (glucocorticoid) decreases CSE expression in macrophages treated with LPS (lipopolysaccharide) (Zhu et al., 2010). Hypoxia led to increase of CSE expression in mammalian cells (Wang et al., 2014). The transcription and cytoprotective factors, such as Nrf2 led to an elevation of CSE expression in rat mesangial cells (Hassan et al., 2012) and mouse hearts (Calvert et al., 2009). Moreover, human CSE was reported as a target for sumoylation *in vitro* (Agrawal and Banerjee, 2008). However, this study did not measure the activity of the sumoylated CSE. Nitrosylation of CSE by NO led to a decrease of CSE activity *in vitro* (Mustafa et al., 2011). Application of isoproterenol to produce infarction-like condition in rat hearts led to a decrease of CSE activity to about 80 % and H₂S levels in the myocardial tissue and plasma to about 40 % of controls (Geng et al., 2004). Therefore, they suggested that down regulation of H₂S pathways may lead to cardiac pathology.

1.3.3. H₂S Biosynthesis and regulation via MST

H₂S can also be synthesised in the CNS, liver, trachea, bronchi, and blood vessels by a PLP independent enzyme, MST. MST is a monomer protein that has a MW about 33 kDa. First, CAT produces 3-mercaptopyruvate from L-cysteine in the presence of α -ketoglutarate. MST then causes desulfuration of mercaptopyruvate to produce H₂S (figure 1.1) (Shibuya et al., 2009; Chan and Wallace, 2013; Rashid et al., 2013). Therefore, MST along with CAT produce H₂S. It has been proposed that MST interacts with 3-mercaptopyruvate in the presence of α -ketoglutarate and causes transfer of sulfur atom from 3-mercaptopyruvate to MST to form persulfide on MST active site (Whiteman et al., 2011). Therefore, MST needs a reducing agent, such as thioredoxin and dihydrolipoic acid (DHLA) *in vivo* and dithiothreitol (DTT) *in vitro* (Rashid et al., 2013; Kimura, 2014; Cao et al., 2019) and alkaline pH in order to desulfhydrate MST and produce H₂S (Kimura, 2014). MST has two rhodanese domains in its structure, which might help in the detoxification of cyanide and H₂S breakdown (Nagahara et al., 1999; Whiteman et al., 2011).

MST was expressed in rat aorta (Shibuya et al., 2009), human coronary and pulmonary arteries endothelial cells, rat and mouse coronary arteries (Kuo et al., 2015), PCA (Donovan et al., 2017; Donovan et al., 2018), mouse hearts (Kondo et al., 2013), porcine myocardium and PVAT (Donovan et al., 2018), and pig bronchioles, trachea, pulmonary arteries and rat liver (Rashid et al., 2013). MST was also expressed in brain and liver of mice and rats (Shibuya et al., 2009; Kuo et al., 2015). In human RBCs (red blood cells), CBS and CSE expressions were undetectable, while MST was expressed (Vitvitsky et al., 2015). Therefore, the biological role of MST needs more studies to be clarified, especially in human diseases of organs that have abundant mitochondria such as heart, liver, brain and lung. This is because about one-third of MST is reported to be in the cytosol and two-thirds in the mitochondria, which indicates the importance of MST in H₂S production inside the mitochondria (Lavu et al., 2011; Módis et al., 2013; Fräsdorf et al., 2014; Wen et al., 2018). Therefore, in these tissues, such as the heart, liver, brain, and lung the MST may have an important physiological role. The role of H₂S in the mitochondria will be discussed later.

MST activity is inhibited under some states, such as oxidising circumstances, which may be due to dimerisation (Nagahara, 2008) and also at increased intracellular calcium level (Mikami et al., 2011).

H₂S can also be synthesised from D-cysteine by D-amino acid oxidase (DAO) through the formation of 3-mercaptopyruvate. DAO is PLP-independent enzyme present in the kidney, cerebellum/brain and liver (Shibuya et al., 2013). Thus, DAO may be considered as a minor source of H₂S.

1.3.4. Comparison of variable tissue distributions of CBS, CSE and MST

These enzymes CBS, CSE and MST have variable tissue localisations. For example, CBS was reported to be present predominantly in the liver, kidney and brain (Abe and Kimura, 1996; Wen et al., 2018). CBS is thought to be the predominant enzyme for endogenous synthesis of H₂S in the rat brain. On the other hand, CSE was reported to be abundant in the vasculature (Hosoki et al., 1997) since it was reported that CBS had low expression in the blood vessels, such as rat portal vein and aorta (Hosoki et al., 1997), and mouse aorta (Al-Magableh and Hart, 2011) by normalisation of CBS expression with GAPDH (glyceraldehyde 3-phosphate dehydrogenase) expression (as a loading control). Therefore, it was suggested that CSE is the principal enzyme for H₂S synthesis in the blood vessels.

The distribution of CBS and CSE between tissues is varied across different studies. CBS expression in rat liver was about 100-fold higher than rat brain (Erickson et al., 1990; Swaroop et al., 1992). Although CSE was reported to be abundant in the vasculature, Donovan's study found that all three H₂S-synthesising enzymes CBS, CSE, and MST were expressed in PCA (Donovan et al., 2017). They found that CBS plays an important role in the ischaemic hypoxic relaxation response in PCA (Donovan et al., 2017). In another study, CSE was not detected, while CBS and MST were expressed in perivascular adipose tissue (PVAT) around PCA (Donovan et al., 2018). Both CSE and CBS were expressed in rat liver (Fiorucci et al., 2005), human umbilical vein endothelial cells (HUVECs) (Lin et al., 2013) and human embryonic kidney-293 cells (HEK-293 cells) (Dai et al., 2019). MST and CSE are detected in rat microglial cells (Lee et al., 2006). Similarly, only MST and CSE were detected in rat lung tissues (Madden et al., 2012). Although H₂S production in the brain of CBS-knockout (KO) mice was comparable to wild-type mice via MST (Shibuya et al., 2009), CBS-knockout in mice led to death within about one month due to cardiovascular and hepatic pathologies (Watanabe et al., 1995). Moreover, CSE was expressed in human astrocytes and neurons (Ichinole et al., 2005; Lee et al., 2009) and mice astrocytes and glial cells (Enokido et al., 2005). These results might be due to a compensatory increase of MST or CSE expression due to CBS-KO or that CBS may not be the major enzyme for H₂S production in the brain.

It might be challenging to determine if one enzyme only is responsible for endogenous H₂S production in tissue. For example, CBS and CSE were together expressed in the liver and kidney of rats (Stipanuk and Beck, 1982; Teng et al., 2013) and mice (Al-Magableh and Hart, 2011), rat ileum tissues (Hosoki et al., 1997), and HEK-293 cells and rat carotid artery (Telezhkin et al., 2009). In another study, both CSE and MST were expressed in human pulmonary artery endothelial cells, rat liver,

rat aorta, and mice aorta (Kuo et al., 2015). Moreover, there is no selective and potent inhibitor for CBS, CSE and MST in order to confirm or exclude the specific involvement of each enzyme's role in endogenous H₂S production. The use of knockout (KO) animals by genetic manipulation of H₂S-synthesising enzyme could help to determine which one is the most important enzyme in endogenous H₂S production, but knockout of one enzyme may increase the others to compensate.

The localisation, expression and activity of these enzymes CBS, CSE and MST, could be dependent on tissue types, species variation and conditions, such as redox state and stress, such as hypoxia. These factors could lead to a change of production and translocation of CBS and CSE to the mitochondria depending on the cellular environment (Fu et al., 2012; Teng et al., 2013). For example, CBS and CSE are cytosolic enzymes and widely expressed in tissues, such the liver, brain and blood vessels. It has been claimed that CBS and CSE may migrate to the mitochondria under stress states, such as oxidative stress and ischaemia or hypoxia. This observation might be due to alteration of mitochondrial permeability under stress circumstances, such as ischaemia or hypoxia and ovary and colon malignant tumours (Fu et al., 2012; Szabó et al., 2013; Teng et al., 2013; Beltowski, 2015). The purpose of this migration of CBS/or CSE from the cytosol to the mitochondria might be due to a homeostatic response of tissue to stress conditions and that H₂S may play an important role in control of stress conditions and exert cytoprotection via H₂S antioxidant, mitochondrial-protection and prevention of calcium accumulation effects.

Although CBS is a cytosolic enzyme, Teng's work found that CBS proteins are accumulated in the mitochondria of rat liver and human hepatoma Hep3B cells under hypoxia (Teng et al., 2013). Interestingly, with the return of oxygen level to the physiological level, the accumulated CBS was degraded within about 10 min to terminate sustained CBS action and avoid overload of H₂S in the mitochondria (Teng et al., 2013). Another support for this observation, Fu's study reported that CSE proteins were translocated to the mitochondria of vascular smooth muscle cells (SMCs) under stress conditions using stress-inducers, such as A23187, tunicamycin and thapsigargin to increase intracellular calcium levels (Fu et al., 2012). Some pathological conditions can lead to changes in H₂S synthesising enzyme expression or H₂S production levels. For example, CSE and MST remained normal in cells in colon cancer, while CBS is overexpressed (Hellmich and Szabó, 2015).

There are additional factors which may influence endogenous H₂S production, such as tissue type or gender. H₂S generation in rat aorta was higher than rat ileum and portal vein (Hosoki et al., 1997). In another study, Zhao and colleagues reported that H₂S generation in different rat vascular tissues was different; it was highest in the tail artery and lowest in the mesenteric artery, while aorta was in between. (Zhao et al., 2003). They also demonstrated that H₂S generation in the rat liver was higher than vascular tissues and ileum. Similarly, H₂S generation was the highest in mice liver compared to mice aorta and kidney (Al-Magableh and Hart, 2011). H₂S production in the mice heart was higher than aorta (Yang et al., 2008). H₂S production in rat liver was higher than human internal mammary artery (Webb et al., 2008). H₂S level was the highest in the murine kidney, then liver and pancreas and the lowest level was in the lung (Ang et al., 2012). The rate of absorption of applied Na₂S (Sodium sulfide, H₂S salt) in the rat heart and liver tissues was higher than the brain (Ishigami et al., 2009). The reason for this variation is unknown, but it might be due to different stores of H₂S, biological significance and metabolism of H₂S in each tissue.

H₂S endogenous production could also be affected by gender. Eto and Kimura's study in mice reported that H₂S production and CBS activity in mouse brain tissues were higher in males compared to females and also orchidectomy of males resulted in a decrease of H₂S production (Eto and Kimura, 2002). In line with this result, additionally, the concentration of H₂S in the blood of human female participants was lower than male participants (Bucci et al., 2009; Brancaleone et al., 2015). This effect may be due to testosterone augmenting effect on H₂S endogenous production possibly by increasing enzymatic transformation of cysteine to H₂S via stimulation of the enzymatic activity of CBS in mouse brain (Eto and Kimura, 2002), CBS/CSE in rat aorta (Bucci et al., 2009), and CSE in rat aorta (Brancaleone et al., 2015). Moreover, in CSE-deficient mice, hyperhomocysteinemia was about 9 fold greater in females than males (Yang et al., 2008). Therefore, testosterone and oestrogen may have an influence on H₂S production.

CBS, CSE and MST are reported to have different cellular distribution, which may lead to different effects of H₂S in the brain and blood vessels. For example, CBS was present in the neurons (Ishigami et al., 2009), while CSE was present in both neurons and astrocytes (Enokido et al., 2005; Ichinole et al., 2005; Lee et al., 2009). Similarly, MST was present in both neurons and astrocytes (Nagahara et al., 1998). In blood vessels, CSE was detected but not CBS in the endothelium of rat aorta, pulmonary, mesenteric and tail arteries (Zhao et al., 2001). CBS and CSE were widely detected in the smooth muscle and endothelium of PCA, while MST was detected in

the endothelium and some areas of smooth muscle (Donovan et al., 2017). CAT is present in endothelial cells, while MST is present in both endothelial and smooth muscle cells (Shibuya et al., 2009; Kimura, 2011).

1.4. Metabolism of H₂S

There are several metabolic pathways for quick clearance of H₂S, which involve transsulfuration of the sulfur atom or reaction with other molecules (Wang, 2012; Wen et al., 2018). These metabolic pathways of H₂S maintain rapid removal of H₂S from the cells and tissues in order to maintain suitable physiological concentrations. Oxidation and methylation pathways are reported to prevent toxicity of H₂S (Reiffenstein et al., 1992; Tangerman et al., 2009; Whiteman et al., 2011).

1.4.1. Oxidation

Oxidation of H₂S is the primary most important metabolic pathway for H₂S in order to decrease the availability of biologically active H₂S. The oxidation pathway involves the oxidation of sulfur in the mitochondria (desulfurisation) via mitochondrial enzymes, such as sulfide quinone oxidoreductase (SQOR), persulfide dioxygenase, rhodanese (thiosulfate: cyanide sulfurtransferase, TCST), and sulfite oxidase. These enzymes oxidise H₂S to polysulfides, including thiosulfate (S₂O₃²⁻) and sulfite (SO₃²⁻) and then, to sulfate (SO₄²⁻) (Li et al., 2011; Whiteman et al., 2011; Beltowski, 2015; Wen et al., 2018). MST in the mitochondria might metabolise some H₂S to polysulfides in rat brain, and these polysulfides might mediate H₂S signalling effects in the mitochondria of brain (Kimura et al., 2013; Kimura et al., 2015). Therefore, polysulfides might be a signalling mediator or storage form of H₂S (sulfane sulfur), and this could indicate an important role of MST in H₂S synthesis and/or sulfide metabolism and thus, MST might be considered as a key checkpoint for H₂S metabolism. This oxidation of H₂S might also be produced via non-enzymatic reactions, such as by interaction with reactive oxygen species (ROS) (Prieto-Lloret et al., 2018; Wen et al. 2018), and then to sulfite then to thiosulfate and eventually to sulfate (figure 1.3).

Sulfate has been reported as the principal end-product of H₂S metabolism (Kajimura et al., 2010; Shen et al., 2014), although thiosulfate may be another metabolic end-product of H₂S (Beltowski, 2015). In a radio-labelling study using Na₂³⁵S in rats demonstrated a difference in H₂S metabolic products in different tissues (Bartholomew et al., 1980). They found that H₂S is principally metabolised by the liver to sulfate, whereas thiosulfate is mainly formed in the lung and by the kidney to a blend of thiosulfate and sulfate (Bartholomew et al., 1980; Whiteman et al., 2011; Shen et al., 2015). These transformations of H₂S metabolic pathways might

be a focus of future research studies as a cardioprotective signalling mechanism via the formation of different endogenous metabolites (Shen et al., 2015) possibly because some metabolic end products, such as thiosulfate may work as an endogenous store for H₂S, which may be a releasable form of H₂S (Sakaguchi et al., 2014; Snijder et al., 2014). Therefore, the H₂S biological effects might be related to H₂S metabolites in addition to H₂S itself. Therefore, H₂S could be stored in thiosulfate, which is considered as H₂S prodrug. For example, application of thiosulfate leads to attenuation of inflammatory damage in mouse lung (Sakaguchi et al., 2014). Similarly, application of thiosulfate in rats is reported to decrease angiotensin II-provoked hypertension, and renal insult and these effects are comparable to NaHS effects (Snijder et al., 2014).

1.4.2. Methylation

H₂S may be catabolised by the methylation pathway as a detoxification pathway in the cytosol which produces methanethiol and then a less toxic molecule, dimethyl sulfide (DMS) via thiol S-methyltransferase (MST) (Li et al., 2011; Wen et al., 2018) (figure 1.3).

1.4.3. Miscellaneous

Additionally, H₂S can react with haem proteins, such as haemoglobin (Hb), or myoglobin, and cytochrome c. Interaction of H₂S with haemoglobin leads to sulfhemoglobin formation, which likely acts as a metabolic sink for H₂S (Li et al., 2011; Beltowski et al., 2015) (figure 1.3).

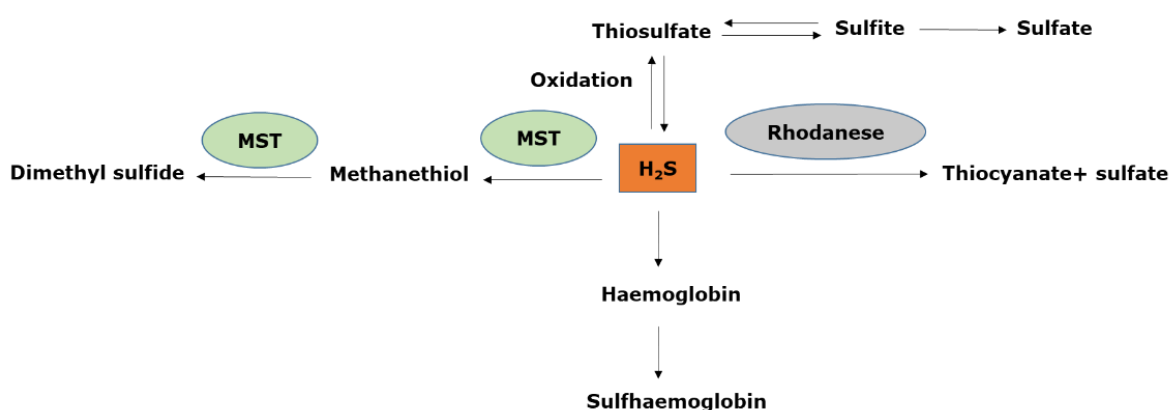


Figure 1.3: Metabolic pathways of H₂S via oxidation, methylation by MST (S-methyl sulfurtransferase), haemoglobin, and rhodanese. Thiosulfate (S₂O₃²⁻), sulfite (SO₃²⁻) and sulfate (SO₄²⁻) (Kolluru et al., 2013; Yu et al., 2014).

1.5. Endogenous H₂S concentrations

Different concentrations of H₂S have been reported in biological fluids of mammals. H₂S concentrations depend on cell or tissue type and location, method of measurement and conditions employed. For example, 20-300 µM in blood was claimed as a range of safety (Olson, 2009; Kashfi and Olson, 2013) using old methods, such as the methylene blue assay and sulfide ion selective electrode (SISE), although about 1 µM H₂S level in tissues and blood was reported to be physiologically relevant using new methods, such as a polarographic H₂S sensor (PHSS) (Whitfield et al., 2008) and the monobromobimane assay (Wintner et al., 2010; Shen et al., 2011).

High H₂S concentration in the serum of patients with obstructive asthma was reported high about 600 µM in serum and sputum of asthmatic patients using a SISE (Saito et al., 2013). Moreover, H₂S concentration in rat pulmonary artery and rat plasma was reported about 301 µM using the methylene blue assay (Chunyo et al., 2003), whereas H₂S concentration in rat, bovine and human brains was reported to be 50-160 µM using the methylene blue assay (Warenycia et al., 1989; Abe and Kimura, 1996; Hosoki et al., 1997; Whiteman et al., 2005; Whiteman and Moore, 2009). In other studies, the measured H₂S concentration in rat and human plasma was about 10-100 µM using the methylene blue assay (Richardson et al., 2000; Zhao et al., 2001; Li et al., 2005; Li et al., 2011; Wen et al., 2018). About 1 µM was measured in human plasma using the gas chromatography (GC) (Polhemus et al., 2014).

On the other hand, Furne's research found that H₂S concentration in mouse brain and liver homogenates were a lot lower, about 14 and 17 nM, respectively using the GC (Furne et al., 2008) and concentrations of around 100 pM were measured in human blood (Tangerman et al., 2009). Presence of a range of reported levels of H₂S might be due to differences in the detection methods, tissues, and/or measuring H₂S or other forms of H₂S, such as thiosulfate, sulfate and sulfane sulfur as an indicator of H₂S production.

It has been suggested that the concentration of H₂S measured in the plasma is lower than the tissue level (Li et al., 2011). This might be due to most of the synthesised H₂S being utilised (autocrine or paracrine) or stored or metabolised at cellular or tissue level, and therefore, the plasma (circulating/systematic) H₂S level in the plasma might be excess from H₂S synthesis at the cellular or tissue level, and it may be produced from different tissues/organs (Li et al., 2011; Wen et al., 2018; Cao et al., 2019). In other words, there would be less H₂S in plasma if much of synthesised H₂S is stored in tissue. Thus, H₂S levels in blood/plasma may be less reliable than H₂S levels in tissues. In line with this observation, Whitfield et al's study found that there was no detectable H₂S in the blood of many species (mouse, rat, pig, cow, lamprey and trout) (Whitfield et al., 2008). Whitfield's research also revealed that the measured H₂S (using Na₂S, sodium sulfide, H₂S salt) quickly disappeared *in vitro* with the addition of 5 % bovine serum albumin (BSA). Moreover, the addition of antioxidant-alkaline buffer leads to release of H₂S from 5 % BSA *in vitro* (Whitfield et al., 2008). Therefore, this result might suggest rapid clearance of H₂S may be via storage in bound forms, such as persulfide (sulfhydrated protein). Therefore, most of the biological H₂S may be stored as a bound form, which may be releasable under specific conditions. Thus, during H₂S measurement, it could be important to detect and consider these stored forms of H₂S, such as sulfane sulfur, which releases stored H₂S under reducing or alkaline conditions, and acid-labile, which releases stored H₂S under acidic condition (Ubuka et al., 2002; Whitfield et al., 2008; Ishigami et al., 2009; Shibuya et al., 2009). These forms could affect the level of H₂S detection and tell us how much H₂S is stored, but not the activity of the enzymes.

There are some issues with H₂S level measurement. For example, there is little agreement about the toxic and physiological levels (Ang et al., 2012). Moreover, the difference between the physiological and toxic levels is narrow. For instance, the toxic level for H₂S in the brain tissue was about double that of the physiologically reported level 50-160 µM (Warenycia et al., 1989).

Overall, the H₂S concentration in the body or tissues is the balance of biosynthesis and oxidation (Olson, 2008; Li et al., 2011; Yu et al., 2014). Several endogenous quantities of H₂S were detected and produced from various vascular tissues with different levels. For instance, Zhao's group found that rat aorta tissues produced more H₂S than rat portal vein tissues (Zhao et al., 2001).

-1.6. Measurement of H₂S

There is an increasing body of evidence that suggests multifaceted biological actions of H₂S within the body. However, H₂S detection and measurement are problematic and challenging because H₂S is unstable and gaseous/volatile in solution. Moreover, H₂S is vulnerable to oxidation and interaction with other molecules (Tangerman et al., 2009; Ang et al., 2012). Furthermore, it is difficult to measure the local concentration of H₂S at the site of synthesis or action. NO is commonly oxidised to nitrite and nitrate as stable metabolic end products of NO. Therefore, the measurement of nitrite and nitrate levels have been commonly used as a biomarker for NO availability (Li et al., 2011; Pikhova et al., 2016). In contrast to NO, H₂S is without specific metabolic end-products that could be measured easily as a biomarker (Li et al., 2011). These properties will lead to difficulties and challenges in the detection of H₂S in biological samples (Shen et al., 2011; Olson et al., 2014).

H₂S measurement in samples (tissues homogenate or plasma) does not necessarily indicate and even may underestimate the actual level of H₂S at the cellular and subcellular levels as active sites of H₂S biosynthesis. For example, it was reported that H₂S level in brain tissue was about three-fold higher than plasma level (Zhao et al., 2001). As such, there is little agreement about the precise measurement of H₂S regarding its active form, concentration-response, and molecular targets (Kolluru et al., 2013) and thus, this dilemma remains a scientific challenge.

Therefore, the availability of a precise, accurate, sensitive, real-time, consistent and reliable method to measure H₂S without disruption of cells or tissue (Kimura, 2011) is vital in order to get sufficient comprehension of H₂S production, level, homeostasis and its biological profile (Tangerman et al., 2009; Li et al., 2011). This may allow exploitation of the potential medical applications of H₂S concentrations in the diagnosis and treatment of some diseases, particularly CVD (Olson et al., 2014). Therefore, development and improvement of available assays for H₂S measurement could be useful to monitor the changes in H₂S concentrations in CVD and after application of H₂S donor drugs (H₂S sources).

Generally, the typical method for H₂S measurement should be specific, sensitive, reliable, cheap/available, simple, consistent, dynamic, and biocompatible. The selection of H₂S measurement method is governed by the facilities available, sensitivity required, complexity of methodology, variability, type of tissues and conditions to be tested.

A typical measurement of H₂S could clarify the toxic and therapeutic level of H₂S at the tissue and cellular level (Whiteman and Moore, 2009; Yu et al., 2014). There are many methods for H₂S measurement. Unfortunately, most of them have advantages and disadvantages and require more refining and optimisation.

1.6.1. Colorimetric assay: The methylene blue assay

The methylene blue assay is the most common and oldest method for H₂S measurement. This assay depends on trapping of free H₂S with zinc to form stable zinc sulfide and followed by reaction with ferric chloride (FeCl₃, oxidising agent) and dimethyl phenylenediamine sulfate (DMPD) under acidic conditions leading to the formation of methylene blue (figure 1.4) (Hughes et al., 2009). The methylene blue is measurable using a spectrophotometer at 670 nm or high-performance liquid chromatography (HPLC) (Whiteman et al., 2011; Sen et al., 2012). The change in methylene blue colour reflects the change in H₂S production and H₂S synthesising enzymes activity (H₂S from L-cysteine via CBS/CSE or H₂S from mercaptopyruvate via MST) (Abe and Kimura, 1996; Hosoki et al., 1997; Zhao et al., 2003; Geng et al., 2004; Yang et al., 2011; Rashid et al., 2013; Candela et al., 2016; Untereiner et al., 2016; Donovan et al., 2017; Donovan et al., 2018).

The aggressive acidic pH of the methylene blue assay could liberate various forms of acid-labile sulfur rather than free H₂S. Therefore, it could lead to erroneously high results. Also, there is a possibility of interaction with coloured materials in the biological samples, which may be similar to sulfide reaction with DMPD and FeCl₃ (Kolluru et al., 2013). Moreover, the H₂S detection range of the methylene blue assay is reported to be from 8 µM (minimum) to 301 µM (maximum) and therefore, it is non-linear with high concentrations of H₂S, such as > 301 µM H₂S (Whiteman and Moore, 2009; Li et al., 2011; Papapetropoulos et al., 2015; Wen et al., 2018). Therefore, it lacks specificity and sensitivity, especially in tissues with low H₂S concentration. Thus, it is unsuitable for detection of low H₂S concentration (< 8 µM H₂S) (Li et al., 2011; Kolluru et al., 2013; Stein and Bailey, 2013; Papapetropoulos et al., 2015). Moreover, the methylene blue assay leads to permanent destruction of the sample and therefore, it is a single-point method and unsuitable for measuring H₂S level in a real-time pattern (Olson et al., 2014).

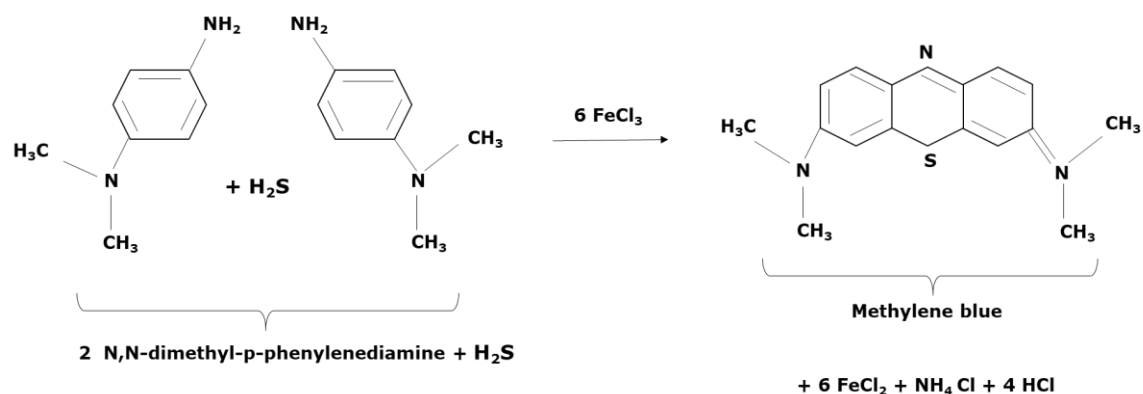


Figure 1.4: The methylene blue assay chemical reactions for the detection of H₂S. The reaction requires one part of H₂S and two parts of the reagent N,N-dimethyl-p-phenylenediamine (DMPD) for stoichiometry in the presence of oxidising agent FeCl₃ (ferric chloride) and acidic pH (Hughes et al., 2009; Shen et al., 2011).

1.6.2. Sulfide ion-selective electrode (SISE)

In this method, a SISE is put into a sulfide ion-containing sample. Also, required is an isolated reference electrode. Both of the SISE and the isolated reference electrode are connected to a millivoltmeter in order to make the electrochemical connection. Sulfide ions diffuse across the sulfide ion-selective membrane of the SISE and cause voltammetric changes and currents. These currents are measured by the millivoltmeter and are proportional to the sulfide ion concentration in the sample. The sulfide concentration is calculated via measuring voltages of the standard sulfide concentrations (Khan et al., 1980; Zhao et al., 2001; Searcy and Peterson, 2004; Madden et al., 2012; Leucker et al., 2017). Then, the sulfide ion concentration potentially represents H₂S concentration (Khan et al., 1980; Searcy and Peterson, 2004; Mustafa et al., 2009; Yu et al., 2016). Using SISE, 1 mM H₂S concentration in organ bath was decreased to about 150 μM after about 30 min (Zhao and Wang, 2002). SISE was also used to measure the changes in H₂S production and concentration in CSE-knockout mice aorta and heart (Yang et al., 2008).

SISE has a detection range from about 34 μM (minimum) to 75 μM (maximum) (Wang, 2012; Wen et al., 2018). Moreover, SISE is sensitive only to sulfide anion (S²⁻), and free H₂S should be fully dissociated. Therefore, SISE requires highly alkaline and antioxidant conditions. Furthermore, the presence of antioxidant may lead to the release of H₂S by desulfhydration of sulfhydrated proteins (Wang, 2012). This method has low reproducibility because it is sensitive to temperature changes, noise and interferences with ions, such as iron and mercury and biological molecules, such as thiols (Nagy et al., 2014; Olson et al., 2014). Moreover, SISE needs a large

volume of sample, frequent cleaning and calibration. Therefore, it is unsuitable for detection of low H₂S levels.

1.6.3. Polarographic H₂S sensor (PHSS)

The polarographic H₂S sensor (PHSS) has three parts, cathode, anode and electrolyte. These three parts are isolated from the sample solution components by a H₂S-permeable membrane. H₂S in the sample solution passes through this membrane and dissociates to HS⁻. Then, HS⁻ reduces ferricyanide to ferrocyanide, which donates electrons to the anode and causes voltammetric (amperometric) changes and currents, which are measured by a millivoltmeter and are proportional to H₂S concentration in the sample (Doeller et al., 2005; Koenitzer et al., 2007; Whitfield et al., 2008; Kuo et al., 2015).

This method measures sulfide ion level, which potentially reflects H₂S level (Doeller et al., 2005; Koenitzer et al., 2007; Al-Magableh and Hart, 2011; Nagy et al., 2014; Kuo et al., 2015; Wen et al., 2008). PHSS has been developed to decrease the interference of SISE with biological molecules, such as thiol (Nagy et al., 2014).

PHSS is sensitive for H₂S at nanomolar concentrations (Whitfield et al., 2008; Olson et al., 2014) in cells and tissues. PHSS has a detection range from about 10 nM (minimum) to 500 μM (maximum) (Doeller et al., 2005). Moreover, PSSH has a real-time property for measuring changes in H₂S levels in biological samples rapidly at the site of synthesis in rat aorta (Koenitzer et al., 2007; Olson et al., 2014). PHSS was useful for measuring H₂S in brain tissue (Hu et al., 2010). Using PHSS to measure H₂S concentrations in organ bath, application of NaHS delivered H₂S in a non-linear manner and 1 mM NaHS led to delivery of about 0.1 mM H₂S in organ bath (Al-Magableh and Hart, 2011). PHSS has been reported to measure about 10 nM H₂S in mammalian tissues, such as heart, liver, brain, aorta, lung and kidney (Doeller et al., 2005, Koenitzer et al., 2007; Whitfield et al., 2008; Mustafa et al., 2009). Furthermore, PHSS could be used to measure sulfide in vertebrate blood or plasma samples (Olson, 2009). Moreover, it could be useful for a large number of samples because it requires a small volume of sample, but this may lead to difficulty in handling of very small samples (Olson et al., 2014). However, PHSS also has disadvantages, such as it needs cleaning and calibration, but less frequent than SISE; it is liable to pressure and temperature changes and interference with reducing agents, such as dithiothreitol (DTT) (Nagy et al., 2014; Olson et al., 2014).

1.6.4. Fluorescent probes

There is a lack of methods that measure H₂S at the cellular level with precise, sensitive, selective and real-time properties (Nagy et al., 2014; Lin et al., 2015; Cao et al., 2019). Recent sensitive methods including fluorescent probes, such as monobromobimane (MBB), combined with gas chromatography (GC) and analyte-filled carbon nanotubes (CNT) have been developed to exclude previous limitations (Olson et al., 2014). Novel methods that measure H₂S at an intracellular level such as fluorescent probes combined with CNT (Wu et al., 2006) in the living system at near-physiological conditions (Lippert et al., 2011; Lin et al., 2013; Lin et al., 2015; Candela et al., 2016; Cao et al., 2019) show relatively promising properties, yet with a need for more optimisation and development (Yu et al., 2014; Wen et al., 2018).

In contrast to previous methods, such as the methylene blue assay and SISE, the fluorescent probes have advantages, such as that they are sensitive to H₂S over biological thiols, such as glutathione and cysteine (Lin et al., 2013; Lin et al., 2015). The experiments of selectivity demonstrated that fluorescent probes, such as SF7-AM (sulfidefluor7-acetoxymethyl ester, azide-based fluorescent probe) have higher reactivity toward H₂S than other biological thiols (Lin et al., 2013; Lin et al., 2015).

Moreover, the detection range of fluorescent probes was reported to be from about 0.5 μM (minimum) to 100 μM (maximum) (Lin et al., 2013; Guo et al., 2015; Wen et al., 2018), and the fluorescent probes could be linear until about 1 mM H₂S concentration (Guo et al., 2015). The fluorescent probes have the possibility of measuring real-time changes in H₂S in living cells and tissues. Also, they do not need extensive steps for sample preparation (no sample destruction), are highly specific, some of them are permeable to most cell membranes, give fast results and a stable signal (Guo et al., 2015; Cao et al., 2019). There are three types of fluorescent probes for H₂S measurement: reduction-dependent, nucleophilic-dependent and metal sulfide-based (Wen et al., 2018).

1.6.4.1. Reduction-dependent fluorescent probes

Sulfidefluor-7 acetoxymethyl ester (SF7-AM), is an enhanced example of the reduction-based fluorescent probe (azide-based). It has been reported to have outstanding cellular trapping ability (cellular selectivity), biocompatibility and good sensitivity for H₂S production in living cells and tissues (Lin et al., 2013; Szczesny et al., 2014; Geró et al., 2016; Lin et al., 2015; Candelaet al., 2016), because the acetoxymethyl ester groups of SF7-AM are specifically cleaved inside the cells via esterases (Lin et al., 2013). The principle of the SF7-AM method is that when SF7-AM (a non-fluorescent azide) reacts with H₂S as a reducing agent, the fluorescent amine product can be quantified by measuring fluorescence and correcting with the tissue blank and expressing as relative fluorescence unit per mg protein (figure 1.5) (Lin et al., 2013; Kuo et al., 2015; Lin et al., 2015).

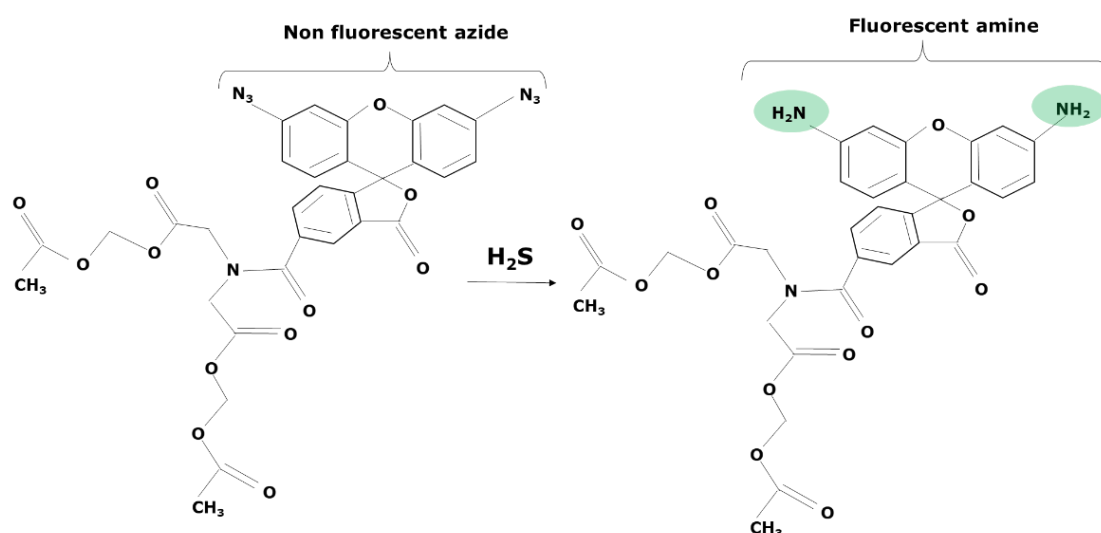


Figure 1.5: The SF7-AM (acetoxymethyl ester) probe reactions for the detection of H₂S. The reaction involves the reduction of a non-fluorescent azide to a fluorescent amine by H₂S acting as a reducing agent (Lin et al., 2013; Lin et al., 2015).

Moreover, some of the fluorescent probes have been developed for targeting particular organelles and addition of various colours. For example, the fluorescent probes, SHS-M1 and SHS-M2 are used for measuring H₂S in the mitochondria through the addition of a mitochondrial-targeted part, triphenylphosphonium (Bae et al., 2013). The probe SHS-M2 changes its colour when contacts H₂S from blue to yellow (Bae et al., 2013).

1.6.4.2. Nucleophilic-dependent fluorescent probes

Nucleophilic-dependent fluorescent probes, in order to detect H₂S, are dependent on the strong hydrophilicity of HS⁻ that is hydrolysed from H₂S under physiological pH conditions (Peng et al., 2014; Wen et al., 2018). This HS⁻ leads to the release of the captured fluorescence by nucleophilic substitution/switch and increase of fluorescence signal (Takano et al., 2017; Wen et al., 2018). These probes have some disadvantages, such as they could interfere with the biological thiols (Winter et al., 2010; Qian et al., 2011; Montoya et al., 2013; Guo et al., 2015). Moreover, these probes require a prolonged time of reaction for H₂S detection about hours (Nagy et al., 2014).

Monobromobimane is an example of a nucleophilic-dependent fluorescent probe (Winter et al., 2010; Cao et al., 2019). In this method, fluorescence HPLC (high-performance liquid chromatography) is used to measure free H₂S and bound H₂S such as sulfane sulfur by using monobromobimane (MBB) (Togawa et al., 1992; Shen et al., 2012; Shen et al., 2015). In non-reducing conditions, this assay has been used to measure free H₂S. In contrast, in the presence of a reducing agent, such as DTT or tris (2-carboxyethyl)phosphine (TCEP) this assay has been used to measure bound H₂S such as sulfane sulfur (reductant-labile sulfur pool/store). First, sulfide reacts with MBB under the alkaline condition to form stable hydrophobic sulfide-dibromobimane, and then sulfide is separated by HPLC and detected by a fluorescence detector attached to the HPLC. This method might be used for measurement of acid-labile sulfide stores under acidic conditions (Nagy et al., 2014). This technique was used to measure H₂S in mouse plasma (Wintner et al., 2010) and rat blood (Shen et al., 2011). This method was reported to measure H₂S in cells, tissues and biological fluids to about 2-5 nM limit, and therefore, it is more sensitive than the methylene blue assay (Shen et al., 2011). In human blood, MBB was more sensitive than PHSS for H₂S measurement (Winter et al., 2010; Olson et al., 2014). However, the MBB method has disadvantages. For instance, it is a lengthy, sophisticated and costly procedure. Moreover, for utmost-results, MBB needs very low-oxygen level and high pH circumstances (Olson et al., 2014). Additionally, it is less convenient for real-time H₂S measurement (Olson et al., 2014) and a large number of samples (Ang et al., 2012).

1.6.4.3. Metal-sulfide based fluorescent probes

Metal sulfide-based fluorescent probes measure H₂S using heavy metals, such as iron and copper to capture the fluorescence. H₂S interacts with iron or copper because of metalophilicity of H₂S, which leads to release of the captured fluorescence and increase of the fluorescence signal (Sasakura et al., 2011; Hou et al., 2012; Guo et al., 2015; Wen et al., 2018). These probes have some disadvantages. For example, thiols could interfere with the signal of these probes (Qian et al., 2011; Sasakura et al., 2011; Montoya et al., 2013; Guo et al., 2015). Also, some of these probes, such as HSip-1 are not permeable through cell membranes (Nagy et al., 2014) and they can only be used for extracellular detection of H₂S (Sasakura et al., 2011).

1.6.5. Carbon Nanotubes (CNT)

Carbon fiber technology such as CNT based H₂S sensors are used for measuring low levels of H₂S at about nanomolar concentrations by adsorption of H₂S in the air or biological solution on the nanotube contact surface (Wu et al., 2006; Zhang et al., 2008; Wen et al., 2018). Some CNT are enhanced with a fluorescent probe (Wu et al., 2006). CNT is relatively selective for H₂S and needs only a small volume of sample and it could be more suitable for measuring H₂S in the air. CNT has disadvantages; for example, it is costly, needs high power, has short-time usability and high-temperature requirement (Tamaki et al., 2000; Zhang et al., 2008).

1.6.6. Gas chromatography

Gas chromatography (GC) is a method described for H₂S measurement (Shibuya et al., 2009; Levitt et al., 2011; Predmore et al., 2012; Kondo et al., 2013). Levitt study found that gas chromatography was useful for the detection of H₂S as free and bound from acid-labile sulfur forms in mouse tissues (Levitt et al., 2011). GC is useful for measuring H₂S in air samples. GC combined with mass-spectrometry technique (GC-MS) was used to measure H₂S in mammalian tissues at about 20-500 μM (Hyšpler et al., 2000; Furne et al., 2008; Kondo et al., 2013). However, GC has disadvantages, such as it needs prolonged times for incubation, it is a sophisticated procedure and requires a large volume of sample (Tangerman et al., 2009). Moreover, GC is less useful for real-time measurement (Nagy et al., 2014; Olson et al., 2014) and a large number of samples (Ang et al., 2012).

1.7. Mechanism of action (signalling pathways) of H₂S

H₂S biological effects are diverse and complex and usually physiological at low concentrations, causing modifications of signalling proteins and ion channels (Li et al., 2011; Paul and Snyder, 2015, Wen et al., 2018). For example, cardiovascular protection is suggested by maintaining endothelial function, ion channel regulation, antioxidation, mitochondrial protection, antiapoptosis, anti-inflammatory and pro-angiogenesis effects (Mancardi et al., 2009; Shen et al., 2014; Kanagy et al., 2017; Wen et al., 2018).

The physiological and pathological effects of H₂S could be due to H₂S or its metabolites (polysulfides and persulfides) via sulfhydrylation, which is a significant post-translational mechanism of action for H₂S similar to phosphorylation and more common than nitrosylation of proteins (Mustafa et al., 2009; Paul and Snyder, 2015). Anti-oxidation is another effect of H₂S (Kimura et al., 2010; Paul and Snyder 2012; Beltowski, 2015). There are several postulated molecular mechanisms for H₂S actions, and most of these mechanisms are overlapped and integrated (figure 1.6).

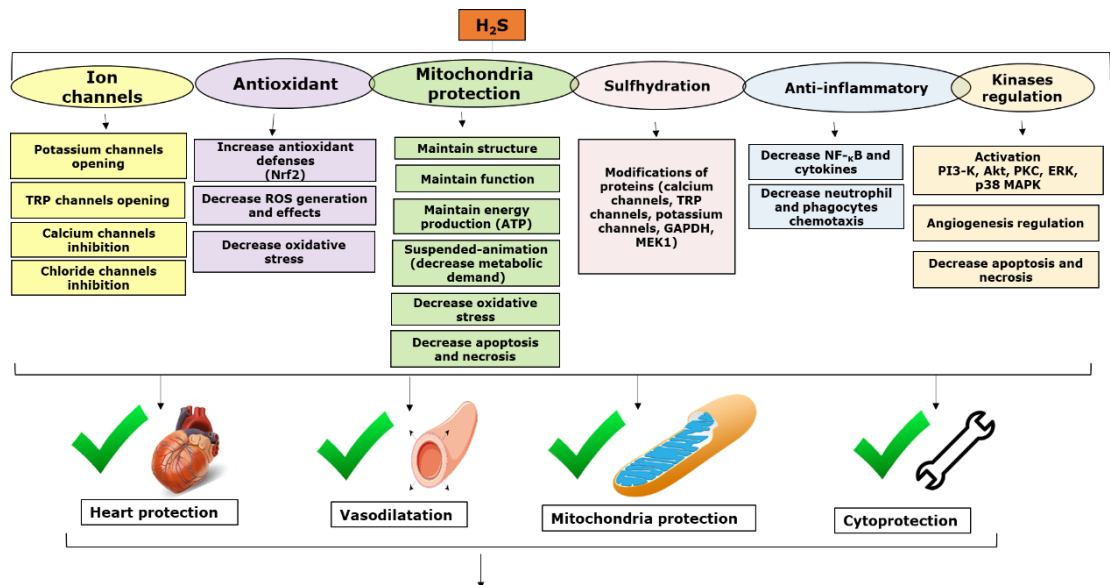


Figure 1.6: Cardiovascular-protective and cytoprotective effects Potential signalling pathways for the cardiovascular-protective and cytoprotective actions of H₂S. TRP channels (transient receptor potential); Nrf2, (nuclear-factor-E2-related factor); GAPDH (glyceraldehyde 3-phosphate dehydrogenase); MEK1 (mitogen-activated extracellular signal-kinase 1); NF- κ B (nuclear factor Kappa Beta); PI3-K (phosphatidylinositol-3kinase), Akt (protein kinase B), PKC (protein kinase C), ERK (extracellular regulated kinase), p38 MAP kinase (mitogen-activated protein kinase) (Szabó et al., 2011; Chan and Wallace, 2013).

1.7.1. H₂S effects on ion channels

1.7.1.1. Potassium channels

H₂S has been claimed to interact with many types of potassium channels, such as ATP-sensitive potassium channels (K_{ATP}) (Zhao et al., 2001; Kubo et al., 2007; Materazzi et al., 2017), calcium-activated potassium channels (BK_{Ca}) (Zhao et al., 2001) and voltage-gated potassium channels (K_v) (Cheang et al., 2010; Hedegaard et al., 2014). The first reported mechanism of H₂S was on K_{ATP} channel. Opening of (K_{ATP}) channels was reported to produce rat aorta smooth muscle relaxation (figure 1.6) (Zhao et al., 2001), which was mimicked by pinacidil (K_{ATP} channel opener). This resulted in a beneficial vasodilatation action during preconditioning (short, repeated, and non-fatal periods of ischaemia or H₂S application before the application of prolonged ischaemia and that may protect the heart against the injury of prolonged ischaemia) to protect rat hearts and atrial and ventricular cardiomyocytes against IRI (ischaemia-reperfusion injury) (Bian et al., 2006; Zhong et al., 2010) and this effect was partially reversed by potassium channel blockers, such as glibenclamide (Kubo et al., 2007; Mancardi et al., 2009).

Similarly, in a study in aortic vascular smooth muscle cells, it was found that H₂S produced membrane hyperpolarisation via K_{ATP} channel opening (Zhao et al., 2001). Therefore, the hyperpolarisation via increase of potassium efflux and decrease of calcium influx may mediate H₂S-induced relaxation (Cheang et al., 2010).

Szabó and his colleagues showed the therapeutic effects of intravenous infusion of NaHS in an experimental model of cardiopulmonary bypass in anaesthetised dogs. They found that the production of H₂S played a beneficial role in myocardial preconditioning and postconditioning responses. Postconditioning is short, repeated, and non-fatal periods of ischaemia or H₂S application after ischaemia and prior the application of prolonged reperfusion may protect the heart against the injury of prolonged reperfusion (Szabó et al., 2011). These responses might help the heart to tolerate prolonged periods of ischaemia and reperfusion injury and might be potentially mediated by H₂S via K_{ATP} channel opening, which might cause hyperpolarisation and decrease calcium influx. Also, this finding might be attributable to maintaining the mitochondrial function, and regulation of cytoprotective and antioxidant factors, such as Nrf2 (figure 1.6).

However, in aorta, K_{ATP} channel opening was reported to play only a partial role in H_2S -mediated relaxation. For example, in mouse aorta, H_2S -mediated relaxation was totally unrelated to K_{ATP} channel opening (Kubo et al., 2007). Large conductance BK_{Ca} channel opening was suggested to be involved in NaHS-mediated relaxation response in rat aorta (Zhao et al., 2001) and rat mesenteric artery (di Villa Bianca et al., 2011; Jackson-Weaver et al., 2011). Furthermore, 0.3 mM NaHS was reported to cause stimulation of BK_{Ca} channels in rat pituitary tumour cells (Sitdikova et al., 2010), while NaHS at 0.1-10 mM concentrations inhibited large conductance BK_{Ca} channels in HEK-293 cells (Human embryonic kidney 293 cells) (Telezhkin et al., 2009). These opposing effects of BK_{Ca} channels may be attributed to differences in H_2S concentration, cell type, species and methodology used in each study.

Moreover, small and intermediate conductance BK_{Ca} channel opening was reported to participate in NaHS-mediated relaxation in rat mesenteric arteries (Cheng et al., 2004), whereas K_v channel opening was reported to participate in H_2S -mediated relaxation response in rat coronary artery (Cheang et al., 2010) and K_v7 channels in porcine coronary artery (Hedegaard et al., 2014). The vasorelaxation effect on mesenteric artery may lead to a decrease of the peripheral vascular resistance (PVR) and result in a decrease in the blood pressure (BP).

1.7.1.2. Transient receptor potential channels

H_2S may also act by the opening of transient receptor potential (TRP) channels (figure 1.6), such as TRPA1 (Ankyrin-1) and TRPV1 (vanilloid-1), which could increase release of some vasodilator neuropeptides, such as calcitonin-gene related peptide (CGRP) or substance P from sensory nerves in smooth muscles of blood vessels, such as mesenteric arteries (Kawasaki et al., 1988). Therefore, it could modulate pain (nociception) and vascular muscle tone (Li et al., 2011; White et al., 2013; Naik et al., 2016; Phan et al., 2020). In 2013, White's study found that NaHS caused smooth muscle relaxation of mesenteric arteries in rats by the opening of TRPA1 channels (White et al., 2013). NaHS led to the activation of TRPV1 channels, contraction and inflammation in guinea pig bronchi via the release of neuropeptide from sensory nerves (Trevisani et al., 2005).

1.7.1.3. Chloride channels

Also, some of H_2S cardio-protective effects, such as control of blood pressure and apoptosis might be attributed to inhibition of chloride channels in rat heart lysosomal vesicles (Malekova et al., 2009). H_2S may cause intracellular acidification via activation of Cl^-/HCO_3^- exchanger in rat aorta smooth muscle cells (Lee et al., 2007), rat aorta (Kiss et al., 2008), and rat glial cells (Lu et al., 2010).

1.7.1.4. Calcium channels

H₂S may cause inhibition of calcium channels (L and T type dihydropyridine voltage-gated calcium channel), and therefore H₂S leads to inhibition of extracellular calcium influx (figure 1.6) (Sun et al., 2008; Al-Magableh and Hart, 2011; Zhang et al., 2012; Tomasova et al., 2015). In 2002, Zhao and Wang study was the first study revealed that H₂S produced direct blockade of calcium channels in rat aorta (Zhao and Wang, 2002). Moreover, it was reported that the relaxation responses of NaHS in mouse and rat aorta was decreased in calcium-free conditions or in the presence of calcium channel blockers, such as nifedipine (Zhao and Wang, 2002; Al-Magableh and Hart, 2011; Zhang et al., 2012; Avanzato et al., 2014). In a similar study, NaHS directly relaxes vascular smooth muscle cells via calcium channel blockade (Dai et al., 2019). NaHS led to inhibition of cardiac functions in isolated rat hearts via calcium channel blockade (Zhang et al., 2012; Mazza et al., 2012). Moreover, NaHS was reported to attenuate excessive stimulation and degeneration of retinal neurons by light via decrease of calcium entry and act as a cytoprotective agent (Mikami et al., 2011).

NaHS also caused inhibition of contraction and had a negative inotropic effect in rat cardiomyocytes and rat hearts by inhibition of L-type calcium channels (Sun et al., 2008; Zhang et al., 2012). Additionally, Xu's work revealed that NaHS reduced calcium influx, which was reversed by BayK8644 (L-type calcium channel agonist) in human atrial fibres (Xu et al., 2011) and also in vascular smooth muscle cells (Dai et al., 2019). In line with these results, Zhang's study found a similar result; they found that NaHS resulted in a suppressive effect on L-type voltage-gated calcium channels currents in rat cardiomyocytes and inhibited cardiac functions in isolated perfused rat hearts (Zhang et al., 2012). In another study, application of NaHS led to relaxation of the myocardial tissue of rats and frogs likely via decrease of intracellular calcium level (Mazza et al., 2012). Moreover, in rat cerebral artery, NaHS caused relaxation via calcium channel blockade but not K_{ATP} channel (Streeter et al., 2012; Tian et al., 2012). AP39 (mitochondria-targeted, H₂S donor) produced a sustained decrease of BP and heart rate via blocking calcium channels in rat hearts (Tomasova et al., 2015).

Application of NaHS in conditions of oxidative stress induced by H₂O₂ leads to decrease of cell death and increase of cell survival via blockade of calcium channels (Avanzato et al., 2014). Therefore, the blockade of calcium channels could participate in H₂S antioxidant effect. In the lung, Na₂S and GYY4137 decreased the intracellular calcium level by attenuation release of calcium from endoplasmic reticulum through InsP₃ (inositol-1, 4, 5 triphosphate) and therefore, it led to smooth muscle relaxation (Castro-Piedras and Perez-Zoghbi, 2013). NaHS application in HEK-293 cells and rat aortic smooth muscle cells resulted in an inhibition of T-type calcium channels using patch-clamp technique (Elies et al., 2014). H₂S might also cause a decrease of the apoptosis and maintenance of the mitochondrial function, permeability integrity, and adjustment of the sarco endoplasmic reticulum calcium-ATPase (SERCA) activity to prevent calcium overload (Polhemus et al., 2014).

Therefore, H₂S may lead to the closure of calcium channels and a decrease in the intracellular calcium concentration, causing a decrease in muscle tone in the blood vessels and heart and decrease of the blood pressure (Yang et al., 2008; Zhang et al., 2015). NaHS application to hepatocyte mitochondria led to inhibition of calcium-activated cytochrome c oxidase leakage from the mitochondria, decreased swelling of mitochondria swelling (Teng et al., 2013).

Increase of intracellular calcium level has been reported to play a pivotal role in IRI (ischaemia- reperfusion injury) because an increase of calcium level leads to leakage of cytochrome c oxidase (complex IV) release from the mitochondria via increase of mitochondrial permeability, swelling of mitochondria, triggering of apoptosis and necrosis (Ott et al., 2002; Boehning et al., 2003). Thus, calcium accumulation may lead to dysfunction of the mitochondria in heart and blood vessels. H₂S decreases intracellular calcium (Zhao and Wang, 2002; Pan et al., 2008; Sun et al., 2008; Al-Magableh and Hart, 2011, Xu et al., 2011, Avanzato et al., 2014). Therefore, a decrease of intracellular calcium level by H₂S may be beneficial to IRI (Morris et al., 1996; Pan et al., 2009) via possibly maintaining calcium homeostasis, preserving mitochondria function and structure, increasing blood flow during ischaemia by relaxation, and decreasing of over-contraction injury during reperfusion.

1.7.2. Antioxidant effects of H₂S

Oxidative stress is an important cause of tissue injury, especially in the heart (Avanzato et al., 2014). H₂S is a reducing agent, and it may act as an antioxidant (figure 1.6) (Geng et al., 2004; Suzuki et al., 2011). These antioxidant effects of H₂S may illustrate some of cytoprotective effects of H₂S in cellular and tissue injury in the heart, blood vessels, liver and kidney (Geng et al., 2004; Li et al., 2011; Suzuki et al., 2011). H₂S decreases oxidative stress by scavenging of ROS (reactive oxygen species) by direct interaction with ROS, such as superoxide and hydrogen peroxide (Geng et al., 2004; Zhuo et al., 2009), neutralisation of ROS (such as increasing in glutathione level by increasing of γ -glutamylcysteine synthetase activity and enhancing of cystine transport) (Kimura et al., 2010) and inhibition of ROS generation (Whiteman et al., 2011; Shen et al., 2015). L-cysteine has been reported a precursor of H₂S, and mice fed with low-cysteine diet have oxidative stress vulnerability (Ishii et al., 2010).

Therefore, the antioxidant action of H₂S decreases DNA breaking, oxidative stress damage, lipid peroxidation and therefore, H₂S repairs endothelial dysfunction. Thus, H₂S acts as a cytoprotective and inflammatory modulator (Szabó et al., 2011; Luo et al., 2014). In line with this idea, the mitochondria-targeted H₂S donor AP39 reduced oxidative stress in murine brain endothelial cells via mitochondria DNA protection and maintenance of cellular energy. Therefore, it could be useful for mitochondria-related pathology (Szczyzny et al., 2014).

Interestingly, in an important *in vivo* study in mice, AP39 (mitochondria-targeted H₂S donor) reduced oxidative stress after cardiac-arrest by antioxidant and mitochondria protective effects, such as decrease of the mitochondrial permeability and ROS production; therefore, AP39 led to cytoprotective effects on mice heart and brain. Moreover, AP39 significantly increased the survival rate after cardiopulmonary resuscitation (Ikeda et al., 2015). Therefore, AP39 protected the heart and led to cardioprotective effects. AP123 and AP39 (mitochondria-targeted H₂S donors) decreased the hyperglycaemia-induced oxidative stress in murine vascular endothelial cells (Geró et al., 2016). The antioxidant effect of AP123 and AP39 was about 100-fold more than Na₂S. AP39 was slightly more effective than AP123 (Geró et al., 2016). The oxidative stress, mitochondrial dysfunction and inflammation play major roles in cardiovascular pathologies. Therefore, these results strongly suggest the cardiovascular protective effects of H₂S via AP123 and AP39, which could be useful to apply in relevant/or similar clinical CVD.

H₂S could activate cytoprotective agents, such as Nrf2 a transcription factor enhancing antioxidant expression at the genetic level, by increasing its localisation in the nucleus in a myocardial ischaemia model in mice hearts (Calvert et al., 2009). Additionally, they demonstrated that Nrf2 mediated cardioprotection activity in mice hearts by pre-treatment with Na₂S, as a preconditioning agent before IRI (Calvert et al., 2009; Calvert et al., 2010). Nrf2 leads to an elevation of CSE expression in rat mesangial cells (Hassan et al., 2012) and mouse hearts (Calvert et al., 2009) and genetic deletion of Nrf2 in mice led to the absence of CSE expression in response to ROS (Hassan et al., 2012). Therefore, H₂S may directly control Nrf2, which might in turn indirectly control H₂S production by enhancing CSE expression likely as a positive feedback loop. Similarly, GYY4137 decreased oxidative stress and inflammation in the aorta of mice with diabetes and atherosclerosis via augmentation of Nrf2 signalling (Xie et al., 2016).

In another study in an *in vitro* and *in vivo* models, AP39 produced antioxidant, anti-inflammatory and renal protective effects by decreasing the oxidative stress and renal damage in rat kidney epithelial cells and renal IRI (Ahmad et al., 2016). Another study demonstrated that administration of NaHS in rats with myocardial damage induced by isoprenaline led to cardioprotective effects by the decrease of oxidative stress, scavenging of ROS and decrease in malondialdehyde (MDA) one end-metabolite of lipid peroxidation which is commonly used as a marker of oxidative stress levels, and improvement of cardiac function (Geng et al., 2004). Moreover, application of NaHS in rat H9c2 cardiomyocytes with H₂O₂-induced oxidative stress leads to an increase of cell viability, survival and decrease of apoptosis (Wu et al., 2015).

Sodha's study found that Na₂S led to an increase in antiapoptosis proteins Bcl-2, proteins of β-cell lymphoma; Bcl-Xl, proteins of β-cell lymphoma-extra-large and decrease in apoptosis-inducing proteins (Bad) in PCA (porcine coronary artery) with IRI (Sodha et al., 2009). Additionally, Na₂S resulted in a beneficial decrease in caspase-3 activity in mice hearts with IRI (Calvert et al., 2009). These results could be attributed to H₂S antioxidant effects, which protect the mitochondria from oxidative stress and preserve mitochondria function and structure.

H₂S could have anti-atherogenic and antithrombotic effects by antioxidant effects, increasing apolipoprotein E like action, decreasing intracellular adhesion molecule-1 (ICAM1), repairing of blood vessels infrastructure and decreasing smooth muscle cell growth, infarct area, calcification, platelet aggregations (such as by a reduction in platelet activating factor, PAF), inflammatory cytokines release, and macrophage accumulation (Shen et al., 2015).

Therefore, H₂S decreases oxidative stress and increases antioxidant defences and therefore, H₂S decreases the heart or blood vessel injury via its antioxidant, antiapoptosis and anti-inflammatory, cardioprotection and cytoprotection actions.

1.7.3. Effects of H₂S on mitochondrial function

The cardioprotective actions of H₂S could be attributed to mitochondrial protection effects (figure 1.6) (Elrod et al., 2007; Susuki et al., 2016; Murphy et al., 2019), especially during ischaemia-reperfusion injury (IRI), including reduce opening of the mitochondrial permeability transition pore (MPTP), decreasing leakage of cytochrome c oxidase complex IV of the electron transport chain from the mitochondria to cytosol, maintaining ATP generation, and decreasing level of apoptosis and necrosis (Karwi et al., 2017) leading to an increase in cardiac repair, lifespan, cellular longevity and survival via increasing of the mesenchymal stem cells (Szczensy et al., 2014; Ikeda et al., 2015; Karwi et al., 2017). Moreover, Na₂S improved mitochondria derangement and increased mitochondrial-rescue in cardiac mitochondria with IRI (Elrod et al., 2007). GYY4137 led to an increase of cell viability, antioxidant and mitochondrial protective effects versus high-glucose levels in H9c2 cells (Wei et al., 2014). In another study, AP39 decreased the oxidative stress, preserved mitochondria infrastructure, MPTP opening, and improved cardiac-survival in rat hearts with IRI (Karwi et al., 2017).

Increase of calcium accumulation, ROS generation and mitochondrial suffering have been reported by many studies as important causes of IRI, especially during reperfusion injury (Shen et al., 2015). Increasing calcium concentration in the mitochondria leads to swelling of the mitochondria, opening of MPTP, the leakage of cytochrome c oxidase, and increasing level of apoptosis and necrosis (Ott et al., 2002; Boehning et al., 2003; Nieuwenhuijs et al., 2006). These effects lead to cardiac injury and application of H₂S can lead to abolishing of these insults and prevention of cardiac injury. For example, in oxidative stress conditions induced by H₂O₂, the application of NaHS leads to decrease of cell death and increase of cell survival via blockade of calcium channels (Avanzato et al., 2014). Therefore, H₂S antioxidant effect may be mediated by blockade of calcium channels, and this effect may lead to a decrease of mitochondrial suffering and dysregulation under oxidative stress and high calcium level circumstances.

H₂S preserves mitochondrial function and ultrastructure (Teng et al., 2013). NaHS application to hepatocyte mitochondria leads to attenuation of calcium-related cytochrome c oxidase leakage from the mitochondria, decreased swelling of mitochondria and apoptosis, and decreased ROS formation in the mitochondria. In rat liver with IRI induced by ischaemia/hypoxia accumulation of CBS in the mitochondria may lead to cytoprotective effects, especially decrease reperfusion injury (Teng et al., 2013). These cytoprotective effects might be due to CBS translocation to the mitochondria, and this translocation leads to increase of H₂S

synthesis in the mitochondria, decrease of calcium-induced cytochrome c oxidase leakage from the mitochondria and ROS production.

H₂S at low concentrations is reported to activate the electron transport of respiratory chain in mitochondria and enhance energy production, whereas H₂S at high concentrations is reported to cause inhibition of the respiratory chain via a competitive and reversible inhibition of cytochrome c oxidase, decrease ATP generation, metabolic inhibition and intracellular acidification (Gubern et al. 2007; Kiss et al., 2007; Módis et al., 2013). ATP is important for muscle contraction, H₂S may lead to decrease of ATP generation via metabolic inhibition and therefore, H₂S leads to muscle relaxation (Kiss et al., 2008). For example, Módis's study reported that about 10-100 µM H₂S stimulated electron transport chain, while about 300-1000 µM suppressed electron transport chain in hepatoma cell line (Módis et al., 2013).

Although H₂S may cause inhibition of the electron transport chain of the mitochondria, Kiss and his co-workers suggested that inhibition of oxidative phosphorylation and decrease of ATP levels by H₂S leads to vasodilation in rat aorta (Kiss et al., 2008) and also in human mesenteric arteries (Materrazi et al., 2017). In line with the same idea, intriguingly, Blackstone's study, found that H₂S may generate fascinating and reversible suspended animation (hibernation-like) phenomenon in mice, a non-hibernating mammal via reversible inhibition of the oxidative phosphorylation and enhancement of mitochondria recovery after reperfusion by reversible inhibition of cytochrome c oxidase (Blackstone et al., 2005; Roth and Nystul, 2005; Blackstone and Roth, 2007; Elrod et al., 2007).

This intriguing phenomenon of suspended animation is characterised by low-oxygen requirement, low-temperature, low heart rate and hypometabolic state during hypoxia-reoxygenation damage. Therefore, this phenomenon may make isolated hearts and cardiomyocytes of mice and rats resistant to lethal hypoxia and allow quick recovery from ischaemia (Blackstone et al., 2005; Roth and Nystul, 2005; Elrod et al., 2007; Szabó et al., 2011). This phenomenon was reported to be useful in mice brain injury and cardiac arrest (Baumgart et al., 2010). This phenomenon induced by H₂S in mice if it could be applicable in human tissues could help during transport of organs and buying extra-time during tissue injury, surgery and organ transplantation as well.

Elrod's study demonstrated that application of Na₂S to mouse hearts with IRI leads to decrease of inflammation, apoptosis and maintenance of mitochondrial function, architecture, and mitochondrial recovery after reperfusion (reoxygenation) (Elrod et al., 2007). Moreover, they reported that H₂S leads to a decrease of the mitochondrial enlargement and enhancement of mitochondrial structure using electron microscopy (Elrod et al., 2007). Therefore, mitochondrial protection could play an important role in H₂S-mediated cardiovascular protection (figure 1.6).

1.7.4. Sulfhydration

Sulfhydration is the process of protein modifications by persulfides formation (-SSH) via interaction of H₂S with cysteine thiols of the targeted protein as PTMs for protein processing (figure 1.6) (Paul and Snyder, 2015; Meng et al., 2017) and it may be similar to phosphorylation. Most of the H₂S-mediated effects are suggested via sulfhydration. Therefore, sulfhydration is a potential signalling pathway for H₂S to control cellular and tissue functions at the molecular level. Nitrosylation of proteins induced by NO commonly results in a decrease of catalytic activity of the proteins, such as GAPDH (glyceraldehyde 3-phosphate dehydrogenase), which is an important enzyme in glucose metabolism and energy production during stress/hypoxia (Hara et al., 2005; Mustafa et al., 2009; Sen et al., 2009; Paul and Snyder, 2012; Meng et al., 2017).

In some proteins, the sulfhydrated protein could be more reactive than the non-sulfhydrated protein leading to the increased biological activity of the sulfhydrated protein, such as GAPDH (Mustafa et al., 2009). Sulfhydration of ATP synthase via NaHS led to an augmentation of energy production in mice livers, HepG2 and HEK-293 cells (Módos et al., 2016). Sulfhydration of Keap1 (Kelch-like ECH-associated protein) by GYY4137 (a slow release H₂S donor) led to a decrease of oxidative stress and inflammation in the aorta of mice with diabetes and atherosclerosis via augmentation of Nrf2 signalling (Xie et al., 2016).

It has been reported that sulfhydration of proteins results in conformational alterations in the targeted proteins, which led to the alteration of protein localisation and activity. For example, sulfhydration of some proteins, such as GAPDH by NaHS may lead to an increase in the enzyme activity of GAPDH by about seven-folds, while sulfhydration of β -actin can lead to polymerisation (Mustafa et al., 2009). Conversely, sulfhydration of some proteins may lead to decreasing of protein activity and closing of ion channels. For example, Zhang's study suggested that sulfhydration is the responsible mechanism for the blockade of calcium channels and negative inotropic effects induced by H₂S, because in rat cardiomyocytes, diamide, oxidant sulfhydryl modifier decreases the availability of free sulfhydryl and therefore, decreases the calcium channel blockade induced by NaHS in rat cardiomyocytes (Zhang et al., 2012). In another study, sulfhydration of phospholamban (PLN) by NaHS led to relaxation of the myocardium of rats and frogs likely via decrease of intracellular calcium level (Mazza et al., 2013). Similarly, sulfhydration may be responsible for NaHS mediated calcium channel blockade in rat vascular smooth muscle cells (Dai et al., 2019).

Similarly, sulfhydrylation of mouse and human platelet proteins via GYY4137 led to an attenuation of platelet activation and thrombogenesis in microvessels (Grambow et al., 2014). Sulfhydrylation of GAPDH and NF- κ B (nuclear factor Kappa enhancer of β -cells) was associated with antiapoptotic, antioxidant and cytoprotective effects (Hara et al., 2005; Filipovic et al., 2015).

Mustafa's study found that about 10-25 % of mice liver proteins were sulfhydrylated and that sulfhydrylation was more dominant than nitrosylation, which represents about 1-3 % (Mustafa et al., 2009). There are many targets for sulfhydrylation such as ion channels, transcription factors, receptors, cellular enzymes and proteins. Examples of these sulfhydrylation-targeted proteins are voltage-gated calcium channels, TRP (transient receptor potential) channels, K_{ATP} (Paul and Snyder, 2012; Moore et al., 2015), NF- κ B (nuclear factor Kappa enhancer of β -cells), MEK1 (mitogen-activated extracellular signal-kinase 1), PKG (protein kinase G), ATP synthase, GAPDH, LDHA (lactate dehydrogenase a), Keap1 (Kelch-like ECH-associated protein1), PLN (phospholamban), SP1, β -tubulin, parkin and actin (Paul and Snyder, 2015; Meng et al., 2017; Sen, 2017).

Therefore, sulfhydrylated proteins would be found in the tissues and they could be considered as a bound form (store) of H_2S , which may be releasable under specific conditions. For example, the presence of reducing agents, such as DTT (dithiothreitol) *in vitro* leads to a decrease or disappearance of sulfhydrylation (Mustafa et al., 2009). In another study in rat cardiomyocytes, the suppressive effect of NaHS on L-type voltage-gated calcium channel currents was reversed by DTT (Zhang et al., 2012). Wedmann's research found that endogenous reductants, such as thioredoxin may be vital in the regulation of endogenous sulfhydrylation level (Wedmann et al., 2016). Thus, it could be important to detect and consider these forms of H_2S during H_2S measurement.

Measurement of sulfhydrylation is not simple. There are several methods for measuring sulfhydrylation. For example, the first and old method is the biotin-switch assay (BSA), which was used by Mustafa's group (Mustafa et al., 2009). In the BSA, the sulfhydrylated protein is labelled with the biotin-HPDP (N-[6-(biotinamido) hexyl]-3-(2-pyridyldithio) propionamide) and next biotin-labelled protein is then extracted (Mustafa et al., 2009). Recently, Sen and his co-workers suggested another assay to measure sulfhydrylated proteins called the red maleimide assay (RMA) by blocking sulfhydrylated protein with red maleimide and then on exposure to DTT leads to a decrease in the fluorescence signal, which indicates the presence of sulfhydrylation (Sen et al., 2012; Mir et al., 2014; Sen, 2017). The RMA showed promising properties

and selectivity over the biotin switch assay, because, in the RMA, it is impossible to detect nitrosylated, oxidised, and disulfide-associated proteins (Sen et al., 2012; Mir et al., 2014; Sen et al., 2017).

1.7.5. H₂S as an oxygen sensor/transducer

Olson reported that there was an inverse relationship between H₂S level and oxygen tension (Madden et al., 2012; Olson, 2013). Therefore, a decrease in oxygen level leads to a decrease in H₂S oxidation and increase in H₂S availability, which may increase the H₂S effect (Olson, 2008). Therefore, the availability of H₂S is the net of H₂S generation minus H₂S oxidation (Olson, 2013). Therefore, the decrease in oxygen level leads to a decrease in H₂S oxidation and increase in H₂S availability, which may increase the H₂S relaxing effect (Olson, 2008). In another study, the hypoxic response in PCA was similar to Na₂S and NaHS response, which was consisted of short (transient) contraction and prolonged (extended) relaxation (Donovan et al., 2017). Therefore, removal of oxygen is suggested to cause relaxation of coronary artery segments and this relaxation response could be mediated via H₂S, at least in part.

In rat aorta pre-contracted with phenylephrine, low H₂S levels (about 5-100 µM) led to a contraction response at high oxygen level (about 200 µM), while a relaxation response was seen at high H₂S levels (about 200-400 µM). Interestingly, in another study using a low oxygen level (about 40 µM) leads to the presence of a relaxation response only (Koenitzer et al., 2007). Therefore, level of oxygen can change the nature of H₂S response, which might be by an increase of the availability of H₂S, or that products of H₂S oxidation at high oxygen level might have a contracting effect to control vascular tone (Koenitzer et al., 2007; Kiss et al., 2008). In line with this idea, Wills's study reported that metabisulfite, which is an oxidation product of H₂S, could lead to vasoconstriction in rat aorta (Wills et al., 1989). Therefore, H₂S could sense the alterations in oxygen level and change its response. Similarly, in rat aorta, H₂S production at low oxygen level is higher than H₂S production at high oxygen level (Doeller et al., 2005). Additionally, there was an enhancement of the relaxation response to IK-1001 (H₂S donor) in rat aorta under low oxygen level conditions (Kiss et al., 2008). In rat lung homogenates and pulmonary arteries, H₂S levels were clearly decreased after the addition of air-gas to the hypoxia-chamber (Madden et al., 2012). H₂S production, particularly via CSE was increased by about 2-fold in the carotid body of mice and rats after hypoxia and mediate the ventilatory response to hypoxia (Peng et al., 2010).

In lamprey aorta, bovine pulmonary artery, rat aorta and pulmonary artery, H₂S was reported to increase the hypoxic response, while H₂S-synthesising enzyme inhibitors decrease the hypoxia response (Olson, 2006). Additionally, they reported that H₂S generation was increased in hypoxia and decreased in hyperoxia (Olson, 2006). Moreover, CBS was reported to play a pivotal role in hypoxia response and AOAA (aminoxyacetic acid, CBS inhibitor) decreased hypoxia response in PCA (Donovan et al., 2017). In porcine myocardium, the H₂S level under hypoxic condition (95 % N₂, 5 % CO₂) is reported to be more than hyperoxic condition (95 % O₂, 5 % CO₂) (Donovan et al., 2018). Application of hypoxia to rat liver led to translocation of CBS from the cytosol to the mitochondria and this translocation led to increase of H₂S production in the mitochondria, antioxidant and mitochondria protective effects (Teng et al., 2013). Similarly, hypoxia in vascular smooth muscle cells led to accumulation of CSE in the mitochondria and maintenance of ATP generation, which was inhibited by PPG (CSE inhibitor) (Fu et al., 2012). Hypoxia led to an increase of H₂S signalling, VEGF (vascular endothelial growth factor) and HIF-1 α (hypoxia inducible factor-1) activity and this led to beneficial pro-angiogenic effects during hypoxia in rat vascular smooth muscle cells (Liu et al., 2010). Hypoxia leads to an increase of H₂S-synthesising enzyme/s expression in about 4 h in human embryonic kidney 293 cells (HEK 293 cells) and hepatocytes (Wang et al., 2014). Therefore, short periods of hypoxia, such as 30 min could be not enough to increase the expression level of H₂S synthesising enzyme/s, but this short period of hypoxia may lead to increase the activity of the available H₂S synthesising enzyme/s, or oxygen levels might also influence stores of H₂S. Therefore, H₂S could participate in tissue response to hypoxia. Thus, hypoxia could lead to increase of H₂S generation/accumulation in tissue and enhancement of H₂S-mediated effects. Therefore, hypoxia could lead to increase of H₂S levels and this could lead to an increase in the blood flow to the ischaemic area (Olson et al., 2008).

1.7.6. H₂S as an inflammatory modulator

The oxidative stress and inflammation are reported to cause detrimental contributions in IRI. Therefore, antioxidant and anti-inflammatory effects of H₂S could be important in decreasing IRI (Shen et al., 2015). Effects of H₂S on inflammation is conflicting. It has been reported that high concentrations of H₂S can lead to pro-inflammatory actions in some cases, such as hemorrhagic and septic shock in Humans, rats and mice (Li et al., 2005). Therefore, in inflammation associated with H₂S level the use of H₂S-synthesising enzyme inhibitors might be a new therapeutic option (Mok et al. 2004; Zanardo et al., 2006; Zhu et al., 2010; Whiteman et al., 2011). It has been reported that high concentrations of inorganic salts, such as NaHS produce pro-inflammatory effects in mouse macrophages (Whiteman et al., 2010).

On the contrary, low concentrations of H₂S could lead to increased cytoprotective and anti-inflammatory effects by a decrease of the immunity responsiveness by decreasing neutrophil and phagocytes chemotaxis, cytokine release, decrease in NF- κ B translocation to the nucleus, apoptosis, necrosis and decrease of TNF- α (figure 1.6). NaHS was reported to decrease the LPS induced inflammation by a decrease of NF- κ B in cultured macrophages (Oh et al., 2006) and microglia (Hu et al., 2007). Sodium thiosulfate (H₂S source) led to anti-inflammatory effects in patients with COVID-19 infection and pneumonia infection by decrease of interleukin-6, TNF- α , and ROS levels (Evgen'ev et al., 2020). Similarly, NaHS reduced inflammation in porcine hearts with IRI (Sodha et al., 2009). A similar result was reported by Li's group. They revealed that GYY4137 use led to decrease of inflammatory mediators, such as TNF- α and NF-Kb and decrease of liver and kidney malfunctions and therefore, GYY4137 protected rats with LPS-induced endotoxic shock (Li et al., 2009). AP39 led to anti-inflammatory effects by lowering interleukin-12 levels in a dose-dependent manner in rat kidney with IRI (Ahmad et al., 2016)

In mouse macrophages, application of LPS to induce inflammation may lead to an increase in CSE expression (Zhu et al., 2010). This increase of CSE expression might be beneficial to produce H₂S anti-inflammatory effect. Moreover, GYY4137 led to attenuation of LPS-induced inflammation in mouse macrophages (Whiteman et al., 2010; Sen et al., 2012). Whiteman's study suggested that H₂S effects on inflammation are complex and they are dependent on H₂S levels and the rate of H₂S release (Whiteman et al., 2010). So the time frame of H₂S release is important.

1.7.7. Effects of H₂S on angiogenesis and kinases

Matrix metalloproteinases are important enzymes involved in cell division and differentiation and proliferation, and tissue remodelling. Angiogenic actions of H₂S are reported to be attenuated at high H₂S concentrations possibly via decrease of VEGF (vascular endothelial growth factor) and activation of matrix metalloproteinase 9, which has antiangiogenic effects. (Cai et al., 2007).

Conversely, angiogenic actions of H₂S are reported to be at low H₂S concentrations. Thus, low H₂S concentration can cause an increase in angiogenesis by improving endothelium repair and increasing release of VEGF (vascular endothelial growth factor) in HUVECs (human umbilical vein endothelial cells) and decreasing anti-angiogenic proteins (Papapetropoulos et al., 2009; Liu et al., 2010). Under hypoxic condition, H₂S application resulted in an increase of VEGF and HIF-1 α (hypoxia inducible factor-1) expression/activity in rat vascular smooth muscle cells and this led to pro-angiogenic actions during hypoxia (Liu et al., 2010). Additionally, H₂S may affect proliferation, apoptosis, vascular remodelling, growth of smooth muscle and decrease of IRI in the heart and blood vessels, such as cells of the aorta in human and rats (Zhao and Wang, 2002; Zhao et al., 2008). For example, it was reported that the administration of Na₂S in mice with heart failure led to an enhancement of the proangiogenic elements, such as matrix metalloproteinase 2, VEGF and activation of kinases, such as PI3-K (phosphatidylinositol-3kinase), Akt (protein kinase B) and these effects led to increase of the cardiac repair and cardioprotection via increase of angiogenesis (Givvimani et al., 2013). Moreover, GYY4137 led to pro-angiogenesis and improvement of vascular remodelling in mouse mesenteric arteries in obese mice via increase of MMP2 synthesis, which resulted in cardiovascular protective effects (Candela et al., 2016).

These pro-angiogenic effects of H₂S may be mediated by activation of kinases, such as PI3-K (phosphatidylinositol-3kinase), Akt (protein kinase B), PKC (protein kinase C), ERK (extracellular regulated kinase) and p38 MAP kinase (mitogen-activated protein kinase) (figure 1.6) (Zhao and Wang, 2002; Cai et al., 2007; Hu et al., 2008; Papapetropoulos et al., 2009; Li et al., 2011; Dai et al., 2019). For example, administration of GYY4137 (slow-release H₂S source) at reperfusion led to decreased myocardial damage of rat hearts with IRI via augmentation of PI3-K/Akt (Karwi et al., 2016). Similarly, application of GYY4137 leads to attenuation hyperglycemic injury in rat cardiomyocytes via stimulation of AMPK (Wei et al., 2014). GYY4137 led to a recovery of the cardiac functions after acute myocardial infarction in rats via pro-angiogenic and antiapoptotic effects (Lilyanna et al., 2016).

These effects could be advantageous for tissue repair; however, it might be detrimental in patients with ovarian and colon cancer (Wang, 2012; Hellmich and Szabó, 2015). For example, CBS was overexpressed in cells in colon cancer (Hellmich and Szabó, 2015), while CSE and MST remained normal. This overexpression of CBS in cancer leads to overproduction of H₂S, and this potentially leads to inflammation, enhancing of mitochondrial energy production via supporting ATP production and maintaining the mitochondrial function, decrease of ROS, activation of kinases, such as PI3-K and Akt, increase of cancer cell multiplication and angiogenesis. Thus, this excessive production of H₂S via overexpression of CBS increases tumour cell energy production, tumour growth and metastasis (Bhattacharyya et al., 2013; Szabó et al., 2013; Szabó and Hellmich, 2013). Therefore, the use of a CBS inhibitor may be a beneficial approach to regulation of cellular energy, and attenuation of tumour growth and metastasis (Bhattacharyya et al., 2013; Szabó et al., 2013; Szabó and Hellmich, 2013; Hellmich and Szabó, 2015).

Although there is inconclusive data about H₂S action on kinases, generally the reported effect of H₂S on kinases is activation (Zhao and Wang, 2002; Mancardi et al., 2009). These variations in H₂S results in studies perhaps due to cells type, condition and H₂S concentration and source used in the study (Li et al., 2011; Wang; 2012).

1.7.8. Miscellaneous effects of H₂S

Yong et al.'s study showed that the use of NaHS in isolated perfused hearts led to attenuation of arrhythmia via various mechanisms, such as a decrease in the activity of the β_1 adrenoceptor through inhibition of adenylyl cyclase and subsequent decrease of cAMP level and maintenance of cardiomyocytes function and survival (Yong et al., 2008).

High concentrations of H₂S may be associated with inhibition of long-term potentiation (LTP, a synaptic model of memory) and Down's syndrome and elevated expression level of CBS (Kamoun et al., 2003). Overproduction of H₂S may impair insulin release and results in diabetes and diabetic cardiomyopathy (Yusuf et al., 2005; Shen et al., 2015).

In contrast, it has been suggested that low concentrations of H₂S (physiological) act as a neuromodulator in the central nervous system (CNS) and could lead to increase in long-term potentiation via activation of N-methyl-D-aspartate (NMDA) receptors, neurotransmission, memory and neuroprotection as an enhancer of glutathione and attenuating glutamate (glutamate is an excitatory neurotransmitter) and ROS levels (Abe and Kimura, 1996). Hence, these previous actions led to beneficial effects on LTP, learning and memory in patients with Alzheimer's disease (Abe and Kimura, 1996; Kimura, 2002; Kimura and Kimura, 2004; Enokido et al., 2005).

1.7.9. Interaction of H₂S with NO

Nitrosylation is a postulated mechanism of action for NO. The interaction between NO and H₂S is complex because it might be synergistic or antagonistic (Zhao and Wang, 2002; Nagpure and Bian, 2016). Moreover, NO could react with H₂S, or free sulfhydryl moiety of reactive cysteine residues of proteins, such as GAPDH and this may lead to the formation of nitrosothiol compounds including thionitrous acid (HSNO), nitroxyl (HNO) and nitrosopersulfide (SSNO) (Beltowski, 2015; Paul and Snyder, 2015). These nitrosothiols might release NO and activate cyclic GMP pathway (Bucci et al., 2010; Paul and Snyder, 2012). However, the interaction between nitric oxide and H₂S may be antagonistic by the formation of inactive active nitrosothiol molecule (Ali et al., 2006; Whiteman et al., 2006). Similarly, the interaction of NO and H₂S led to contraction responses by a direct inhibition of NO synthase activity in rat aorta (Geng et al., 2007), rat aorta and human umbilical vein endothelial cells (HUVECs) (Geng et al., 2007), and human internal mammary artery (Webb et al., 2008).

1.8. The biological significance of H₂S in heart and vascular diseases

H₂S at physiologically relevant concentrations could play important physiological roles. Because there is accumulated evidence that indicates the importance of H₂S to maintain the basal cardiovascular functions and produce cardiovascular protective effects. Therefore, physiological concentrations of H₂S have important physiological impacts, especially in the cardiovascular system, such as control of vascular tone, blood pressure, cardiovascular tissues integrity, antioxidant defences, smooth muscle proliferation. Thus, this evidence emphasise the importance of the H₂S pathway in cardiac and vascular physiology as demonstrated in the following examples:

First, H₂S could control the vascular tone via vasorelaxing effect or via interaction with NO. The interaction of H₂S with NO is complex and it might be synergistic and lead to vasodilatation by activation of NO synthesis and inhibition of cyclic GMP degradation by inhibition of phosphodiesterase in rat aorta (Bucci et al., 2010). Moreover, in rat aorta, it was reported that endothelial-denudation and inhibition of nitric oxide synthase using L-NAME led to a decrease in NaHS relaxation response (Zhao et al., 2001). Additionally, co-administration of GYY4137 with L-NAME led to decrease of cardioprotective effects of GYY4137 in rat hearts with IRI (Karwi et al., 2016).

However, the interaction of H₂S with NO could be inhibitory and results in vasoconstriction and increase of blood pressure (BP) through the formation of inactive nitrosothiol (Ali et al., 2006; Whiteman et al., 2006). A similar result was observed in mouse and rat aorta (Kubo et al., 2007) and human internal mammary artery (Webb et al., 2008). This vasoconstriction may be a biological mechanism to prevent a continuous vasodilatation effect of NO via controlling the availability of NO to control vascular tone (Ali et al., 2006). Furthermore, nitrosylation of proteins by NO is suggested to decrease protein activity (Hara et al., 2005; Sen et al., 2009; Paul and Snyder, 2012). Another example, nitrosylation of CBS/CSE by NO led to decreased activity of recombinant human CBS (Taoka and Banerjee, 2001), rats CBS (Prathapasinghe et al., 2008), and CSE *in vitro* (Mustafa et al., 2011). H₂S led to inhibition of bovine recombinant NO synthase activity (Kubo et al., 2007). Additionally, co-administration of AP39 with the nitric oxide synthase inhibitor L-NAME led to augmentation of the hypotensive effect of AP39 and decrease of heart rate in rat hearts (Tomasova et al., 2014). These results could suggest a physiological antagonism between H₂S and NO.

Generally, the concentration of H₂S donors (sources), such as NaHS needed for vasodilatation was commonly reported to be high (Zhao et al., 2001; Zhao et al., 2002; Fiorucci et al., 2005; Ali et al., 2006; di Villa Bianca et al., 2011). For example, H₂S levels 10-50/kg μM was required to produce a decrease in BP in an *in vivo* model in rats, while high H₂S levels more than 100 μM were reported to produce relaxation of blood vessels and also a decrease in BP and heart rate (Zhao et al., 2001; Zhao et al., 2003; Fiorucci et al., 2005; Ali et al., 2006; di Villa Bianca et al., 2011; Li et al., 2011; Whiteman et al., 2011; Wen et al., 2018). H₂S is important for vascular smooth muscle cells differentiation (Yang et al., 2011). H₂S was reported to inhibit human platelet aggregation *in vitro* in a concentration dependent pattern (Zagli et al., 2007). Similarly, GYY4137 led to a decrease of platelet activation and thrombogenesis in human and mice microvessels (Grambow et al., 2014). Therefore, H₂S could be important in cardiac and vascular physiology and attenuation of thrombosis.

Second, H₂S could maintain the heart and blood vessels integrity and infrastructure of mitochondrial via acting as an electron donor for the respiratory chain, maintaining of energy production (Gubern et al. 2007; Módis et al., 2013; Ikeda et al., 2015; Geró et al., 2016), and improving endothelium function and antioxidant defences and decreasing lipid peroxidation (Candela et al., 2016). Therefore, these effects led to cardiovascular protective actions.

Third, H₂S may decrease the inflammation and lead to cardiovascular protective effects (Li et al., 2009; Sodha et al., 2009; Whiteman et al., 2010; Shen et al., 2015).

Fourth, H₂S could act as an oxygen sensor/transducer, because H₂S could mediate tissue response to hypoxia and activation of ventilation in mice and rats (Peng et al., 2010). Hypoxia leads to increase the expression/activity of the H₂S synthesising enzyme/s and enhancement of H₂S-mediated effects (Wang et al., 2014).

Fifth, application of L-cysteine (H₂S precursor) led to relaxation of rat mesentery artery and this relaxation was inhibited PPG by and this indicates the potential physiological importance of H₂S in control of BP (Cheng et al., 2004).

Sixth, physiological concentrations of H₂S are important for the cardiovascular functions and production of cardiovascular protective effects. Therefore, very low concentrations of H₂S have been reported to be associated with many pathophysiological impacts, especially in the cardiovascular system. Therefore, H₂S could be contributed to cardiac and vascular pathology as demonstrated in the following examples:

Firstly, altered (disturbance) levels of H₂S and homocysteine are commonly linked with cardiovascular diseases. For instance, a genetic mutation of CBS in human led to low H₂S level and hyperhomocysteinemia, which led to oxidative stress and atherosclerosis and cardiovascular pathologies (Polhemus et al., 2014; Shen et al., 2015; McCully, 2015). H₂S levels in plasma has been reported by many studies to be very low in patients with coronary artery disorders, myocardial infarction and heart failure compared to age-matched controls (Jiang et al., 2005; Kovačić et al., 2012; Polhemus et al., 2014). In the thoracic artery of SHR (spontaneously hypertensive rats) H₂S levels were very low, and administration of NaHS led to decrease of blood pressure (Yan et al., 2004; Zhao et al., 2008). Similarly, H₂S level and CSE expression were markedly lower in patients with cardiovascular diseases (CVDs) compared to controls (Jiang et al., 2005) and in mice with a chronic heart failure (Sen et al., 2008). Moreover, H₂S generation was reported to be very low and associated with more atherosclerosis progression in obese mice compared to non-obese mice (Candela et al., 2016; Leucker et al., 2017). Low production of H₂S in vasculature has reported to be associated with vascular pathologies, such as hypertension (Zhong et al., 2003; Yan et al., 2004; Yang et al., 2008), impairment of endothelium function (Mustafa et al., 2011), hyper-proliferation of smooth muscle cells and atherogenesis (Mani et al., 2013), and oxidative stress and atherosclerosis risk (Yang et al., 2010; Leucker et al., 2017). Administration of isoproterenol to produce infarction-like condition in rat hearts led to a decrease of CSE activity to about 80 % and H₂S levels in the myocardial tissue and plasma to about 40 % of controls (Geng et al., 2004). Therefore, they suggested that down regulation of H₂S pathways may lead to cardiac pathology. Treatment with H₂S donors (sources), such as NaHS resulted in beneficial effects on these cardiovascular pathologies. Nonetheless, whether cardiovascular disease is the result or a cause, remains less clear. Generally, the use of H₂S-synthesising enzyme inhibitors led to worsening of these cardiovascular pathologies, while the use of H₂S sources led to beneficial results and improvement of these cardiovascular pathologies.

Secondly, a decrease of the blood supply to tissues leads to tissue injury or dysfunction (ischaemia)/hypoxia, the application of exogenous H₂S using H₂S sources (H₂S salts and donors) in ischaemic heart conditions can lead to beneficial and anti-ischaemic effects (Polhemus et al., 2014; Shen et al., 2015; Yu et al., 2014; Li et al., 2018; Wen et al., 2018). Moreover, the application of NaHS in rats with induced heart failure leads to an enhancement of the left ventricular function (Pan et al., 2009). Furthermore, application of Na₂S in pigs with myocardial IRI results in a decrease of the infarct size and enhancement of contractility (Sodha et al., 2009). Additionally, the application of Na₂S in mice with myocardial IRI also results in a

decrease of the infarction size and enhancement of the left ventricular function (Calvert et al., 2010). GYY4137 led to a recovery of the cardiac functions after acute myocardial infarction in rats (Lilyanna et al., 2016). Similarly, GYY4137 decreased the cardiovascular remodelling in obese mice (Candela et al., 2016). Therefore, H₂S could mediate the cardiac and vascular protective effects against hypoxic/ischaemic injury (Blackstone et al., 2005; Olson et al., 2008).

Thirdly, it has been suggested that H₂S reduces angiotensin II levels, which results in a decrease of bradykinin degradation and an increase of bradykinin level and a decrease of blood pressure (BP) and this effect could indicate the importance of H₂S to maintain physiological BP (Ahmad et al. 2015; Li et al., 2018). To support this effect, NaHS inhibited angiotensin II effects and this led to decreased endothelial dysfunction, decreased the proliferation of vascular smooth muscle, attenuated collagen formation, decreased hypertrophy and remodelling of the myocardium, attenuated binding of angiotensin II to angiotensin II type1 receptor, and decreased hypertension in spontaneously hypertensive rats (SHR) (Zhao et al., 2008). Similarly, in mice with high angiotensin II levels NaHS decreased blood pressure and oxidative stress, while use of H₂S synthesising enzyme inhibitors, such as PPG led to worsening of blood pressure and oxidative stress (Al-Magableh et al., 2015). NaHS also attenuated the activity of angiotensin converting enzyme (ACE) in a dose-dependent pattern in human umbilical vein endothelial cells (HUVECs) and umbilical veins *ex vivo* may be via H₂S chelating with Zinc at the active part of angiotensin converting enzyme (Laggner et al., 2007). Moreover, NaHS attenuated the development of heart failure in rats after administration of isoproterenol via decrease of the release of renin from mast cells (Liu et al., 2014). Additionally, the administration of sodium thiosulfate (H₂S source, STS, Na₂S₂O₃) led to decrease of angiotensin II induced hypertension, angiotensin II induced heart failure, proteinuria and renal injury in rats, and then STS increased the cardiovascular and renal protection (Snijder et al., 2014; Snijder et al., 2015).

Many cardiovascular diseases, such as hypertension, ischaemic heart diseases, and heart failure were reported to be associated with very low H₂S levels. Whether cardiovascular diseases is the result or cause of very low H₂S level remains less clear although cardiovascular diseases may be the result of very low H₂S level, because the use of H₂S-synthesising enzyme inhibitors led to deleterious effects, while the administration of H₂S sources in angiotensin II mediated cardiovascular pathologies led to beneficial results.

Additionally, H₂S was suggested to produce a relaxation response in mice smooth muscle by activation of myosin-light chain phosphatase (Dhaese et al., 2010). H₂S was reported to decrease oxidative stress and repair endothelial dysfunction, which may lead to an increase in intracellular NO synthase activity and then it leads to an increase in NO synthesis and relaxation of smooth muscles of blood vessels, bronchi, uterus, urinary bladder, corpus cavernosum and gastrointestinal system (Zhao et al., 2001; Dunn et al., 2015).

Fourthly, the application of H₂S synthesising enzyme inhibitors to decrease endogenous H₂S level and define the contribution of each of H₂S-synthesising enzymes in ischaemia and IRI led to deleterious effects, while application of H₂S sources led to disappearance of these deleterious effects (Webb et al., 2008; Li et al., 2011; Papapetropoulos et al., 2015; Wen et al., 2018).

Fifthly, knockout study of H₂S synthesising enzymes, in particular, CBS in mice led to death within a month due to cardiovascular damage (Watanabe et al., 1995), while CSE-KO (knockout) mice are associated with low H₂S level, hypertension, reduced endothelial-dependent vasodilatation and high peripheral vascular resistance. Interestingly, administration of H₂S to these mice restores normal blood pressure (Yang et al., 2008; Mustafa et al., 2011). The approaches to studying H₂S biology via application of H₂S synthesising enzyme inhibitor/s and knockout (genetic modifications) studies will be discussed in more details next.

Generally, an overview of the literature shows the cardiovascular protective actions of different types of H₂S sources, such as NaHS, Na₂S, GYY4137 and AP39 in cultured cells, isolated perfused hearts, mesenteric artery beds, aorta rings of various rodent and animal models of hypertension, myocardial ischaemia and heart failure (Mancardi et al., 2009; Szabó et al., 2011; Polhemus et al., 2014; Shen et al., 2015; Yu et al., 2014; Li et al., 2018, Zhang et al., 2018). Therefore, in the heart and vasculature, there is accumulating evidence about the potential contribution of H₂S pathways in the physiology and treatment of cardiovascular diseases (CVD).

1.9. Approaches to studying H₂S biology

It has been suggested that H₂S plays crucial roles in various physiological and pathological processes (Wang, 2012; Andreadou et al., 2015). However, most of the specific signalling events of H₂S regarding these processes are still unclear and require more research. Thus, a comprehensive understanding of H₂S generation, regulation, measurement and signalling is pivotal to understand the physiological significance of H₂S and potential H₂S contribution in diseases. Scientific approaches to understanding H₂S biology usually rely on three approaches.

First, the use of H₂S donors (sources, drugs) as an exogenous H₂S application. Second, the administration of H₂S synthesising enzyme inhibitors as pharmacological tools to decrease endogenous H₂S level and define the contribution of each of the H₂S-synthesising enzymes in H₂S generation (Li et al., 2011; Papapetropoulos et al., 2015; Wen et al. 2018). The availability of selective and potent CBS/CSE/MST enzyme inhibitors is necessary to understand H₂S biology and to confirm or exclude the involvement of specific enzyme in endogenous H₂S production. PPG is a suicide inhibitor (irreversible) for CSE by limiting the entrance of cysteine to the catalytic pocket of CSE (Whiteman et al., 2011; Asimakopoulou et al., 2013). AOAA is a non-selective inhibitor for CBS. AOAA is non-selective as it also inhibits CSE at around the same concentration (Wang, 2012; Papapetropoulos et al., 2015). There is no selective inhibitor for MST, although recent studies in our laboratory have identified a candidate MST inhibitor, 1-(3,4-Dihydroxyphenyl)-2-[(4-hydroxy-6-methyl-2-pyrimidinyl)sulfanyl] ethanone. Moreover, metabolic products and analogues, such as pyruvate, α -ketobutyrate (Whiteman et al., 2011) and aspartic acid (AA, aspartate) have been tried as MST inhibitors (Madden et al., 2012; Donovan et al., 2017; Donovan et al., 2018). However, these tools lack selectivity, specificity, sensitivity, potency and drug-like properties, such as pharmacokinetic profile.

Third, genetic modification experiments, such as studying knockout (KO) mice through inactivation of H₂S synthesising enzyme genes. Unfortunately, this could lead to different pathologies and phenotypes. Therefore, they are non-applicable for human (Yang et al., 2008; Beltowski, 2015). For example, Watanabe's study demonstrated that CBS- knockout mice usually die within five weeks and have short stature with hyperhomocysteinemia (Watanabe et al., 1995). Therefore, the lethal phenotype of CBS-KO and absence of selective and potent CBS inhibitors leads to difficulty in the study of CBS-KO.

CSE-KO mice are associated with hypertension, reduced endothelial-dependent vasodilatation and high peripheral vascular resistance in an age-dependent pattern because endogenous H₂S production in the aorta and heart of CSE-KO was about one-quarter of the wild-type mice (controls) (Yang et al., 2008). Interestingly, the administration of H₂S to these mice restored normal blood pressure (BP). Therefore, these results indicate an important role of H₂S production via CSE in control of vascular tone and BP. In another study, endogenous H₂S production in the carotid body of CSE-KO mice was about a half of the wild-type mice (controls) (Peng et al., 2010). Therefore, they suggested that CSE may contribute to about half of the endogenous H₂S production and oxygen sensing in mouse carotid bodies. Similarly, endogenous H₂S production in CSE-KO mice liver was about a one-third of the wild-type mice (controls) (Untereiner et al., 2016). Therefore, they suggested that CSE may contribute to about two-thirds of the endogenous H₂S production and mitochondrial protection in mouse liver. These differences in H₂S production between studies may be attributed to different mouse strain, KO approach, tissue and methodology used in each study. A study of CSE-KO model found that there was atrophy of the skeletal muscles in mice fed with cysteine-restricted nutrition (Ishii et al., 2010).

MST-KO mice are linked with anxiety (Nagahara et al., 2013; Suwanai et al., 2016). Peleli's study found that knocking down of MST in mice led to relatively less cardiovascular issues in adult mice (about 2-3 months), while it results in more cardiovascular issues in older mice (about 18 months). Moreover, there was a decrease of antioxidant defences and increase of oxidative stress in the older mice, and this result could be attributed to differences in antioxidant defences and ROS levels between adult and older mice, potentially in an age-related pattern (Peleli et al., 2020). These differences in phenotypes of MST-KO mice might be attributed to differences in the type of mice and the method of genetic manipulation used, which may lead to different phenotypes of the knockout model of the same enzyme. Therefore, there is plethora of evidence emphasising the significance of the H₂S pathways in cardiac and vascular physiology.

1.10. Therapeutic implications of H₂S releasing agents

Pharmacological regulation of H₂S concentration is usually carried out using H₂S donors (sources, drugs) to augment H₂S levels and enzyme inhibitors to decrease endogenous H₂S production. These are promising goals for therapeutic interventions (Chuah et al., 2007; Zhao et al., 2008; Szabo et al., 2007; Whiteman et al., 2011; Shen et al., 2014; Wallace and Wang, 2015; Wallace et al., 2018; Wen et al., 2018). H₂S may provide a promising target for a drug candidate (druggability) in therapeutic interventions as an alternative/additional or as prophylactic therapy. Therefore, H₂S donors and inhibitors of CBS, CSE and MST may beneficially influence diverse conditions involving dysfunction and inflammation of the heart and blood vessels. In other words, use of H₂S donors where there are low H₂S levels, while the use of inhibitors of H₂S biosynthesis in cases associated with high levels of H₂S (Mancardi et al., 2009; Papapetropoulos et al., 2015). For instance, administration of H₂S synthesising enzyme inhibitors may be a potential therapeutic option for the treatment of diseases associated with high H₂S levels. For example, AOAA may be useful in Down's syndrome (Kamoun et al., 2003), and some ovarian and colon cancer (Bhattacharyya et al., 2013; Szabó et al., 2013; Szabó and Hellmich, 2013; Hellmich and Szabó, 2015; Cao et al., 2019). PPG may be useful in septic and haemorrhagic shock (Mok et al. 2004; Zanardo et al., 2006; Zhu et al., 2010; Whiteman et al., 2011). AOAA/PPG might be useful in diabetes (Yusuf et al., 2005) and obstructive asthma (Saito et al., 2013).

In mammals H₂S is principally produced via CBS, CSE and MST, and in bacteria, similar enzymatic machinery was reported. Furthermore, H₂S production in bacteria can lead to an increase in energy production, resistance to antibiotics and oxidative stress, and decrease the antibacterial activity of antibiotic (Shatalin et al., 2011). Thus, the development of specific and selective inhibitors for bacterial CBS, CSE and MST might lead to a new generation of antibiotics (Paul and Snyder, 2012).

The administration of H₂S donors could be a potential therapy (drug) for the treatment of cardiovascular diseases associated with very low H₂S levels, such as patients with hypertension (Yang et al., 2008; Zhao et al., 2008), coronary artery disorders, myocardial infarction and heart failure (Jiang et al., 2005; Kovačić et al., 2012; Polhemus et al., 2014), atherosclerosis (Yang et al., 2010; Candela et al., 2016; Leucker et al., 2017), endothelial dysfunction (Mustafa et al., 2011), and hyper-proliferation of smooth muscle cells (Mani et al., 2013).

Therefore, the administration of H₂S sources has promising pharmacotherapy potential (druggability) for many cardiovascular diseases (CVDs), such as hypertension, myocardial infarction, angina, arrhythmia and heart failure, gastrointestinal diseases including peptic ulcers, inflammatory disorders, COVID-19 and pneumonia infection, and neurological diseases, such as Parkinson's, Alzheimer's disease, and erectile dysfunction.

There are many types of H₂S donors (sources, drugs) (Beltowsky, 2015; Wen et al., 2018); these include:

(1) Inorganic sulfide salts, such as NaHS and Na₂S are commonly used in H₂S studies. These salts release a large amount of H₂S during a short time, a fast release H₂S salt. Therefore, they have a rapid onset and short duration of action and could lead to supra-physiological (toxic) levels due to uncontrollable H₂S release. Physiological endogenous H₂S generation was reported to be in small amounts with a slow release rate (Li et al., 2008). Therefore, H₂S release from these salts may not be similar to physiological H₂S generation. These salts have liability for oxidation and impurity (Beltowski, 2015), which can be confirmed by the presence of polysulfides as yellow colour (Hughes et al., 2009; Wen et al., 2018). These properties of inorganic H₂S salts could lead to inconsistent and less reliable results. H₂S salts, such as NaHS and Na₂S could be regarded as the classical H₂S sources used in many previous studies.

IK-1001 is a modified parenteral form of Na₂S from IKARIA company with a special pH and isotonicity formulation (Kiss et al., 2008). IK-1001 used in phase II studies. For example, IK-1001 used in clinical studies for some cases of congestive heart failure, heart remodelling and coronary artery transplantation to stimulate healing and revascularisation (Wen et al., 2018) and also used to decrease IRI in mice liver (Jha et al., 2008). SG-1002 is sodium polysulthionate used as H₂S prodrug in phase I studies, which led to positive results in mice with induced-heart failure and patients with congestive heart failure via controlled/prolonged H₂S and sulfane sulfur release (Kondo et al., 2013; Li et al., 2018; Wallace et al., 2018; Wen et al., 2018).

Sodium thiosulfate (STS, Na₂S₂O₃) is an FDA (Food and Drug Administration) approved H₂S source for treatment of some pathological cases, such as cyanide poisoning (Berbata et al., 2017) and lung injury (Zhang et al., 2019). STS releases H₂S slower than Na₂S and NaHS. The application of STS in patients with COVID-19 and pneumonia infection led to positive results, which could be due to anti-inflammatory, antioxidant and antiviral/antibacterial effects of STS (Evgen'ev and Frenkel, 2020). Moreover, STS led to antioxidant and cardioprotective effects in mice with chronic heart failure (Sen et al., 2008) and cardiovascular and renal protection effects in rats with angiotensin II induced cardiovascular and renal injuries (Snijder

et al., 2014; Snijder et al., 2015). Another example, STS led to decrease of LPS-induced inflammatory insult in mouse lung and HUVECs (Human umbilical vein endothelial cells) via attenuation generation of cytokines, NF- κ B and ROS (Sagaguchi et al., 2014).

(2) Synthetic organic slow-releasing H₂S sources, such as GYY4137 [morpholin-4-ium 4-methoxyphenyl (morpholino) phosphinodithioate], which is water-soluble, and has a slow onset of action due to a slow hydrolysis, a slow-release H₂S source (Karwi et al., 2016). In other words, GYY4137 releases small amounts of H₂S over prolonged time *in vitro* and *in vivo* (Li et al., 2008). Thus, H₂S release from GYY4137 could be more physiologically relevant and controllable than inorganic H₂S salts (Li et al., 2008; Whiteman et al., 2011). Whether, GYY4137 releases H₂S directly inside or outside the cells is unknown. Moreover, GYY4137 is impassable to the blood-brain barrier (Yu et al., 2010). The chemical structure of GYY4137 (figure 1.7) (Li et al., 2008; Huang et al., 2016).

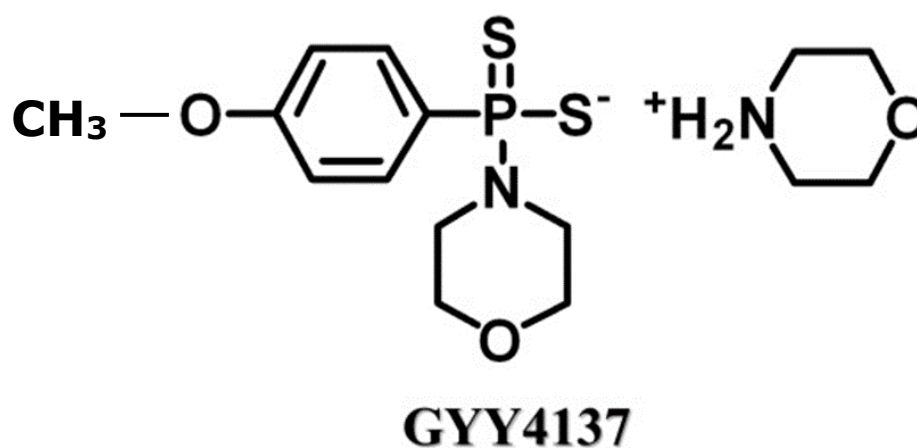


Figure 1.7: The chemical structure of GYY4137 (Li et al., 2008; Huang et al., 2016).

GYY4137 is reported to produce sustained vasorelaxation of rat aorta and decrease of blood pressure in normal and SHR (spontaneously hypertensive rats) in an *in vivo* and *ex vivo* models compared to the short duration relaxation response induced by NaHS (Li et al., 2008). Therefore, GYY4137 was reported to have more potent hypotensive and vasorelaxant effects than NaHS (Li et al., 2008). GYY4137 has promising results in many experimental models of inflammation compared to inorganic salts NaHS (Li et al., 2009; Whiteman et al., 2010; Sen et al., 2012) and cardiovascular pathologies in rat aorta and perfused kidney of SHR as a vasodilator and antihypertensive, which results in a hypotensive action (Li et al., 2008). Similar results of cardioprotective effects of GYY4137 are reported in rat heart with ischaemic injury (Lilyanna et al., 2015) and rat heart with IRI (Karwi et al., 2016). Furthermore, GYY4137 led to improvement of vascular remodelling in mouse mesenteric arteries

in obese mice (Candela et al., 2016). Moreover, GYY4137 led to a relaxation of phenylephrine-precontracted bovine ciliary artery (Chitnis et al., 2013). GYY4137 led to anti-inflammatory and antiviral effects in an *in vitro* model of human respiratory syncytial virus (Bazhanov et al., 2017; Bazhanov et al., 2018). These anti-inflammatory and antiviral activities of GYY4137/H₂S could be useful in the treatment of COVID-19 infection pandemic (Evgen'ev et al., 2020).

Another example of slow-releasing H₂S sources is FW1256 (3-dihydro-2-phenyl-2-sulfanylenebenzo[d][1,3,2]oxazaphosphole), which releases H₂S over hours and decreases inflammatory response in an *in vivo* model and mouse macrophages treated with LPS (Huang et al., 2016).

(3) Mitochondria-targeted organic H₂S sources, such as AP123 and AP39 [10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl)phenoxy)decyl) triphenylphosphonium bromide]. Thus, AP123 and AP39 release H₂S in the mitochondria (Szczensy et al., 2014; Karwi et al., 2017). AP39 consists of two parts, one part is triphenylphosphonium (TPP⁺), which is a mitochondrially targeted part and the other part is dithiolethione, which is the H₂S donor part, and these two parts are connected by an aliphatic linker, as illustrated in the Chemical structure of AP39 (figure 1.8) (Tomasova et al., 2015; Gerő et al., 2016)., whether the biological effect is due to only the H₂S-releasing part or whether both parts are biologically active is unclear.

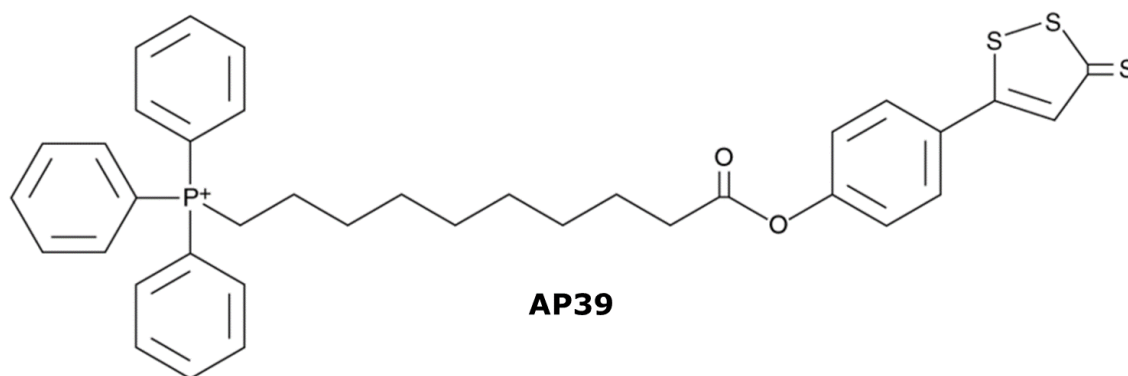


Figure 1.8: The Chemical structure of AP39 (Tomasova et al., 2015;; Gerő et al., 2016).

Localisation of H₂S in the mitochondria using mitochondria-targeted H₂S sources AP123 and AP39 could be beneficial because it could lead to about 100 fold enhancement of H₂S antioxidant effect against oxidative stress compared to H₂S salts (Gerő et al., 2016). Moreover, H₂S has a short half-life, and other H₂S sources, such as H₂S salts and GYY4137 are not specific in delivering H₂S to the mitochondria due to off-target effects. Therefore, H₂S localisation in the mitochondria could be

beneficial to maintain mitochondrial function and energy production and protect mitochondria against oxidative stress. AP39 is slightly more potent than AP123. AP123 and AP39 release H₂S slower than Na₂S (Geró et al., 2016). Therefore, AP123 and AP39 could be useful for the treatment of chronic cardiovascular diseases (CVD), rather than H₂S salts. GYY4137 and AP39 could be considered as new generation H₂S sources in recent studies.

(4) Analogues of cysteine, including S-propyl-cysteine (SPC), S-allyl-cysteine (SAC), S-propargyl-cysteine (SPRC) and N-acetyl-cysteine (NAC). These compounds are suggested to be relatively selective substrates for CBS and CSE compared to L-cysteine, which can be used by alternative pathways, such as glutathione biosynthesis. Moreover, these compounds may enhance CBS and CSE expression via an unidentified mechanism and increase H₂S level (Beltowski et al., 2015). These compounds are reported to generate H₂S slower than inorganic H₂S salts and have antioxidant effects (Li et al., 2018). For example, NAC use in COVID-19 infection was useful as an adjuvant therapeutic option due to potential antiinflammatory, antioxidant, and mucolytic effects of NAC (De Flora, et al., 2020).

Application of SPC, SPRC, and SAC leads to a positive outcome in rat hearts and cardiomyocytes with ischaemic/hypoxic damage via cardioprotective effects mediated by antioxidant effects (Wang et al., 2009; Wang et al. 2010). Similarly, SAC attenuated IRI in rat heart through H₂S-dependent mechanism via antioxidant effect, reduce of remodelling, possibly enhancement of H₂S/CSE activity and CSE expression (Chuah et al., 2007).

(5) H₂S-producing drugs, which consist of H₂S donating molecule and drug to form a hybrid drug, H₂S-producing drug. These H₂S-producing drugs usually have enhanced activity and lowered adverse effects, such as non-steroidal anti-inflammatory medicines (NSAIDs), including S-aspirin, S-diclofenac, and S-naproxen. The administration of these drugs produce a beneficial decrease of gastric toxicity and enhanced activity more than the ordinary NSAIDS (Wallace et al., 2007; Chan and Wallace, 2013; Wen et al., 2018). Another interesting example, the application of aspirin-ACS14 in rats with an experimental metabolic syndrome led to cardiovascular protective effects, such as decrease of hypertension, IRI and endothelial impairment (Rossini et al., 2010). S-Zofenoprilat, as a sulfhydrated angiotensin converting enzyme inhibitor (ACEI), the use of S-Zofenoprilat in SHR produced vascular protective effects, such as normalisation of H₂S levels in plasma and tissue (aorta and carotid arteries), decrease of hypertension and endothelial impairment (Bucci et al., 2014). Therefore, S-Zofenoprilat, in compararison with enalapril as a classical

ACEI, has been reported to produce additional beneficial effects in the cardiovascular system unrelated to inhibition of ACE but related to H₂S generation.

(6) Natural food-releasing H₂S sources as plant-derived polysulfides, H₂S stores. Derivatives of garlic, such as DADS, diallyl disulfide; DATS, diallyl trisulfide and ajoene. Other natural sources, such as broccoli and cabbage (sulforaphane) and onions are considered as natural food-releasing H₂S (Benavides et al., 2007; Wen et al., 2018). Epidemiology research reported that garlic use was linked with decrease morbidity and increase of longevity and health advantages (Mulrow et al., 2000; Sobenin et al., 2010). For example, administration of DATS, which released H₂S slower than Na₂S, led to a decrease of IRI in mouse hearts (Predmore et al., 2012). DADS and DATS application to rat and mouse liver cells leads to enhancement of cell viability and antioxidant defences. Therefore, DADS and DATS led to a decrease of oxidative stress (Chen et al., 2004; Fukao et al., 2003; Wu et al., 2004; Zeng et al., 2008; Predmore et al., 2012).

Therefore, garlic derivatives, such as DADS and DATS might have cardioprotective and cytoprotective effects, such as antioxidant, decrease oxidative stress, lipid-lowering, antiplatelet, anti-inflammatory, antibacterial and antiviral effects potentially through the release of H₂S (Chen et al., 2004; Fukao et al., 2003; Wu et al., 2004; Benavides et al., 2007; Zeng et al., 2008; Predmore et al., 2012). Moreover, the administration of garlic in patients with coronary artery and heart diseases led to cardiovascular-protective effects and clinical improvements, such as exercise tolerance and lipid-lowering and decrease of cardiovascular pathologies (Verma et al., 2005; Sobenin et al., 2010).

The oral and parenteral administrations of H₂S donors as therapy are recommended to be released slowly rather than bolus administration, which may lead to non-physiological and toxic levels of H₂S as well as the short duration of action. In other words, the bolus administration gives too high concentration during a short period (Shen et al., 2014; Beltowsky, 2015).

In short, H₂S druggability (H₂S replacement therapy) may be beneficial for patients who have cardiovascular diseases with decreased H₂S concentrations as an alternative/additional therapeutic or prophylactic option (Shen et al., 2015). Nonetheless, there are still some obstacles facing translatability of H₂S sources (or inhibitors) in the clinical field, such as safety cautions and long-term effects. For instance, there is little consensus regarding the therapeutic (clinical) or pathological (toxic) concentration for different tissues, diseases and species and the active form of H₂S.

1.11. Aims of the study

H₂S appears to play a role in the regulation of vascular tone. Therefore, there is accumulated evidence regarding the role of H₂S in the vasculature, while there is little known about H₂S role in the heart. Although many signalling pathways for H₂S have been proposed, they are still unclear. Therefore, there are three main aims of this study:

First, the measurement of endogenous H₂S production in the heart by investigating the expression level of H₂S synthesising enzymes (CBS, CSE and MST) and parallel measuring of the activity of these enzymes via development of an assay.

Second, the development of an assay to measure the sulfhydrylation level in the heart, which could be regarded as an index for H₂S production and as a potential signalling mechanism for H₂S.

Third, investigating the effects of three different types of H₂S donors on vascular tone in PCA using organ-bath as a functional study. All these aims could help to understand role of H₂S in the heart and blood vessels, and they would hopefully help in the development and transferring of H₂S-relating sources as potential drugs/druggability for treatment of some cardiovascular diseases and allow future studies to measure any changes in cardiac dysfunction.

Chapter Two

Measurement of Hydrogen Sulfide Generation in the Heart

2.1. Introduction

In recent years, evidence that H₂S may have an influential role in several important biological processes has increased. Many studies have reported the production of H₂S within the body. However, the detailed mechanisms of H₂S generation, regulation, and effects in the heart are still unclear and need to be clarified. H₂S detection and measurement are problematic because H₂S is unstable and is volatile in solution. Moreover, there is no known specific metabolic end-product that could be easily measured as a biomarker (Shen et al., 2011; Shen et al., 2012; Olson et al., 2014).

The measurement of H₂S generation in the heart is important because a decrease of H₂S production is reported to be associated with many cardiovascular pathologies. For example, H₂S generation was reported to be low in obese mice compared to non-obese mice (Candela et al., 2016). Moreover, low production of H₂S in the vasculature is reported to be associated with vascular pathologies, such as hypertension (Yang et al., 2008), impairment of endothelium function (Mustafa et al., 2011), hyperproliferation of smooth muscle cells (Mani et al., 2013), and atherosclerosis risk (Yang et al., 2010).

There is increasing evidence for generation and activity of H₂S in the vasculature, but there is much less information about the role of H₂S in the heart. The measurement of H₂S production in the heart could lead to more understanding of biological role of H₂S in the heart. The aim of this chapter was to elucidate further the regulation of H₂S synthesis in different parts of the heart by determining enzyme expression and parallel activity.

In this chapter, two techniques have been applied. First, Western blotting was carried out to detect the expression level of H₂S synthesising enzymes, CBS, CSE and MST. The generation of H₂S was then determined using both the methylene blue assay and the fluorescent probe SF7-AM (sulfidefluor-7 acetoxymethyl ester). Changes in the generation of H₂S were measured under different conditions, such as with inhibitors (such as aminooxyacetic acid, AOAA; propargylglycine, PPG; phenylpyruvic acid, PPA) and activators (such as dithiothreitol, DTT; S-adenosyl methionine, SAM), different conditions (pH; degassing) and biological factors, (such as atria/ventricles; gender males/females) in order to evaluate the effects of these conditions on regulation of H₂S generation in the heart.

2.2. Materials

The reagents used in these experiments were prepared as indicated (Table 1)

Table 1: reagents and stock solutions

Compound	Catalogue & Source	Concentration	Solvent
L-cysteine	C7352, Sigma-Aldrich, UK	100 mM	Milli-Q water
Pyridoxal 5-phosphate, PLP	82870, Sigma, UK	1 mM	Tris-EDTA pH 7.4
DL-propargyl glycine (PPG, CSE inhibitor)	P7888, Sigma, UK	100 mM	Tris-EDTA 7.4
Aminoxyacetic acid (AOAA, O-carboxymethyl hydroxylamine, CBS inhibitor)	13408, Sigma-Aldrich	100 mM	Tris-EDTA pH 7.4
Sodium sulfide (Na ₂ S, H ₂ S salt, as standard)	208043, Sigma-Aldrich, UK	10 mM	Dissolved immediately before the experiment in Milli-Q water, kept tightly closed and on ice
Zinc acetate	Z0625, Sigma, UK	6% w/v	Milli-Q water
Dimethylphenylenediamine sulfate (DMPD)	D176605, Sigma-Aldrich, UK	0.1% (w/v)	5 M HCl
FeCl ₃	157740, Sigma-Aldrich, UK	50 mM	1.2 M HCl
Sodium hydroxide	1091369025, Sigma-Aldrich, UK	0.5 M	Milli-Q water
L-Aspartic acid	11195, Sigma-Aldrich, UK	10 mM	Tris-EDTA pH 7.4
Dithiothreitol (DTT)	D0632, Sigma, UK	10 mM	Tris-EDTA pH 7.4
S-adenosyl methionine (SAM, an allosteric activator/modulator of CBS)	A7007, Sigma-Aldrich, UK	10 mM	Tris-EDTA pH 7.4
Sodium mercaptopyruvate	G2915, Santa Cruz Biotechnology, USA	3 mM	Tris-EDTA pH 7.4
Dimethylsulfoxide (DMSO)	D5879, Sigma-Aldrich, UK		
Phenylpyruvic acid (PPA, MST inhibitor)	286958, Sigma-Aldrich, UK	1 M	DMSO
Sulfide fluor-7 acetoxymethyl ester (SF7-AM)	748110, Sigma, UK	5 mM	DMSO
Bovine serum albumin (BSA)	BP9702, Fisher Scientific, UK		Deionised water

2.3. Methodology

2.3.1. Western blotting

2.3.1.1. Sample preparation

Pig heart tissues (from males and females, age < 6 months, ~ 50 kg) were isolated at the abattoir and transported to the laboratory in ice-cold Krebs'-Henseleit buffer ([mM]: NaCl 118, KCl 4.8, CaCl₂ 1.3, NaHCO₃ 25, KH₂PO₄ 1.2, MgSO₄ 1.2, glucose 11.1) (Fisher Scientific, Loughborough, UK). Sections of the left atrium and left ventricle were dissected and tissue frozen at -80 °C. When the tissue was thawed, part of the tissue was weighed and homogenised in lysis buffer (mM: 20 Tris, 1 EGTA, 320 sucrose, 1 NaF, 10 β-glycerophosphate and 0.1% Triton X100, [pH 7.6], plus protease inhibitor mix [Sigma-Aldrich, UK, P8340]) to give 100 mg of tissue per ml of lysis buffer (10 % w/v). Samples were mixed on ice for 15 min and homogenised using a hand-held Ultra-Turrax homogeniser on full speed for 30 s; the sample was centrifuged at 3300 x g for five min (Sigma 3-18K refrigerated centrifuge) at 4 °C. Then, the supernatant layers were transferred to new Eppendorf tubes, and the pellets were discarded. 150 µl of the supernatant layer was mixed with 30 µl of 6x solubilisation buffer (SB: 1.5 M Tris, 30 % glycerol, 24 % SDS, 5 % β-mercaptoethanol, 2.5% bromophenol blue). 50 µl of each sample was frozen at -20 °C for assaying protein content.

2.3.1.2. Lowry protein assay

The samples were thawed gently and diluted in deionised water. Serial dilutions of bovine serum albumin (BSA, 0.05 to 0.5 mg/ml) were prepared in duplicate for the standard curve. After preparation of the standards and samples, Lowry AB solution was prepared by mixing 100 µl of 2% sodium potassium tartrate and 100 µl of 1% copper sulphate (solution B) to 20 ml of solution A (Lowry A solution, % w/v: 0.4 NaOH, 0.2 SDS and 2 sodium bicarbonate). 1 ml of Lowry AB solution was added to Eppendorf tubes containing either BSA standards, unknown samples or blanks (just DW). All the tubes were incubated for 10 min at room temperature. After this, Folin and Ciocalteu's reagent was diluted 1:1 with milli-Q deionised water. Then, 100 µl of this mixture was added to each Eppendorf tube and thoroughly mixed and then incubated for 45 minutes at room temperature. Finally, 200 µl from each tube was transferred to 96-well plate in duplicate, and the absorbance was measured at 750 nm using a spectrophotometer (SPECTRA MAX 340pc, USA) microplate reader and software SOFTMAX pro 2.6.1 (Lowry et al., 1951).

2.3.1.3. Polyacrylamide Gel Electrophoresis: sample loading and running phase

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a pre-cast 4-20 % gradient gel (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). The gel was placed in a Bio-Rad mini-protean 3 electrophoresis tank, and the reservoir was properly prepared to prevent leakage. Running buffer was poured into the tank (running buffer 1x: 190 mM glycine, 3.5 mM SDS and 19 mM tris). Next, the samples were heated at 95 °C for five min. Then, all the sample tubes were thoroughly mixed and centrifuged at 13000 rpm on a bench-top centrifuge for one min. 1 µl of molecular weight markers (Precision Plus Protein All Blue Standards, 161-0373, Bio-Rad Laboratories, UK) was loaded into the first well, and different amounts of proteins were carefully loaded (30 µg for CBS; 30 µg for CSE; 20 µg for MST) into the wells of the gel. Lids were placed on the tank and gels run at 175 V for 40 min.

2.3.1.3.1. Western blotting

Proteins were transferred onto nitrocellulose membrane in a Bio-Rad mini-transfer tank. Briefly, gels were soaked in transfer buffer (TB: 1.9 M glycine, 20 % v/v methanol and 0.19 M tris) for a few min and then placed onto the filter paper. Nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was placed onto the gel and bubbles removed smoothly using a roller. Next, the second filter paper was added to the sandwich, and again the bubbles were removed gently by a roller. After that, the cassette was closed and placed into the tank. Proteins were transferred at 100 V for 60 min.

After transfer, Ponceau S staining solution (Sigma-Aldrich, UK) was added to the nitrocellulose membrane to check the success of transferring protein bands and equal loading. The nitrocellulose paper was quickly washed with milli-Q water and then in Tris-buffered saline-Tween 20 (TBST: 25 mM Tris, 125 mM NaCl and 0.1 % v/v Tween 20). The nitrocellulose was then blocked in 30 ml of 5 % w/v fat-free milk powder in TBST at room temperature for 60 min on a shaker platform.

2.3.1.3.2. Antibody incubation

After blocking, the nitrocellulose was incubated with the appropriate primary antibody (see table 2) in 10 ml of 5% fat-free milk in TBST. The nitrocellulose was then incubated overnight at 4 °C with shaking. The properties of the primary and secondary antibodies, which were used in Western blotting, are in the following table based on the producer's datasheets.

Table 2: The properties of the primary and secondary antibodies

Primary antibodies	Catalogue number	Company	Species Raised in	Type	Anticipated MW (kDa)	Dilution
Anti-MST	HPA001240	Sigma, UK	Rabbit	polyclonal	31-35	1:1000
Anti-CBS	H0000875-D01P	Abnova, UK	Rabbit	polyclonal	61-63	1:1000
Anti-CSE	Ab80643	Abcam, UK	Rabbit	polyclonal	44-46 (45)	1:1000
Anti-GAPDH	G8795	Sigma, UK	mouse	monoclonal	37	1:10,000
Secondary antibodies						
Infrared dye, IR680CW(red)/ IR800CW (green)		Licor, Cambridge UK	Goat			1:10,000

Next day, the primary antibody was removed, and the nitrocellulose membrane was washed with TBST as follows: three times for 5 min and three times for 15 min at room temperature (RT). Subsequently, the nitrocellulose membranes were incubated with secondary antibodies; goat anti-rabbit IR800CW (emitting a green fluorescence) and anti-mouse IR680CW (emitting a red fluorescence) (Licor, Cambridge, UK) both 1:10,000 dilution in 5 % fat-free milk. The nitrocellulose membranes were then incubated for 60 min at RT, with shaking. The secondary antibodies were then discarded, and membranes washed with TBST before proceeding with the next washing with TBST, three times for 5 min and three times for 15 min at room temperature. Finally, nitrocellulose membranes were washed with Milli-Q water and visualised using an Odyssey Licor infrared scanner using the following settings: Image studio software programme version 3.1.4., 700/800 channels, intensity 4, mu 84, and Q lowest. Quantification of the bands was performed by densitometry analysis of the fluorescent signals using Image Studio version 3.1.

2.3.2. Enzyme activity assays

2.3.2.1. Determination of sulfide (as hydrogen sulfide) by the methylene blue assay

The methylene blue (MB) assay outlined below, for the determination of hydrogen sulfide, is based on iron (Fe^{3+}) catalysed reaction of zinc-trapped sulfide with dimethyl phenylenediamine sulfate (DMPD) under acidic conditions leading to the generation of MB which can be detected spectrophotometrically at 670 nm. The change in methylene blue colour reflects the change in H_2S production and H_2S synthesising enzyme activity (H_2S from L-cysteine via CBS/CSE or H_2S from mercaptopyruvate via MST). The method outlined below is essentially that described by Chen and Mortenson (1977), which itself was modified from Gilboa-Garber (1971).

2.3.2.1.1. Sample preparation

The heart tissue was thawed slowly and weighed and then homogenised (1 g in 10 ml, 10 % w/v) in Tris- EDTA buffer (0.1 M tris, 1 mM EDTA, pH 7.4) using a hand-held Ultra-Turrax homogeniser on full speed for 30 s on ice. After that, the sample homogenate was centrifuged at 1000 x g for 20 min at 4 °C (Sigma 3-18K refrigerated centrifuge). The supernatant layers and the pellets were subsequently collected. The supernatant layers were centrifuged at 30,000 x g for 1 h at 4°C. Then, the resultant supernatants were dispensed into 1.5 ml aliquots in Eppendorf tubes, labelled and stored at -80°C until use. The pellets were resuspended as 1 g in 2 ml of Tris- EDTA buffer (0.1 M tris, 1 mM EDTA, pH 7.4) and represented as a mitochondrial fraction (particulate) and dispensed into 1.5 ml Eppendorf tubes labelled and stored at -80 °C freezer until use.

2.3.2.1.2. Measuring CBS activity

The methylene blue assay was performed according to a published method (Webb et al., 2007) with some modifications. Rat liver cytosol (RLC) was used as a positive control for assay development and it was diluted 1:4 with Tris-EDTA buffer (0.1 M tris, 1 mM EDTA, pH 7.4) with 100 μM PLP (pyridoxal 5-phosphate) and the final concentration of PLP was 75 μM in RLC. Porcine heart cytosol (PHC) and rat heart cytosol (RHC) was diluted 1:1 with Tris-EDTA buffer (0.1 M tris, 1 mM EDTA, pH 7.4) with 200 μM PLP and the final concentration of PLP was 100 μM in PHC and RHC. H_2S synthesising enzyme inhibitors (AOAA or PPG 10 mM) were added in 10 μl per 10 ml of diluted cytosol and preincubated for 15 min at 37 °C to give final concentration of 100 μM AOAA or PPG. 270 μl of diluted cytosol were distributed, to which 30 μl of 100 mM L-cysteine substrate were added, before incubation for 60 min in a shaking

water bath at 37 °C. After the end of the incubation period, 75 µl of NaOH was added to each Eppendorf tubes to stop the reactions and then 500 µl of zinc acetate. To parallel tubes, incubation blanks were prepared, which were exactly the same ingredients but were not incubated at 37 °C.

Then, all the tubes were left for 30 min at room temperature. After this, 300 µl of freshly mixed DMPD-FeCl₃ mixture (2 volumes of 0.1 % w/v DMPD plus 1 volume of 50 mM FeCl₃) was added to all the tubes and allowed to stand for 30 min. Next, all tubes were thoroughly mixed and centrifuged at 3000 x g for 5 min. As a standard, 3 µl of 10 mM Na₂S, 270 µl Tris buffer and 30 µl cysteine were mixed, then 75 µl NaOH, 500 µl zinc acetate and 300 µl of DMPD-FeCl₃ were mixed as above. Then, 250 µl of each tube was transferred into individual wells of a 96-well plate, and the optical density was read at 670 nm using spectrophotometer (SPECTRA MAX 340pc, USA) microplate reader using software SOFTMAX pro 2.6.1. Enzyme activity was calculated using the following equation:

$$\text{Enzyme activity (nmoles/ mg protein)} \\ = \frac{30 \text{ nmoles}}{\text{Amount of protein (mg)}} \times \frac{\text{Absorbance of test} - \text{absorbance of blank}}{\text{Absorbance of standard} - \text{absorbance of blank}}$$

Where 30 nmoles is the amount of Na₂S standard assessed (derived from 100 µM Na₂S standard in 0.3 ml of Milli-Q water as a reaction volume). Data were expressed as nmoles per mg protein.

2.3.2.1.2.1. Effects of dithiothreitol

In some experiment, after dilution of the cytosol with tris-EDTA buffer (0.1 M tris, 1 mM EDTA, pH 7.4), DTT (dithiothreitol) was then added in order to determine the effect of 0.5 mM DTT on the time course of CBS activity during different incubation times (0, 15, 30, 45 and 60 min).

2.3.2.1.2.2. Effects of aspartic acid on H₂S production

Aspartic acid (AA, aspartate) can be used to inhibit the MST pathway as it is an alternative substrate and competitive inhibitor for CAT. In order to determine whether the CAT/MST pathway contributed to H₂S production from L-cysteine, the cytosol was preincubated with 10 mM aspartic acid for 15 min.

2.3.2.1.2.3. Effects of degassing on H₂S production

In some experiment, in order to determine the effect of degassing on the CBS activity degassing of the tris-EDTA buffer (0.1 M tris, 1 mM EDTA, at pH 7.4 or pH 9) was carried out using vacuum chamber before the experiment.

2.3.2.1.3. Measuring MST activity

The methylene blue assay was performed according to published methods (Shibuya et al., 2009; Rashid et al., 2013) with modifications. RLC was used as a positive control and diluted 1:10 with Tris-EDTA buffer (0.1 M tris, 1 mM EDTA, at pH 7.4 or pH 9.0) and then 0.5 mM DTT was added. Porcine heart cytosol (PHC) and rat heart cytosol (RHC) were diluted 1:5 with Tris-EDTA buffer (0.1 M tris, 1 mM EDTA, at pH 7.4 or 9.0) and then 0.5 mM DTT was added. Phenylpyruvic acid (PPA), which was tested as a MST enzyme inhibitor (10 mM final concentration) was added in some experiments. 270 μ l of diluted cytosol were distributed, to which 30 μ l of the substrate 3-mercaptopyruvate, (final concentration 0.3 mM) was added, and then the mixture was incubated for 30 min in a shaking water bath at 37 °C. As above for CBS activity, reactions were stopped and estimation of methylene blue production assessed.

2.3.2.2. SF7-AM Fluorescence

2.3.2.2.1. Quantification of H₂S using SF7-AM

The detection of H₂S is an essential factor in determining enzyme activity. Therefore, it is crucial to develop and investigate alternative detection methods, such as fluorescent probes (Candela et al., 2016). The development of a sensitive and accurate method for H₂S measurement is a challenge. In recent years, a new fluorescent probe has appeared, sulfidefluor-7 acetoxymethyl ester (SF7-AM), which has been reported to have outstanding cellular trapping ability, biocompatibility and good sensitivity for H₂S generation in live cells and tissues (Lin et al., 2013; Candela et al., 2016). The principle of the SF7-AM method is that when SF7-AM (a non-fluorescent azide) reacts with H₂S as a reducing agent, the fluorescent amine product can be quantified by standard methods, fluorescence readings were measured and expressed as relative fluorescence units (RFU)/ per mg protein.

2.3.2.2.2. Sample preparation

Rat liver cytosol (RLC) and pig heart tissues (left atrium and left ventricle) were prepared as for the methylene blue assay. In this study, H₂S production was measured in pig heart cytosol (PHC) and particulate (including mitochondrial) fractions. In order to measure H₂S synthesis in a 96 well plate format, each well contained 180 μ l of tissue sample with final concentrations: 5 μ M SF7-AM fluorescent probe, 10 mM L-cysteine and 100 μ M PLP for CBS/CSE pathways or 5 μ M SF7-AM fluorescent probe and 0.3 mM sodium mercaptopyruvate for the MST pathway. In experiments containing enzyme inhibitors, AOAA and PPG or PPA were pre-incubated

with samples for 15 min before addition of SF7-AM and substrate. The final volume was 200 µl in each well. All experiments were carried out at 37 °C using a plate reader for 90 min (single end-point assay style). After incubation, plates were read on a Fluoroskan Ascent TM plate reader at 485 nm excitation and 538 nm emission wavelengths. Readings were obtained in triplicate and expressed as means. Tissue blank values were then subtracted from test samples and data were expressed as relative fluorescence units/ per mg protein.

2.3.2.2.2.1. The effect of tissue dialysis

In some experiments tissue dialysis was carried out in order to remove any possible particles which may interfere with the SF7-AM assay. First, tissue samples were split into two parts; one part was subjected to dialysis, while the other part was kept for the same period and temperatures, without being dialysed. For dialysis, the sample was pipetted into dialysis tubing, size of tubing about 1 ml (Sigma) in 10 mM Tris buffer (pH 7.4) and stirred overnight at 4 °C.

2.4. Statistical analysis

Data analysis and statistics were performed using Graph-Pad PRISM 6 (Software version 6, USA). All values were expressed as means ± standard error of the mean (SEM). Comparisons between more than two data groups were made using one-way ANOVA (analysis of variance) followed by Sidak's post-hoc test. For comparisons between two data sets, a two-tailed unpaired Student's t-test was used, or for paired data, a two-tailed paired Student's t-test. The P-value <0.05 was considered statistically significant between the data sets. * represents P-value <0.05, ** represents P-value <0.01, *** represents P-value <0.001, where n= number of experiments from separate heart and liver samples.

2.5. Results

2.5.1. Western blotting

Western blotting was performed in order to assess the expression level of CBS, CSE and MST in the heart. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, house keeping protein) was used as a loading control, while rat liver cytosol (RLC) was used a positive control as recommended by many studies in the literature as a good positive control for CBS, CSE and MST expression and activity.

2.5.1.1. Cystathionine β -synthase (CBS) expression

Western blotting results indicated that CBS immunoreactivity was expressed in porcine heart cytosol (PHC) of left atria and ventricles as bands at about 63 kDa (a high molecular weight band) and at lower intensity at 48 as (as a low MW band) with different amount of protein loaded (figure 2.1).

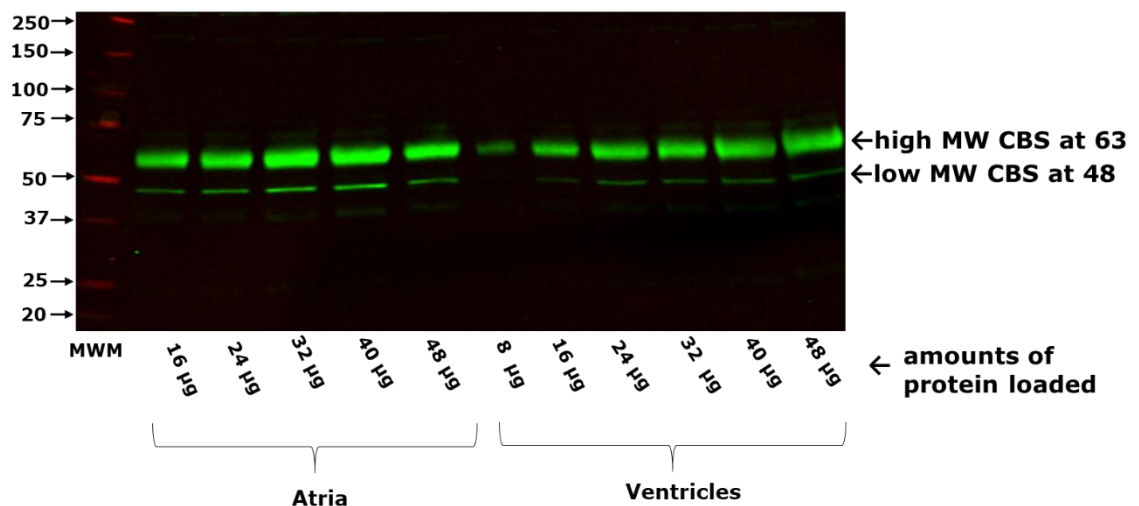


Figure 2.1: Western blot analysis showed CBS (cystathionine β -synthase) expression in porcine heart cytosols (PHC) (left atria and ventricles) as high molecular weight (MW) at 63 kDa and as low MW at 48 kDa. MWM: molecular weight marker. Different amounts of protein loaded; (n=1).

In order to validate the process, the linearity of detection of CBS immunoreactivity compared to protein loading was assessed as a pilot experiment to determine optimal protein loading. Linear regression analysis for porcine ventricular and atrial cytosol fractions demonstrated a linear pattern and there was a good correlation for the atria (r^2 was 0.546) and for the ventricles (r^2 was 0.877) (figure 2.2), and so 30 μg protein was loaded in subsequent studies.

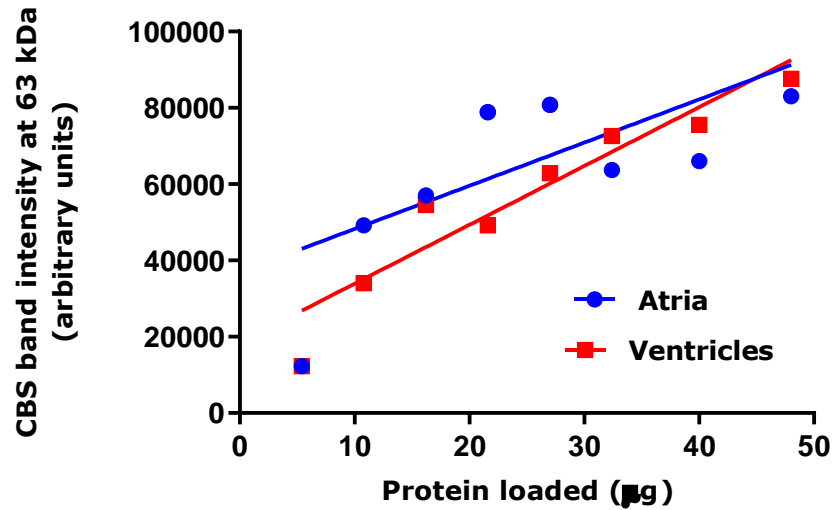


Figure 2.2: Western blot analysis demonstrated a linear regression pattern between the amounts of loaded protein and CBS signal at 63 kDa (as high MW band) in left atria and ventricles of porcine heart cytosols (PHC). The X-axis represents the amount of protein loaded. The Y-axis represents CBS band intensity at 63 kDa quantification (arbitrary units); (n=1).

Having established the appropriate amount of protein to load onto the gel, the Western blot was repeated in samples from different animals. As before, Western blot analysis showed two bands of CBS immunoreactivity at 63 and 48 kDa in comparison to the loading control GAPDH (glyceraldehyde 3-phosphate dehydrogenase, housekeeping protein, loading control) at 37 kDa in porcine heart cytosol (PHC) of three different animals. Using rat liver cytosol (RLC) as a positive control, the low MW CBS immunoreactivity was more obvious. There were also faint bands at 63 kDa in the RLC preparations, as well as multiple, fainter, higher MW bands (figure 2.3).

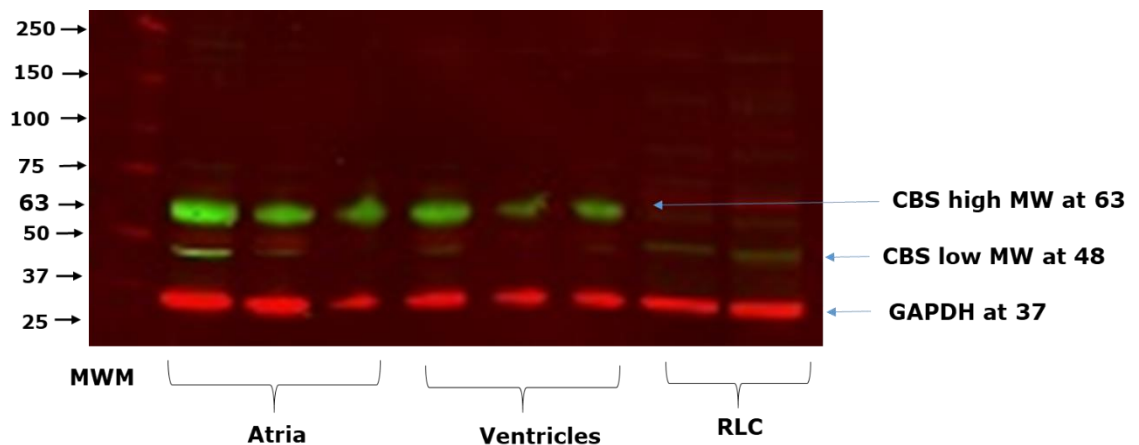


Figure 2.3: Western blotting analysis showed CBS (cystathionine β -synthase) expression in cytosols of porcine left atria and left ventricles from three different animals at high MW (63 kDa) and at low MW (48 kDa). MWM: molecular weight marker. GAPDH (glyceraldehyde 3-phosphate dehydrogenase, housekeeping protein, loading control) expression at 37 kDa. 30 μ g amount of protein loaded. Rat liver cytosol (RLC) was obtained from two different animals.

Using GAPDH as a housekeeping protein, a comparison was made of expression levels of CBS (63 kDa band) in porcine atria and ventricular cytosol fractions and compared to the positive control rat liver cytosol (figure 2.4: A and B). The normalised expression level (relative abundancy) for pig atria and ventricles appear higher than in the rat liver fraction (figure 2.4: A), while the ratio of the low/high MW CBS is lower in pig atria and ventricles than the rat liver fraction (figure 2.4: A and B).

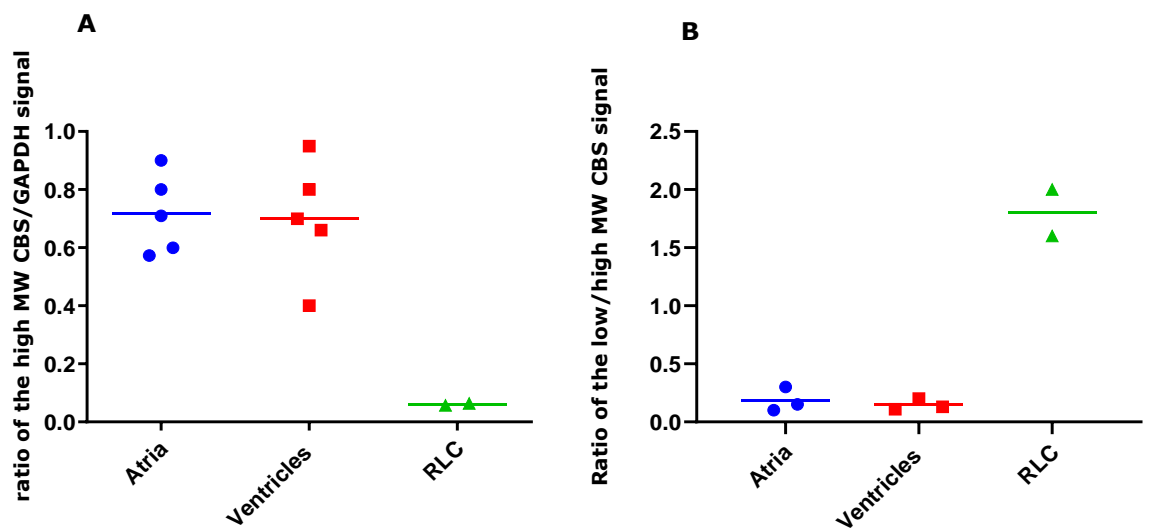


Figure 2.4: (A) Normalised ratios between high MW (63 kDa) CBS signal and GAPDH signal, left atria and ventricles of different samples of porcine heart cytosol (PHC) of three different animals. The X-axis represents different tissues (atria, ventricles and RLC). The Y-axis represents the ratio of CBS band intensity at 63 KDa/GAPDH band intensity at 37 kDa quantification. (B) Quantification of ratio of the low/high MW CBS band intensity in PHC of left atria and ventricles, and RLC (rat liver cytosol). RLC of two different animals. Non-significant $P > 0.05$ paired Student's t-test atria compared to ventricles, (n=5).

In order to assess whether expression of the high and low MW bands of CBS immunoreactivity were independently regulated, linear regression analysis was conducted (figure 2.5). There was a significant relationship between the low and high MW forms of CBS.

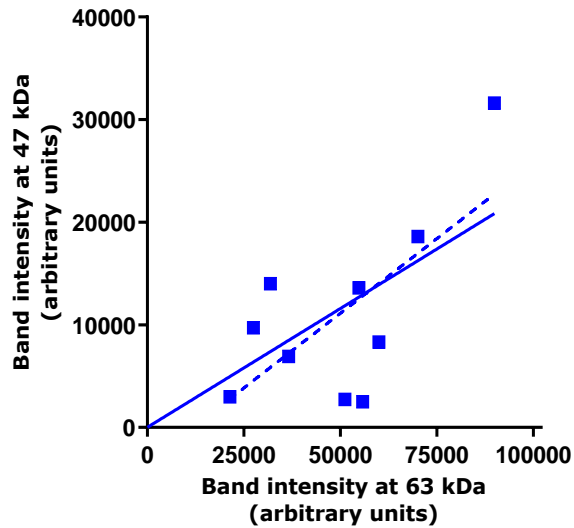


Figure 2.5: Linear regression analysis comparing low (47 kDa) and high MW (63 kDa) of CBS bands in left atria and ventricles of porcine heart cytosols (PHC) of five different animals (n=5). Indicated is the line of best fit (dashed line) and a line forced through the origin (solid line) with R^2 values of 0.464 and 0.442, respectively.

2.5.1.2. Cystathionine γ -lyase (CSE) expression

Western blotting indicated a non-detectable bands CSE immunoreactivity in cytosols of left atria and ventricles of porcine heart at 45 kDa in different amounts of protein loaded as a pilot experiment to determine optimal protein loading (figure 2.6). Similar observations were found in three separate experiments.

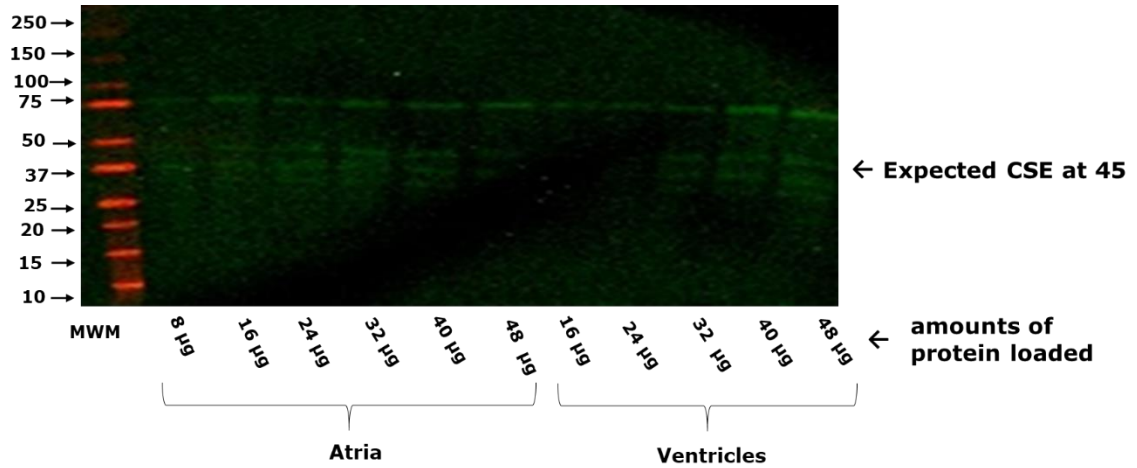


Figure 2.6: The western blotting analysis showed CSE expression in porcine heart cytosols (PHC) (left atria and ventricles) as non detectable bands at 45 kDa. MWM (molecular weight marker). Different amounts of protein loaded; (n=1).

Western blot analysis showed obvious detectable bands of the housekeeping protein (GAPDH, loading control) at 37 kDa in PHC of atria and ventricles and RLC (positive control). There were strong of CSE bands at 45 kDa in RLC (figure 2.7). In contrast, there were non-detectable bands of CSE at 45 kDa in PHC from atria and ventricles (figure 2.7).

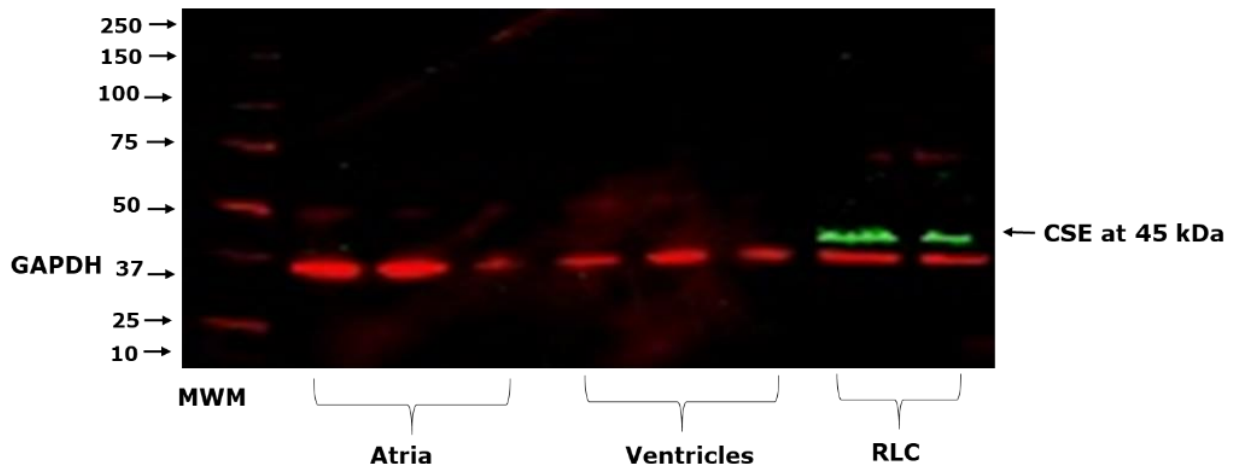


Figure 2.7: Western blotting analysis demonstrated non detectable CSE (cystathionine γ -lyase) expression in cytosols of porcine left atria and ventricles from three different animals at 45 kDa, while there were obvious bands in rat liver cytosol (RLC) obtained from two different animals at 45 kDa. MWM (molecular weight marker). GAPDH (glyceraldehyde 3-phosphate dehydrogenase, housekeeping protein, loading control) expression at 37 kDa. 30 μ g amounts of protein loaded.

Using GAPDH as a housekeeping protein, a comparison was made of expression levels of CSE at 45 kDa band in porcine atria and ventricular cytosol fractions and compared to the positive control rat liver cytosol (figure 2.8). The normalised expression level (relative abundancy) for pig atria and ventricles appear lower than in rat liver fractions.

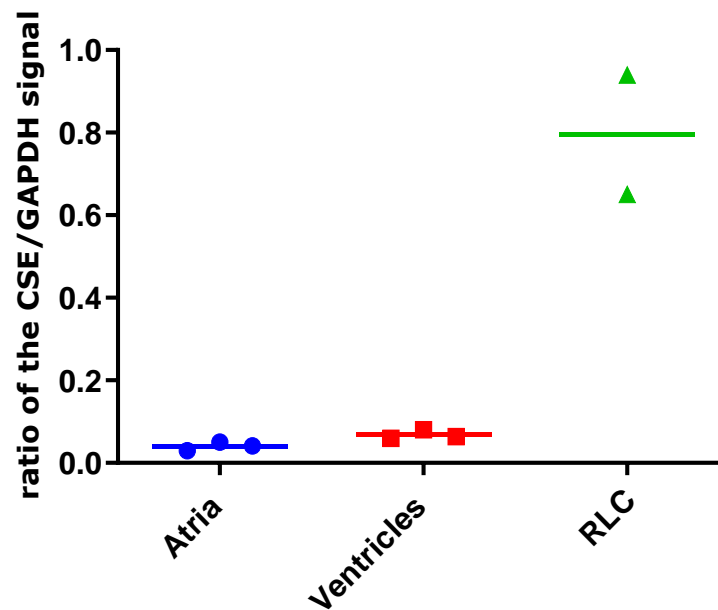


Figure 2.8: Normalised ratios between CSE signal and GAPDH signal, indicating high expression of CSE in RLC and non-detectable CSE expression in PHC. The X-axis represents different tissues (atria, ventricles and RLC). The Y-axis represents the ratio of CSE band intensity at 45 KDa/GAPDH band intensity at 37 KDa quantification. Left atria and left ventricles of porcine heart cytosol (PHC) of three different animals. RLC (rat liver cytosol) of two different animals as a positive control. Non-significant $P > 0.05$ paired Student's t-test atria compared to ventricles, (n=3).

2.5.1.3. Mercaptopyruvate sulfurtransferase (MST) expression

Western blotting indicated the presence of MST immunoreactivity in left atria and ventricles of porcine heart cytosols (PHC) as clear bands at 30 kDa with different amount of protein loaded (figure 2.9).

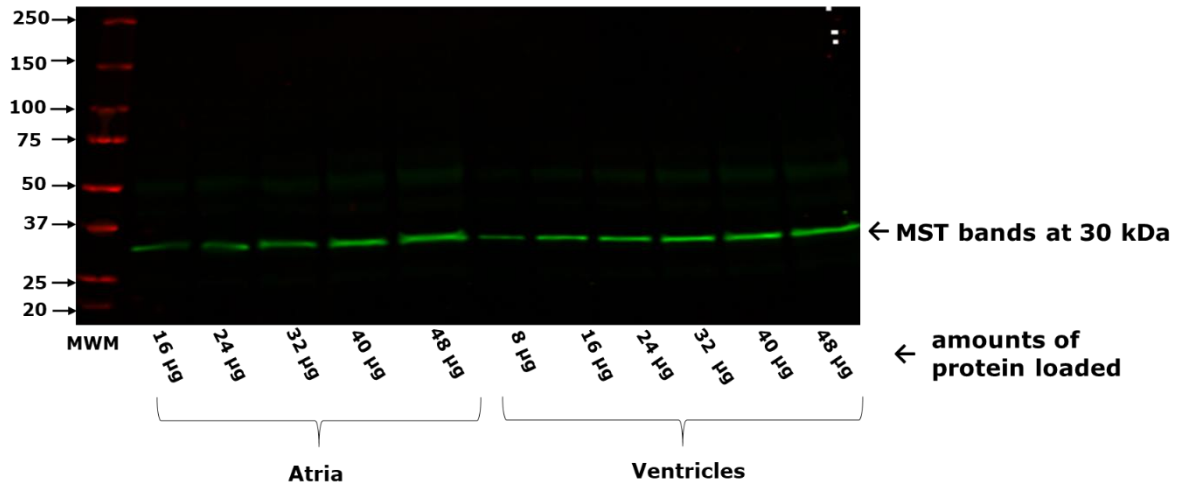


Figure 2.9: Western blotting analysis showed MST (mercaptopyruvate sulfurtransferase) expression as clear bands at 30 kDa in porcine heart cytosols (PHC) (left atria and ventricles). MWM: molecular weight marker. Different amounts of protein loaded, (n=1).

In order to validate the process, the linearity of detection of MST immunoreactivity at 30 kDa compared to protein loading was assessed as a pilot experiment to determine optimal protein loading. Linear regression analysis for porcine ventricular and atrial cytosol fractions demonstrated a linear pattern and there was a good correlation for the atria (r^2 was 0.947) and for the ventricles (r^2 was 0.987) (figure 2.10), and so 20 μ g protein was loaded in subsequent studies.

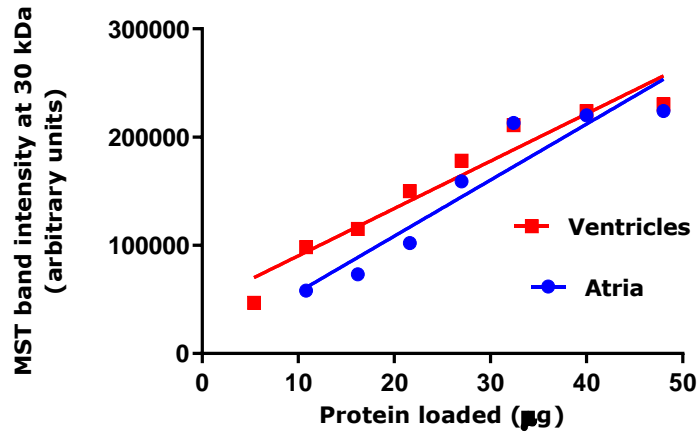


Figure 2.10. Western blotting analysis showed a linear regression pattern between the amounts of protein loaded and MST signal at 30 kDa in left atria and ventricles of porcine heart cytosols (PHC). The X-axis represents the amount of protein loaded. The Y-axis represents MST band intensity at 30 kDa quantification (arbitrary units); (n=1).

Having established the appropriate amount of protein to load onto the gel, the Western blot was repeated in samples from different animals. As before, Western blot analysis showed distinct bands of MST immunoreactivity at 30 kDa in comparison to the loading control (GAPDH) at 37 kDa in porcine heart cytosol (PHC) of three different animals. Using rat liver cytosol (RLC) as a positive control, the MST immunoreactivity was more obvious in RLC at the same apparent MW (figure 2.11).

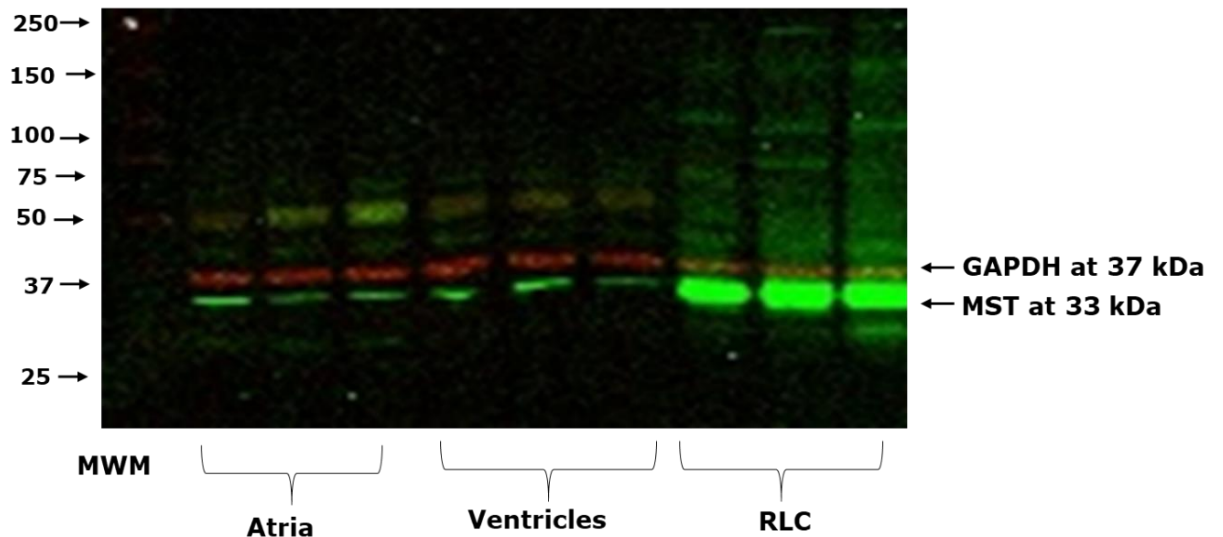


Figure 2.11: Western blotting analysis showed MST (mercaptopyruvate sulfurtransferase) expression in cytosols of porcine of left atria and left ventricles from three different animals at 30 kDa. MWM (molecular weight marker). GAPDH expression (glyceraldehyde 3-phosphate dehydrogenase, housekeeping protein, loading control) at 37 kDa. 20 μ g amount of protein loaded. Rat liver cytosol (RLC) was obtained from three different animals.

Using GAPDH as a housekeeping protein, a comparison was made of expression levels of MST at 30 kDa band in porcine atria and ventricular cytosol fractions and compared to the positive control rat liver cytosol (figure 2.12). The normalised expression level (relative abundancy) for pig atria and ventricles appear lower than in rat liver fractions.

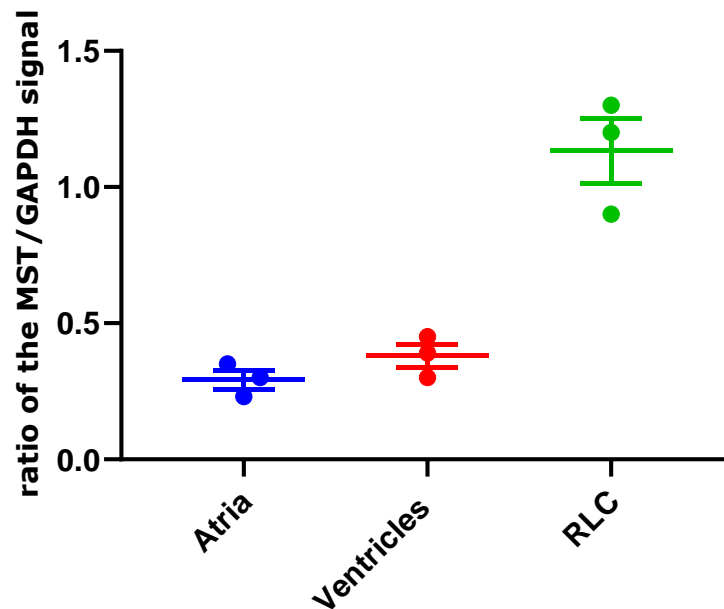


Figure 2.12: Normalised ratios between MST signal at 30 KDa and GAPDH signal at 37 kDa, indicating higher expression of MST in RLC compared to PHC. The X-axis represents different tissues (atria, ventricles and RLC). The Y-axis represents the ratio of MST band intensity at 30 KDa/GAPDH band intensity at 37 kDa quantification. Left atria and left ventricles of porcine heart cytosol (PHC) of three different animals. RLC (rat liver cytosol) obtained from three different animals as a positive control. Non-significant $P > 0.05$ paired Student's t-test atria compared to ventricles, (n=3).

2.5.2. Enzyme assays

2.5.2.1. Measuring CBS activity using the methylene blue detection method

The methylene blue assay (MBA) was conducted to assess the enzyme activity of CBS and MST in the heart. RLC was used as a positive control and for assay development and optimisation. PPG, AOAA and PPA were used in RLC and PHC. Na₂S was used as a standard.

2.5.2.1.1. Selection of blanks

In order to select the most useful blank, we investigated different types of blanks including incubation on ice (low temperature blank), omission of L-cysteine (no substrate blank), omission of enzyme (no tissue blank), omission of PLP (no cofactor blank) or addition of PLP after the reaction, and addition of sodium hydroxide/zinc acetate to stop the reaction at the beginning of incubation period (no incubation blank). The comparison of absorbance (optical density, OD) for the different types of blanks using RLC as the enzyme source demonstrated that the blanks all had similar levels and were lower than the positive control RLC. Then, we selected the blank with the highest value (figure 2.13).

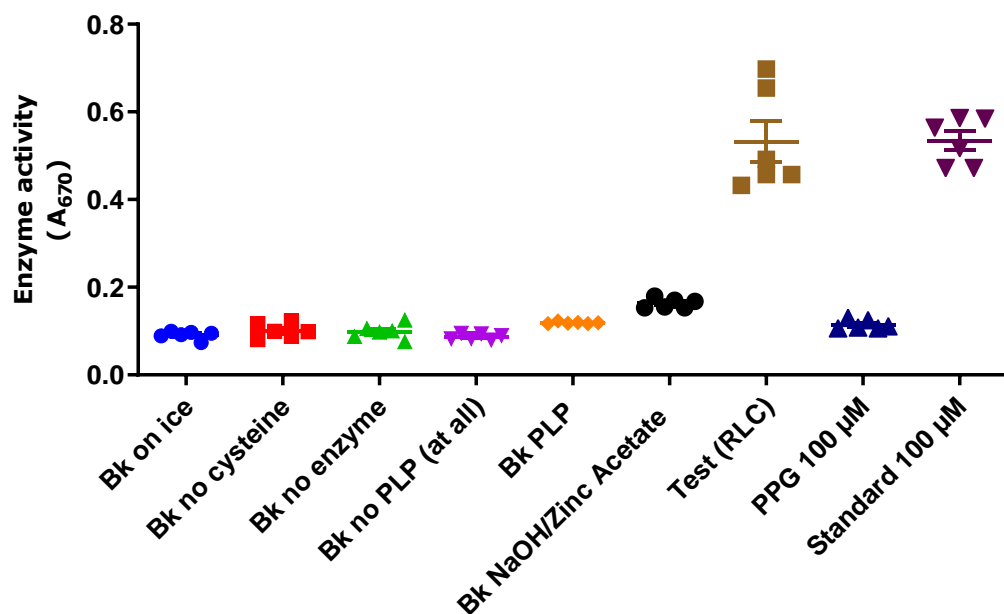


Figure 2.13: Comparison of the optical density for the different types of blanks in rat liver cytosol (RLC, test). Blank (Bk); on ice (incubation of the reaction on ice), no cysteine blank (omission of L-cysteine), no enzyme blank (no tissue), no PLP (no pyridoxal phosphate) or PLP (addition of 100 μ M PLP after 1 hour), NaOH/Zinc Acetate (addition of sodium hydroxide/zinc acetate to stop the reaction at the beginning of incubation period). NaOH (sodium hydroxide); standard (100 μ M Na₂S); PPG (100 μ M, propargylglycine, CSE inhibitor). The X-axis represents different types of blanks. The Y-axis represents the enzyme activity (optical density at 670 nm). Data presented as mean \pm SEM, (n=6).

2.5.2.1.2. Effects of dithiothreitol

DTT is a reducing agent that may decrease oxidation of H₂S and increase H₂S availability in the MST assay. The aim of this experiment was to examine the effect of DTT on the time course of CBS activity. The addition of DTT (0.5 mM) to the CBS assay did not alter enzyme activity in RLC at short incubation times (0 and 15 min). However, DTT increased the level of H₂S detection at longer incubation times 30, 45 and 60 min (figure 2.14).

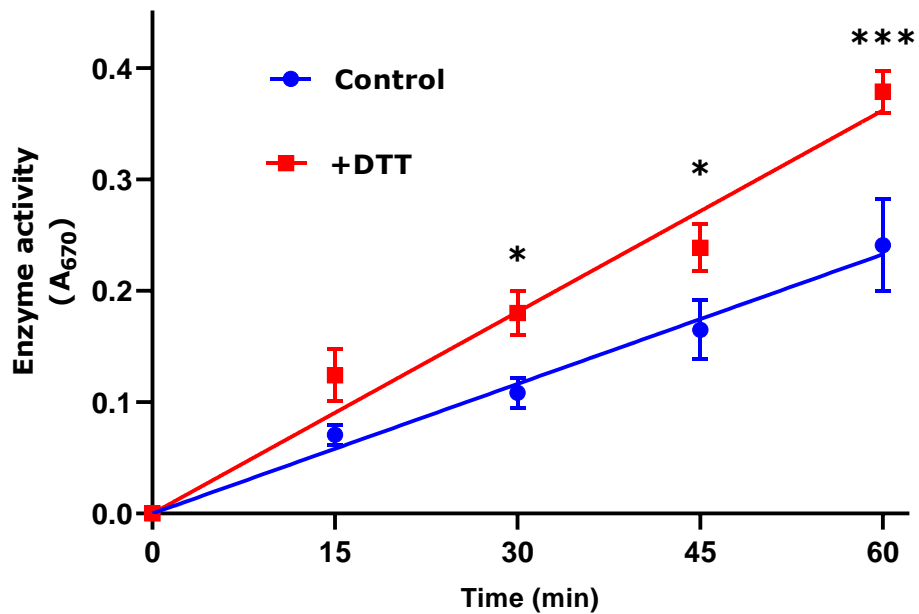


Figure 2.14: The effect of DTT (0.5 mM) on enzyme activity in rat liver cytosol during different incubation times (0, 15, 30, 45 and 60 min), (n=5 different rat cytosols). *P<0.05, ***P<0.001 compared to control using two-way ANOVA with post-hoc Sidak's correction.

2.5.2.1.3. Effect of aspartic acid on H₂S production

Aspartic acid (AA, aspartate) can be used to inhibit the MST pathway as it is an alternative substrate and competitive inhibitor for CAT (Shibuya et al., 2009; Madden et al., 2012; Donovan et al., 2017; Donovan et al., 2018). The aim of this experiment was to determine whether the CAT/MST pathway contributed to H₂S production from L-cysteine in the presence of PLP.

The addition of aspartic acid (AA, 10 mM) did not show any significant differences in H₂S production in the presence of PLP and without/with DTT (0.5 mM) in RLC (figures 2.15).

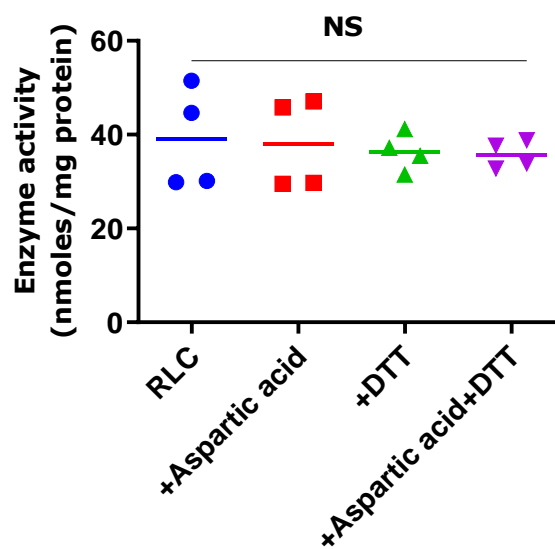


Figure 2.15: Effects of 10 mM aspartic acid without and with DTT (dithiothreitol, 0.5 mM) on H₂S production from L-cysteine in RLC at 60 min. Data presented as mean ± SEM. NS (non-significant) $p > 0.05$ using one-way ANOVA followed by Sidak's multiple comparisons test, $n=4$.

H₂S production was assessed in the absence of PLP, which could demonstrate the MST (PLP-independent enzyme) activity. The addition of aspartic acid (10 mM) evoked a small, but significant reduction in H₂S production in the absence of PLP and DTT in RLC (figure 2.16: A), but not in the presence of DTT (0.5 mM) (figure 2.16: B), following 60 min incubation at 37 °C.

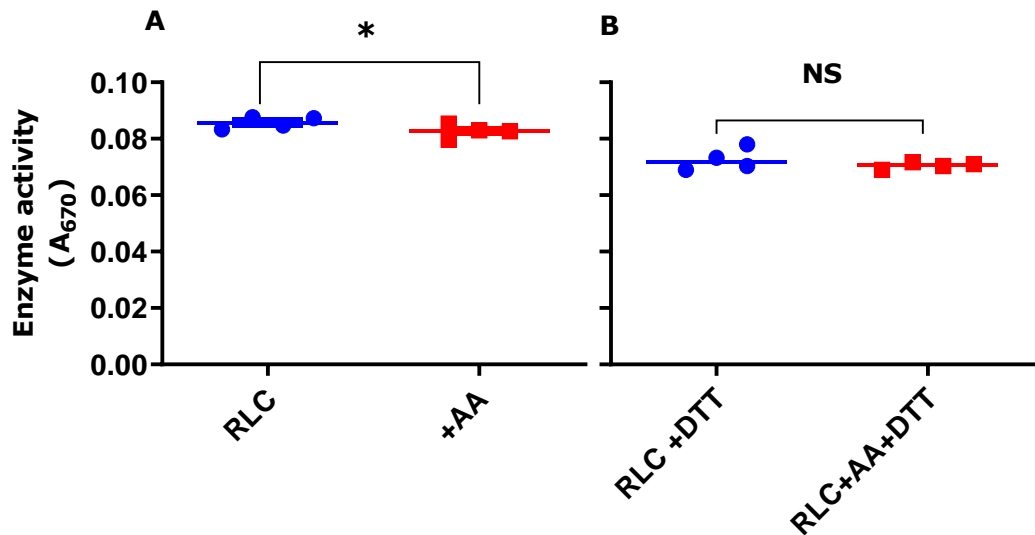


Figure 2.16: Effects of aspartic acid (AA, 10 mM) (A) alone and (B) in combination with DTT (0.5 mM) on enzyme activity in rat liver cytosol (RLC). Data presented as mean \pm SEM. NS (non-significant) $p > 0.05$, * $p < 0.05$ using two-tailed paired Student's t-test, $n=4$.

2.5.2.1.4. The effects of DTT on L-cysteine-derived H₂S production

There was an increasing level of H₂S generation with increasing concentrations of L-cysteine, in the absence or presence of DTT (figure 2.17). The presence of DTT enhanced H₂S production at 1 mM L-cysteine, but evoked an inhibition of H₂S production at 3 and 10 mM L-cysteine.

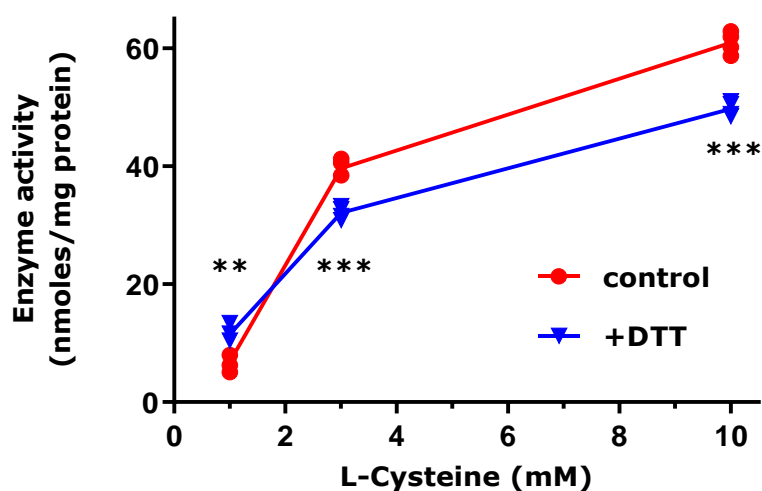


Figure 2.17: Effects of DTT in the presence of different concentrations of L-cysteine on H₂S production (DTT, 0.5 mM). Data presented as mean \pm SEM. ** $p < 0.01$, *** $p < 0.001$ using one-way ANOVA followed by Sidak's multiple comparisons test; (n=4).

2.5.2.1.5. H₂S production in different tissues

H₂S production was low in the pig heart tissue compared to rat liver. A potential interpretation for the low activity could be due to the delay in storage, transport and receiving pig heart tissue from the abattoir. Therefore, a comparison was made with rat heart cytosol (RHC) and rat liver cytosol (RLC), which were processed immediately after death. In this series of experiments, the controls of cytosolic preparations from porcine atria, rat heart and rat liver were significantly high compared to the blanks (figure 2.18: A and B). H₂S production was higher in the rat heart tissue compared to pig heart tissue (figure 2.18: A). H₂S production was higher in the rat liver tissue compared to both pig heart tissue and rat heart tissue (figure 2.18: A and B). H₂S production was about similar in the rat liver tissue and the standard (figure 2.18: B). The presence of the enzyme inhibitors caused significant reductions only in rat liver cytosol preparations (Figure 2.18: B).

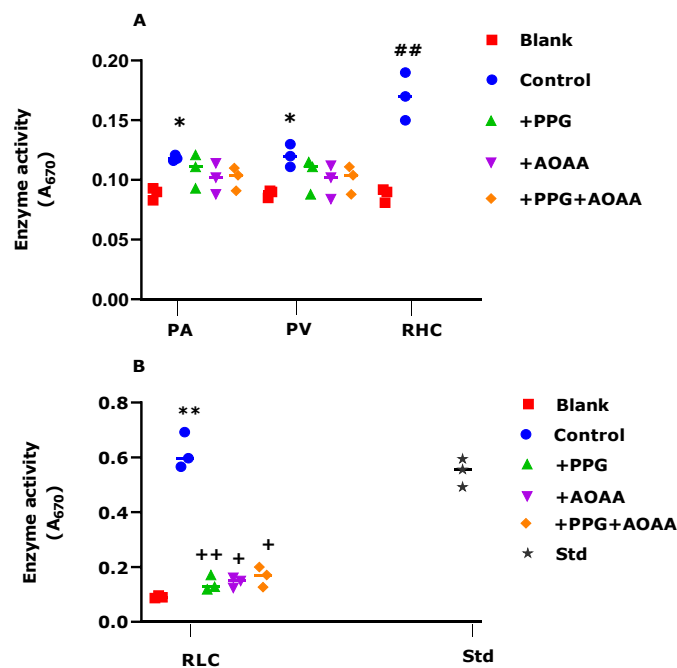


Figure 2.18: Comparison of the enzyme activity (absorbance at 670 nm) for porcine and rat tissues. (A) Porcine heart atria (PA); porcine heart ventricles (PV); RHC (rat heart cytosol). (B) RLC (rat liver cytosol), Std (standard, 100 μ M Na₂S). PPG (100 μ M propargylglycine, CSE inhibitor); AOAA (100 μ M aminoxyacetic acid, CBS inhibitor). Data presented as mean \pm SEM. NS (non-significant) $P > 0.05$, * $P < 0.05$, ** $P < 0.01$ compared to the blank using one-way ANOVA followed by Dunnett's multiple comparisons test, ## $P < 0.05$ paired Student's t-test compared to the blank.

+P<0.05, ++P<0.01 compared to the control using one-way ANOVA followed by Dunnett's multiple comparisons test, (n=3).

2.5.2.1.6. Effect of pH on H₂S production

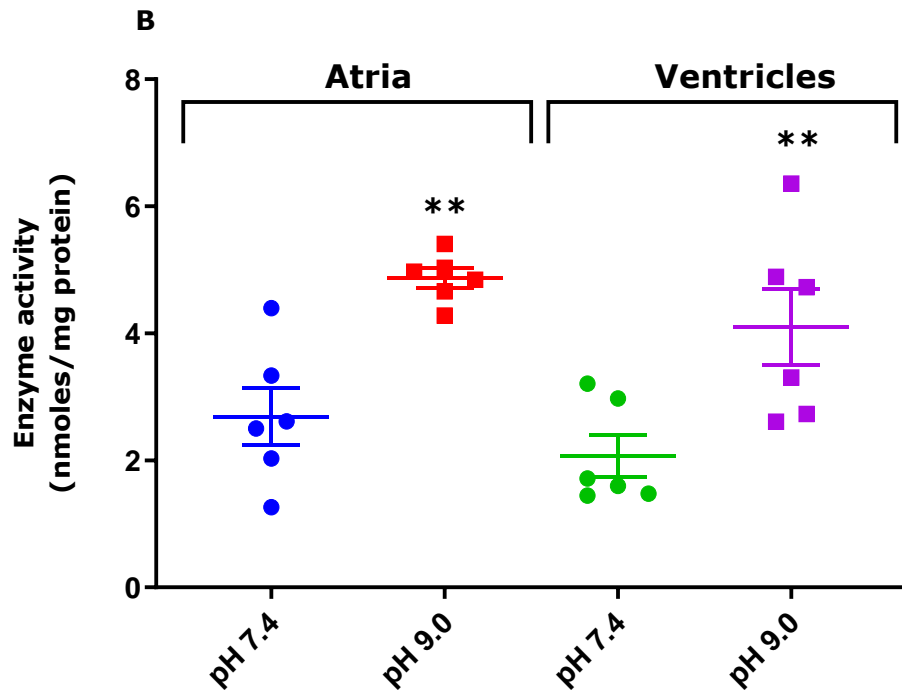
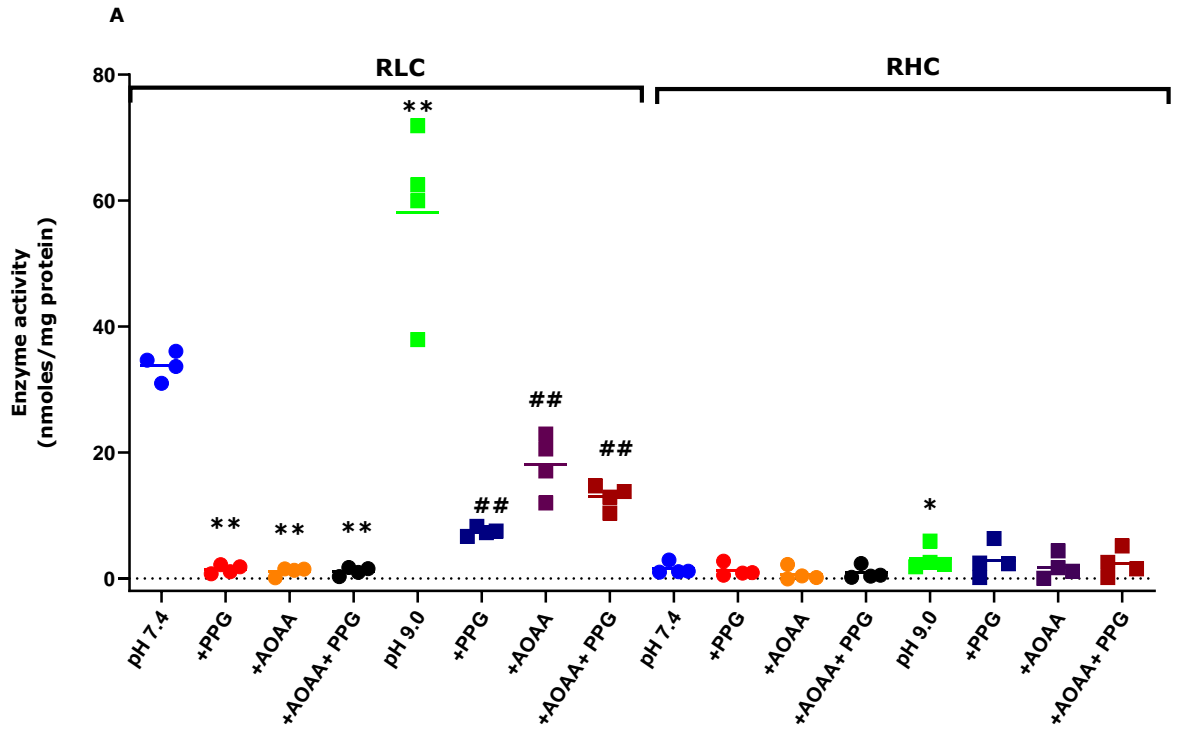
The experiments above indicated low CBS enzyme activity in the heart in comparison to the liver. Given the larger and more consistent levels of H₂S identified in rat heart preparations, subsequent investigations used RHC.

As H₂S in solution is more stable at alkaline pH (as it favours dissociation to hydrosulfide anion), an analysis of the pH sensitivity of the CBS assay was conducted.

Increasing the pH of the enzyme assay to 9.0 caused an increase in the level of H₂S detected in RLC (figure 2.19: A), RHC (figure 2.19: A) and PHC (figure 2.19: B). Therefore, there was an increase in H₂S detected in RHC, and there was a significant increase of H₂S detection produced via CBS at pH 9 in RHC (figure 2.19: A). Furthermore, there was a significant increase of H₂S detection produced via CBS at pH 9 in PHC (figure 2.19: B). Moreover, the pH 9 made the values relatively less variable than at pH 7.4 (figure 2.19: A and B).

PPG and AOAA both caused inhibition of H₂S production in RLC at pH 7.4 and pH 9.0 (figure 2.19: A). However, there was no significant effect on RHC (figure 2.20: A). The combination of PPG with AOAA had no further effect beyond that seen with PPG alone, in RLC (figure 2.19: A).

Furthermore, the combination of PPG with AOAA did not lead to a synergistic inhibition in the rat heart at either pH. Moreover, there was relatively more inhibition by PPG in the liver and by AOAA in the heart; however, both of them were non-significant differences between them, AOAA and PPG (figure 2.19: A). Moreover, at pH 9, there was more inhibition by AOAA than PPG in both atria and ventricles of PHC (figure 2.19: C).



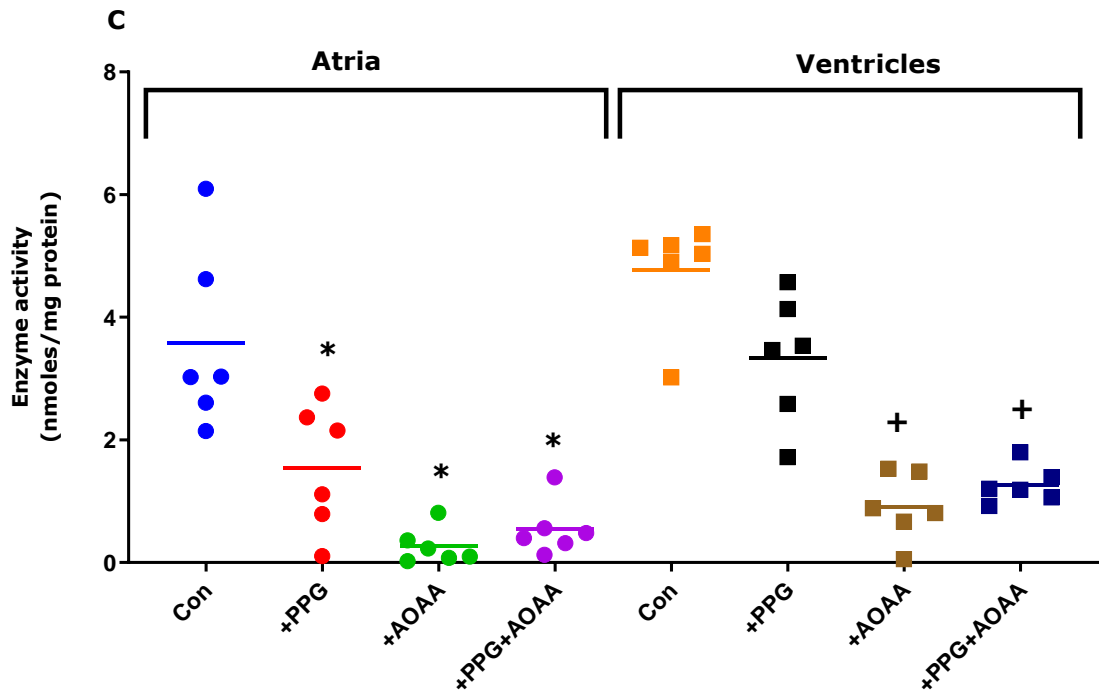


Figure 2.19: Comparison of the effect performing the methylene blue assay at pH 7.4 with pH 9 effects on H₂S production from L-cysteine in (A) rat liver cytosol (RLC) and rat heart cytosol (RHC) of four different animals (n=4). PPG (propargylglycine, CSE inhibitor, 100 μM); AOAA (aminooxyacetic acid, CBS inhibitor, 100 μM). (B) The effects of pH 7.4 vs pH 9.0 in PHC (porcine heart cytosol) (n=6). (C) The effects of PPG vs AOAA at pH 9 in PHC (n=6). The data presented as mean± SEM. ** (pH 7.4 RLC vs +PPG, +AOAA, +PPG+AOAA, pH 9 RLC). ## (pH 9 RLC vs AOAA, PPG, PPG+AOAA). \$ (pH 7.4 RLC vs +PPG+AOAA). * (RHC pH 7.4 vs + pH 9). ** (atria pH 7.4 vs pH 9.0). ** (ventricles pH 7.4 vs pH 9.0). * (atria vs +PPG, +AOAA, +PPG+AOAA). + (ventricles vs +AOAA, +PPG+AOAA). Con (control). NS (non-significant) p > 0.05, *, +, \$ p < 0.05, **, ## p < 0.01 using one-way ANOVA followed by Sidak's multiple comparisons test, (n=6).

2.5.2.1.7. Time profile of H₂S production at pH 9.0

As the data in PHC indicated an increase in H₂S detection at pH 9.0, an analysis of the pH sensitivity of the CBS assay at different incubation times (0, 30, 60 and 90 min) in PHC and RHC was performed. The results indicate that the optimal incubation time was 60 min at pH 9.0 in both PHC and RHC (figure 2.20).

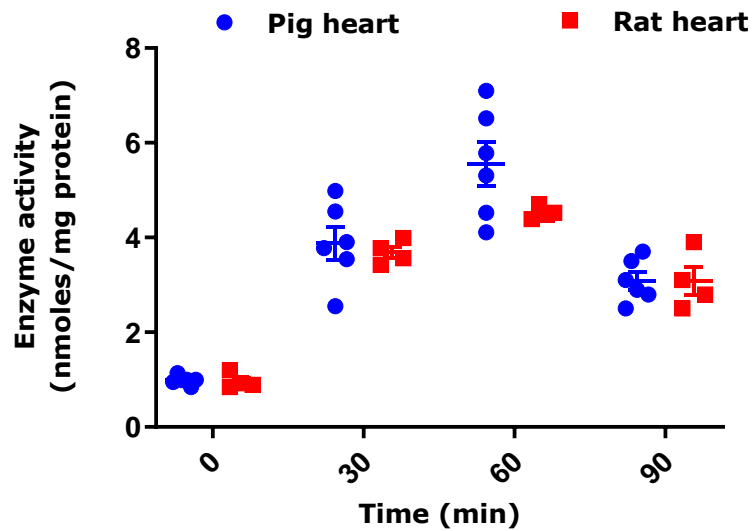


Figure 2.20: Effect of incubation time on detection of H₂S from L-cysteine in PHC and RHC using the methylene blue assay at pH 9.0. Data are expressed mean \pm SEM. PHC (pig heart cytosol) from six different animals (n=6). RHC (rat heart cytosol) from four different animals (n=4).

2.5.2.1.8. Effect of degassing buffers on H₂S production

It has been suggested that oxygen gas dissolved in the buffer may oxidise H₂S and decrease H₂S availability interfering with the detection of H₂S, so the effects of degassing the buffers prior to carrying out the methylene blue assay were investigated

The comparison of the degassing effects on enzyme activity for RLC revealed that there was a non-significant effect by degassing with different concentrations of L-cysteine (1, 3 and 10 mM) (figure 2.21).

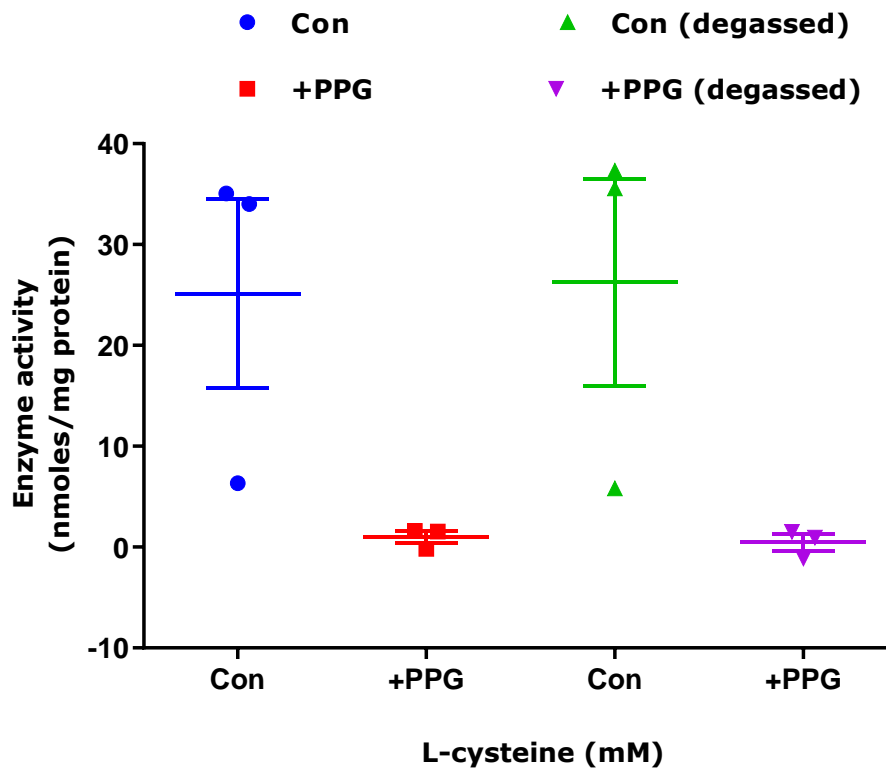


Figure 2.21: Effect of degassing (by vacuum chamber) on H₂S detection from L-cysteine using the methylene blue assay with different concentrations of L-cysteine (1, 3 and 10 mM) in RLC (rat liver cytosol) of three different animals. Data presented as mean \pm SEM. The results indicated non-significant effects of degassing. NS (non-significant) $p > 0.05$ using one-way ANOVA followed by Sidak's multiple comparisons test.

Degassing also had no effect on the level of H₂S detected in RHC at either pH 7.4 or pH 9.0 (figure 2.22). There was no significant effect of AOAA at pH 7.4, but a significant reduction in H₂S detection was seen at pH 9.0 in RHC (figure 2.22). Moreover, degassing had no effect on the detection of H₂S in PHC in either atria or ventricles (figure 2.22). Thus, degassing had no effect on the detection of H₂S in heart tissues either rat or pig hearts.

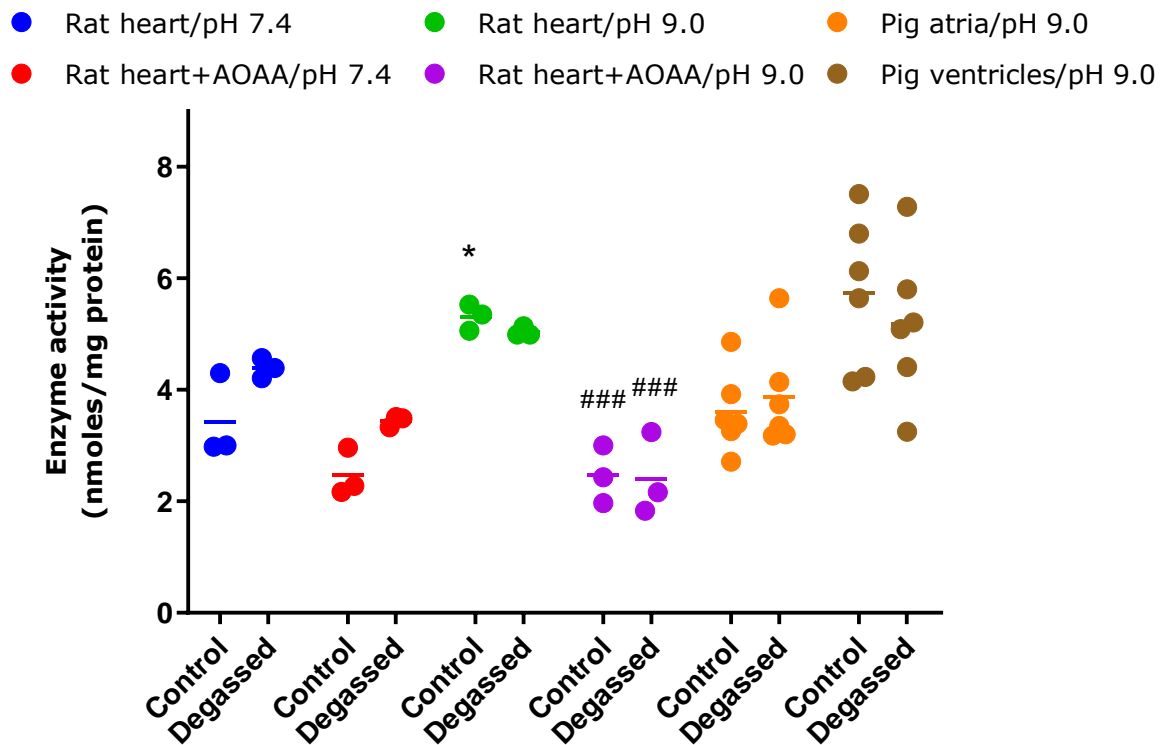


Figure 2.22: Effect of degassing on H₂S detection using the methylene blue assay at pH 7.4 and 9.0 in RHC (rat heart cytosol) of three different animals and in tissue from six pig ventricles and atria. AOAA (aminooxyacetic acid, 100 μM). Data are presented as mean ± SEM. *P<0.05 pH 9.0 vs pH 7.4, ###P<0.001 presence vs absence of AOAA using two-way ANOVA followed by Sidak's multiple comparisons test.

2.5.2.1.9. Effect of S-adenosyl-L-methionine on H₂S production

SAM has been described as a positive allosteric modulator of CBS and activator of CBS activity (Abe and Kimura, 1996), its effects were investigated in RHC and PHC. The effects of SAM (2 mM) on enzyme activity in RHC and PHC demonstrated that there were no significant effects of SAM (2 mM) on enzyme activity in RHC and PHC (figure 2.23).

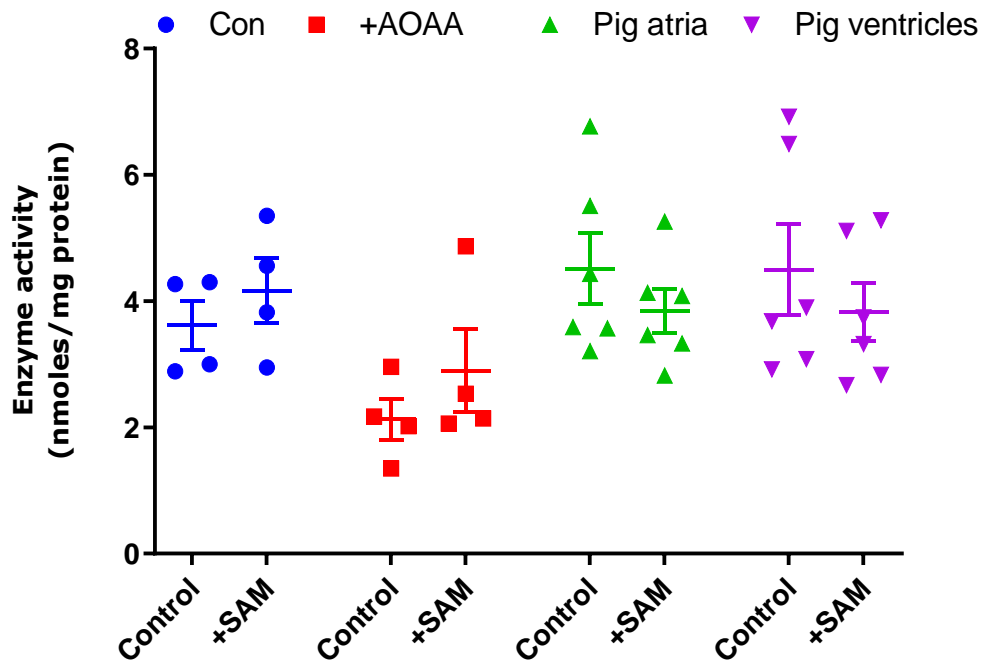


Figure 2.23: Effects of SAM (S-adenosyl-L-methionine, 2 mM) on H₂S produced in the presence of L-cysteine using the methylene blue assay in rat heart cytosol (RHC) of four different animals and PHC (n=6). Data are presented as mean \pm SEM. AOAA (aminooxyacetic acid, 100 μ M). There were no significant effects of SAM identified using two-way ANOVA followed by Sidak's multiple comparisons test.

Co-incubation of SAM (2 mM) with different concentrations of cysteine (1, 3, 10 mM) on enzyme activity in RLC showed that there was a non-significant effect of SAM (2 mM) on enzyme activity in RLC (figure 2.24). Furthermore, there was inhibition by PPG in the absence or presence of SAM (figure 2.24).

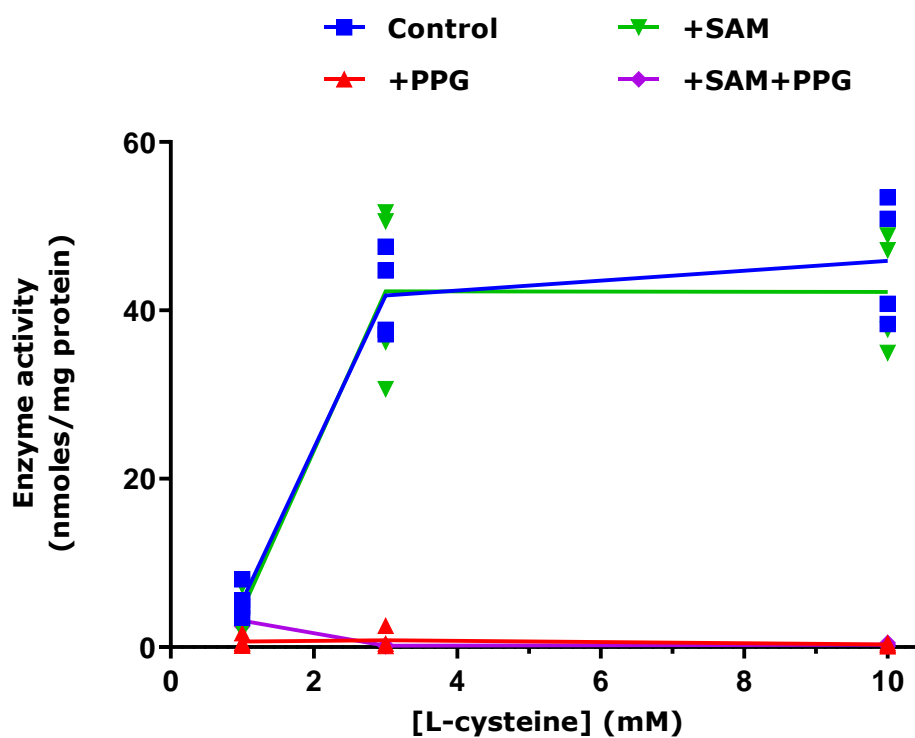


Figure 2.24: Effects of SAM (S-adenosyl-L-methionine, 2 mM) on cysteine-derived H₂S levels (n=4) using the methylene blue assay in rat liver cytosol (RLC). Data presented are mean± SEM. PPG (CSE-inhibitor, 100 μM). NS (non-significant) p > 0.05 using one-way ANOVA followed by Sidak's multiple comparisons test, (n=4).

SAM (2 mM) also failed to alter L-cysteine-dependent enzyme activity in rat brain cytosols (RBC). SAM also failed to alter the inhibitory effect of AOAA in RBC (figure 2.25).

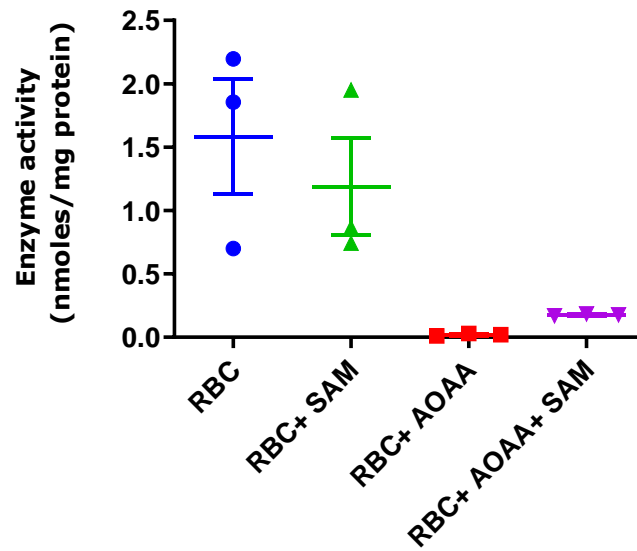
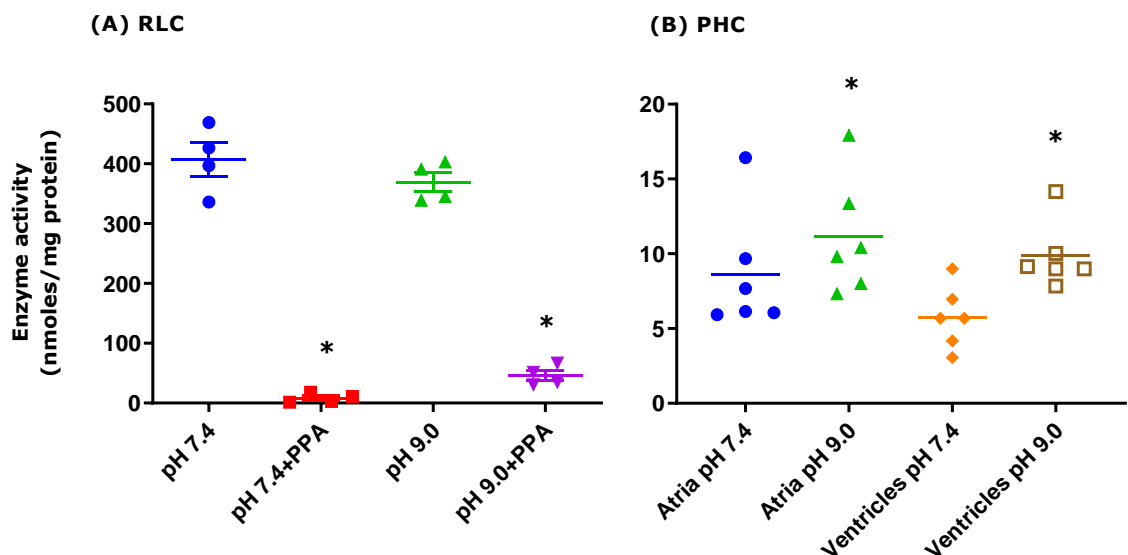


Figure 2.25: Effects of SAM (S-adenosyl-L-methionine, 2 mM) on 10 mM L-cysteine-derived H₂S levels using the methylene blue assay in rat brain cytosol (RBC). Data presented are mean \pm SEM. AOAA (aminooxyacetic acid, 100 μ M). NS (non-significant) $p > 0.05$ using one-way ANOVA followed by Sidak's multiple comparisons test, (n=3).

2.5.2.2. Measuring MST activity using the methylene blue detection method

The results of Western blotting demonstrated that MST was clearly detected in the PHC, but the relative abundance of MST in PHC was low in comparison with RLC. As H₂S in solution is more stable and detectable at alkaline pH (as it favours dissociation to hydrosulfide anion) (Shen et al., 2011; Ang et al., 2012; Shen et al, 2012), an analysis of the pH sensitivity of the MST assay was conducted. PPA (10 mM) was used to investigate the contribution of MST enzyme activity in RLC and PHC (Wing and Baskin, 1992; Shibuya et al., 2009; Rose et al., 2017).

The effect of pH on H₂S detection from mercaptopyruvate in RLC indicated that there was no difference in H₂S detected at pH 9 compared to pH 7.4 (figure 2.26: A). Furthermore, there was significant decrease in H₂S detection from mercaptopyruvate by PPA at pH 7.4 and pH 9 in RLC (figure 2.26: A). On the other hand, there was a significant increase in the level of H₂S detected in atria and ventricles from pig heart at pH 9 compared to pH 7.4 (figure 2.26: B). Moreover, there was no significant difference in the level of H₂S detected in atria compared to ventricles in pig heart at pH 9 (figure 2.26: C). Overall, there was more H₂S production from mercaptopyruvate than L-cysteine.



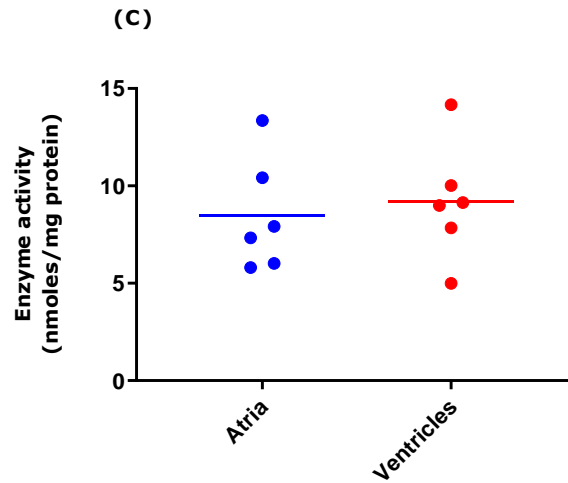


Figure 2.26: The effect of incubation pH on mercaptopyruvate-derived H₂S detected using the methylene blue assay in (A) rat liver cytosol (RLC) (n=4) and also in (B) PHC (porcine heart cytosol) and (C) at pH 9.0 from atria and ventricles (n=6). Data presented as mean. PPA (10 mM, phenylpyruvic acid, MST inhibitor). * p < 0.05 using two-tailed paired Student's t-test.

The aim of this experiment was to determine the effect of the potential (candidate) MST inhibitor PPA on H₂S detected from mercaptopyruvate in RHC, PHC of atria and ventricles, and RLC at pH 7.4. The comparison of the MST enzyme activity for RHC, PHC atria and ventricles with RLC showed that there was a significantly lower MST enzyme activity in RHC in comparison to RLC (figure 2.27: A) and also in PHC atria and ventricles compared to RLC at pH 7.4 (figure 2.27: B). MST activity level of both RHC and RLC was higher than PHC of atria and ventricles (figure 2.27: A and B). Furthermore, there was a significant decrease in H₂S detection from mercaptopyruvate by PPA in RHC (figure 2.27: A), PHC atria and ventricles (figure 2.27: B) and RLC (figure 2.27: A and B).

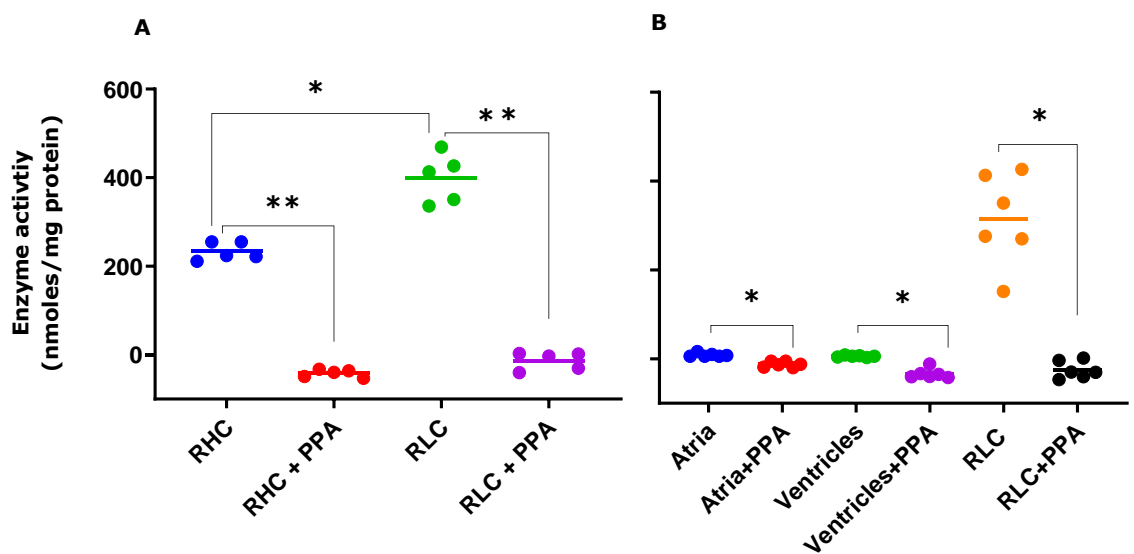


Figure 2.27: Comparison of MST enzyme activity in (A) rat heart cytosol (RHC) of five different animals (n=5) in the absence and presence of PPA (10 mM, phenylpyruvic acid). (B) Comparison of MST enzyme activity in porcine heart cytosol (PHC) atria and ventricles of six different animals (n=6) in the absence and presence of PPA (10 mM, phenyl pyruvic acid). Data presented as mean. ** p <0.01 using two-tailed paired Student's t-test; * p <0.05 using one-way ANOVA vs control followed by Sidak's post-hoc test.

There was a high MST activity level in RHC in comparison to PHC, but both of RHC and PHC MST activity level was low in comparison to RLC (figure 2.27: A and B). About one-third of 3-MST is reported to be in the cytosol and two-thirds in mitochondria (Lavu et al., 2011; Paul and Snyder 2012; Fräsdorf et al., 2014). Therefore, the aim of this experiment was to compare MST activity in mitochondria obtained from rat heart (particulate fraction) in the absence and presence of PPA (10 mM) at pH 7.4.

The comparison of MST activity in RHM (rat heart mitochondria) with RLC showed that there was high level of activity in RHM and RLC at pH 7.4, although the activity was significantly higher in RLC than RHM (figure 2.28). Moreover, this activity was significantly reduced in the presence of 10 mM PPA (figure 2.28).

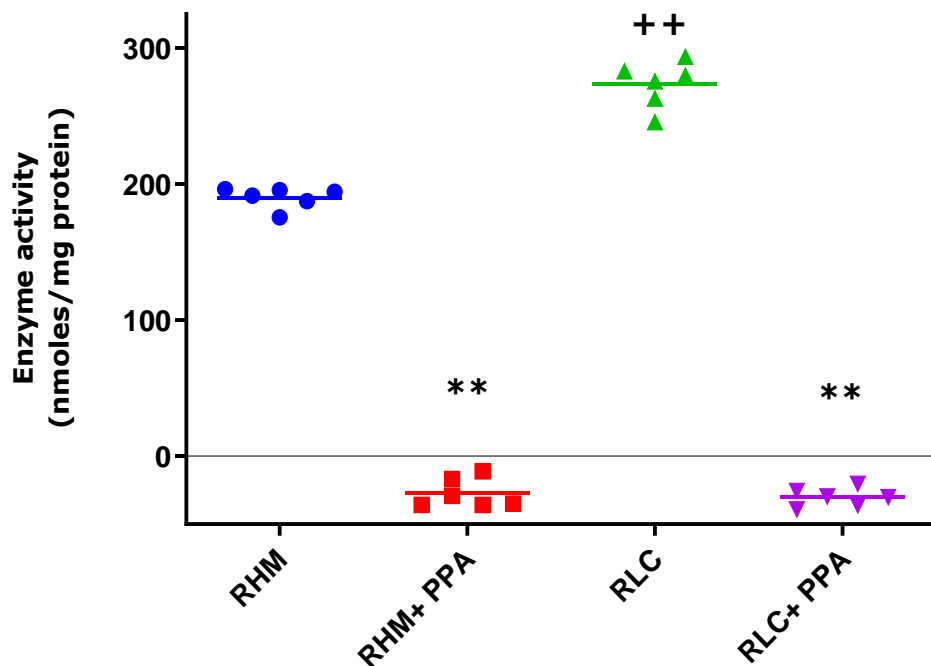
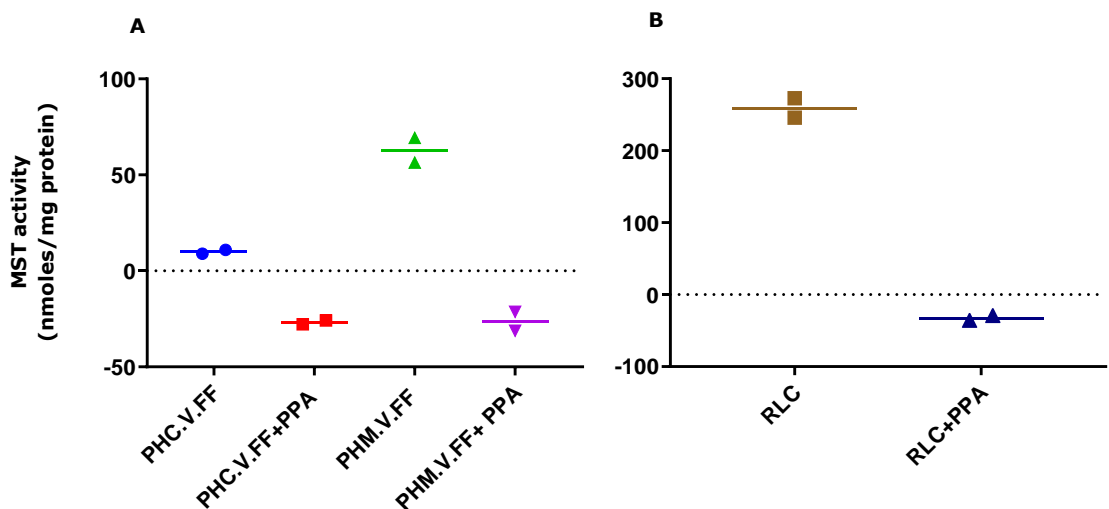


Figure 2.28: Comparison of MST activity in rat heart mitochondria (RHM) of six different animals with rat liver cytosol (RLC). Data presented as mean. PPA (10 mM, phenylpyruvic acid). ** $p < 0.01$ using two-tailed paired Student's t-test. ++ $p < 0.01$ RHM vs RLC using two-tailed unpaired Student's t-test, (n=5).

2.5.2.2.2. MST and CBS activity in porcine ventricles

In order to decrease the possible deterioration of porcine heart tissue by handling, storage and transport and therefore optimising tissue preparation and quality (more fresh), we investigated whether freezing heart tissue on dry ice at the abattoir could improve the signal for both MST and CBS activity in both cytosol and particulate/mitochondria fractions.

The comparison of the MST and CBS activity levels in freshly and rapidly frozen (PH.V.FF) cytosol and mitochondria from pig heart with RLC demonstrated that there was still low MST activity (figure 2.29: A and B) and CBS activity (figure 2.29: C and D) in freshly/rapidly frozen (PH.V.FF) cytosol and particulate/mitochondria (figure 2.29: A and C) in comparison with RLC (figure 2.29: B and D) at pH 7.4. Also, there was more H₂S production from via MST from mercaptopyruvate in mitochondrial fraction than cytosol of freshly frozen left pig heart tissues (figure 2.29: A) and via CBS from L-cysteine in mitochondrial fraction than cytosol of freshly frozen left pig heart tissues (figure 2.29: C).



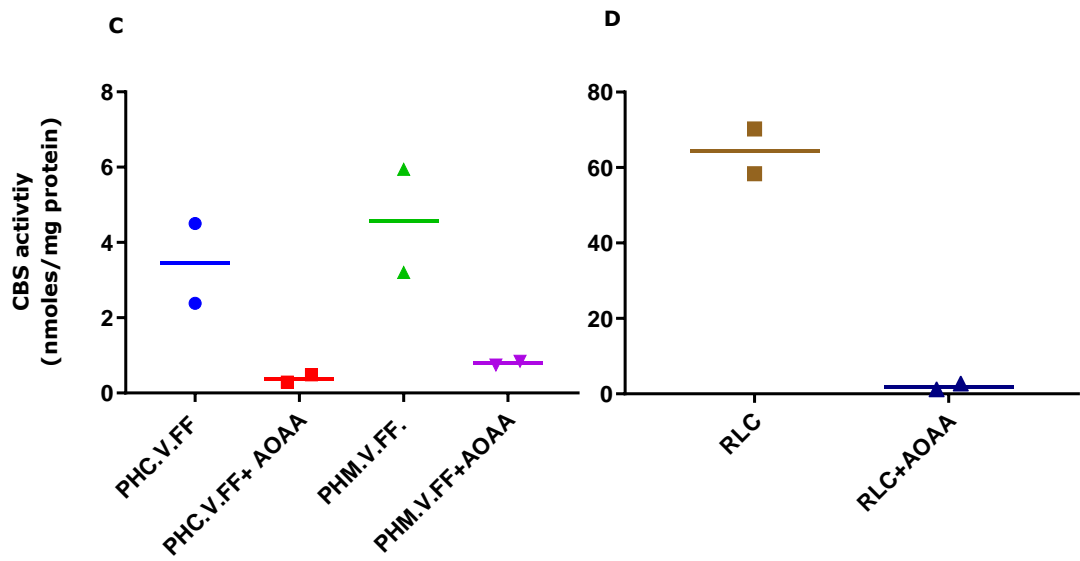
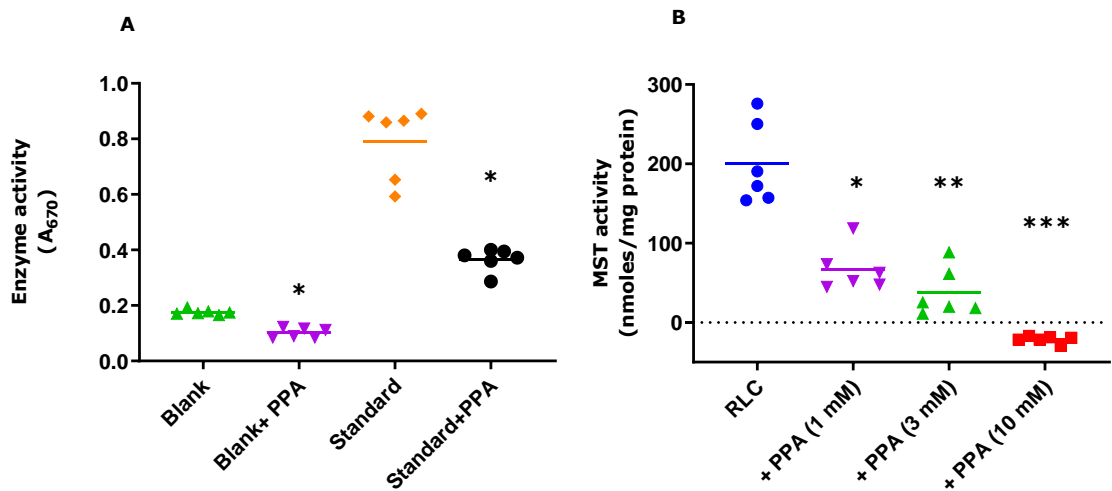


Figure 2.29: Comparison of enzyme activity in porcine heart freshly/rapidly frozen ventricle cytosol (PHC.V.FF) and particulate/mitochondria (PHM.V.FF): (A) MST activity with (B) rat liver cytosol (RLC, positive control) and also (C) (PHC.V.FF) cytosol and particulate/mitochondria (PHM.V.FF) CBS activity with (D) RLC at pH 7.4. (A and B) MST activity; PPA (10 mM, phenylpyruvic acid). (C and D) CBS activity; AOAA (100 μ M, aminoxyacetic acid, CBS inhibitor). Data presented as mean, (n=2).

2.5.2.2.2. Effect of PPA on MST activity

The experiments presented above indicate that phenylpyruvic acid reduced the production of H₂S from mercaptopyruvate. In order to confirm these data, the aim of this experiment was to assess the effect of PPA on the production of H₂S in RLC in comparison with no tissue blank and the standard (Na₂S, 100 μM). PPA caused a concentration-dependent reduction in H₂S detected in both RLC, blank and Na₂S, indicating/suggesting a non-selective effect of PPA, especially at 10 mM (figure 2.30: A, B and C).



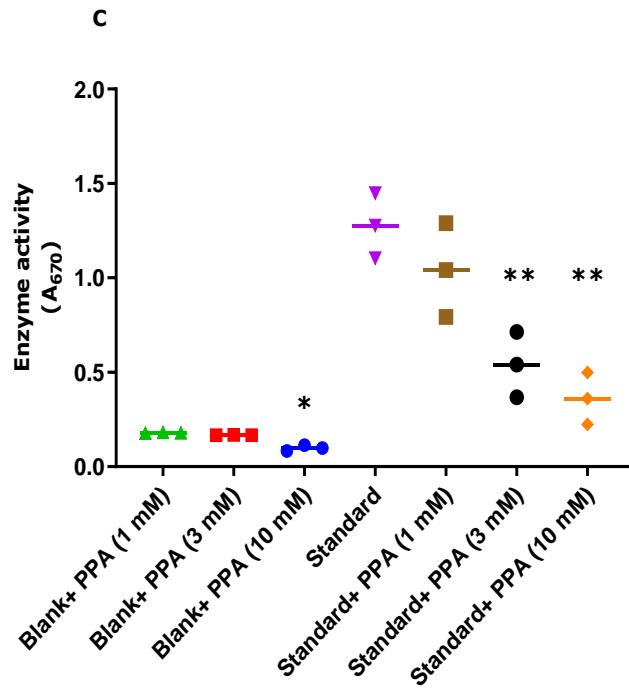


Figure 2.30: Comparison of the effect of PPA (10 mM, phenylpyruvic acid, MST inhibitor) on (A) the blank (tissue blank) and standard (Na₂S, 100 μM) (n=6). (B) PPA effect (1, 3 and 10 mM, phenylpyruvic acid) on RLC (rat liver cytosol) (n=6). (C) PPA effect (1, 3 and 10 mM, phenylpyruvic acid) on the tissue blank and standard (Na₂S, 100 μM) (n=3). Data presented as mean. * p <0.05, ** p <0.01, *** p <0.001 vs respective control using one-way ANOVA followed by Sidak's multiple comparisons test.

2.5.2.3. Monitoring H₂S production using SF7-AM fluorescence

The main aims of these experiments were to determine whether H₂S synthesis could be detected in heart tissue using the fluorescent probe SF7-AM. RLC was used as a positive control. Na₂S was used as a standard.

There was measurable (detectable) but low activity in PHC atria and ventricles at pH 7.4 in comparison with RLC (figure 2.31). There was no significant effect of AOAA and PPG in PHC (figure 3.31). On the other hand, there was significant inhibition of CBS activity by AOAA and PPG in RLC (figure 2.31).

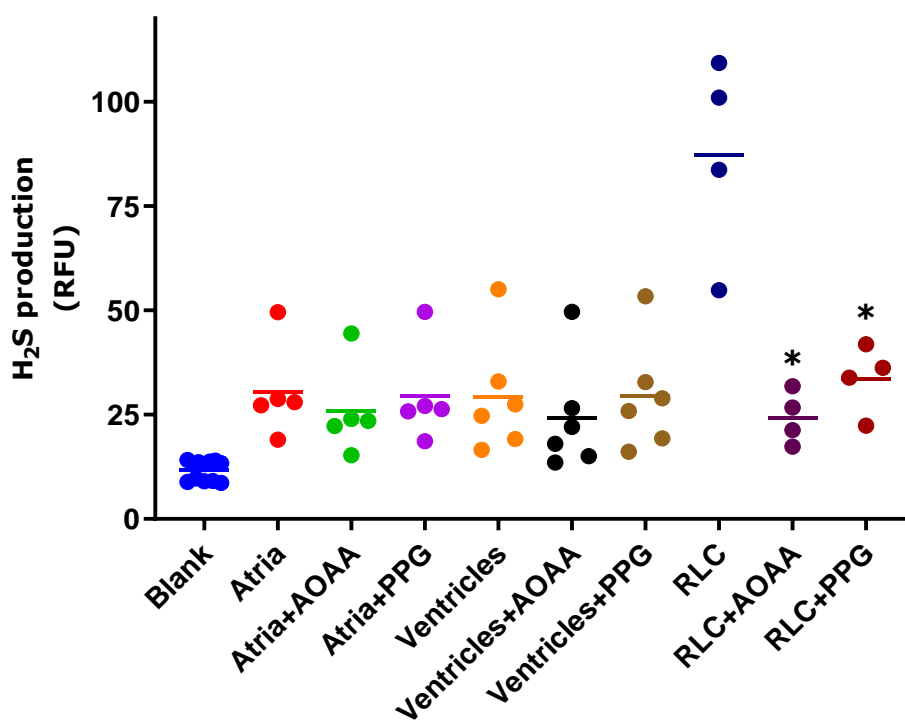


Figure 2.31: Comparison of CBS activity (fluorescence) vs blank (no tissue blank) in porcine heart cytosols (PHC) atria and ventricles with RLC (rat liver cytosol). AOAA (aminoxyacetic acid, 100 μ M CBS inhibitor); PPG (propargylglycine, 100 μ M, CSE inhibitor). The y-axis represents relative fluorescence unit (RFU). Data presented as mean. * $p < 0.05$ vs RLC using one-way ANOVA followed by Sidak's multiple comparisons test, (n=5).

2.5.2.3.1. Na₂S as a standard

In order to validate the SF7-AM assay, Na₂S was used as a standard. There was a linear relationship between the fluorescence measured and Na₂S concentrations (figure 2.32).

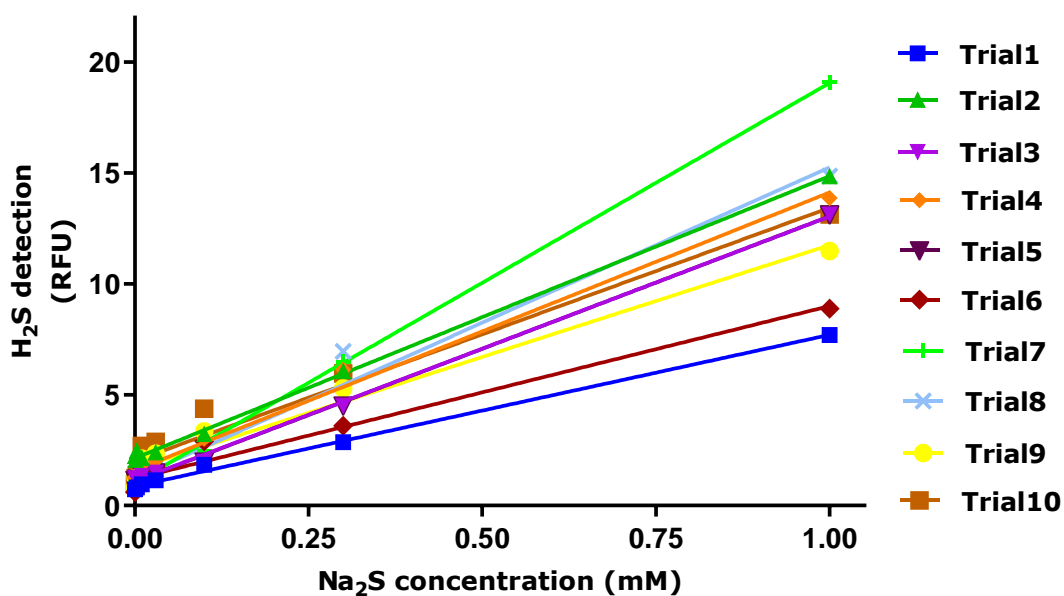


Figure 2.32: A linear relationship between the fluorescence measured by SF7-AM and sodium sulfide (Na₂S; standard) concentrations. The y-axis represents relative fluorescence unit (RFU). (n=10).

2.5.2.3.2. CBS activity in atria and ventricles

The aim of this experiment was to compare SF7-AM-mediated detection of CBS activity in porcine heart cytosol from atria and ventricles with AOAA and PPG at pH 7.4. Also, to compare CBS activity at pH 7.4 vs pH 9.0.

AOAA, but not PPG, evoked a significant inhibition in both PHC atria and ventricles (figure 2.33: A). Also, there was a significant increase of H₂S detection produced via CBS at pH 9.0 in comparison to pH 7.4 (figure 2.33: B).

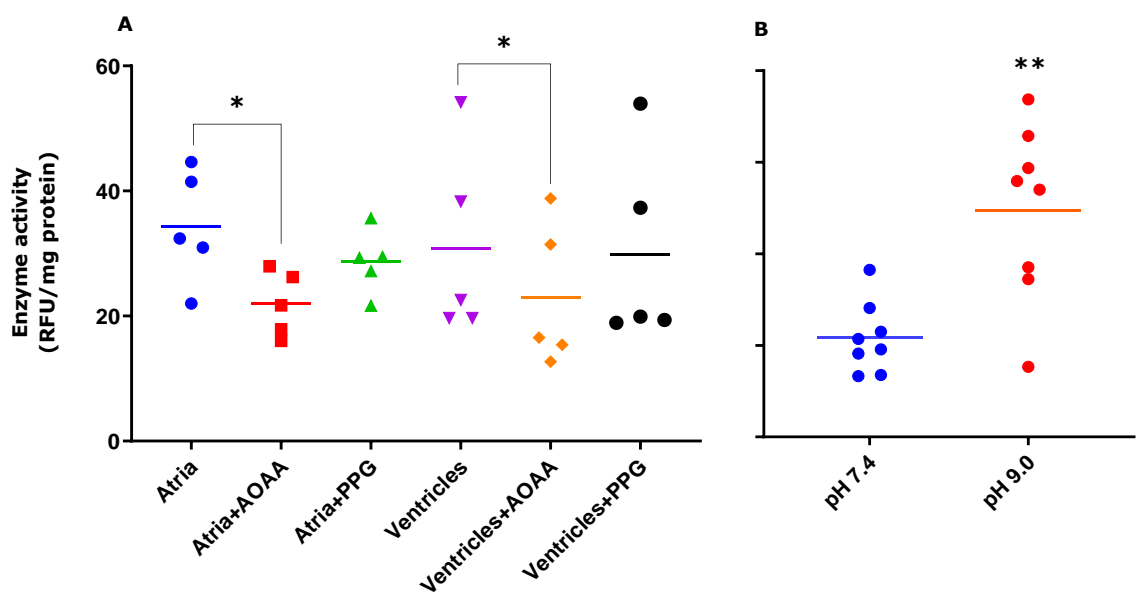
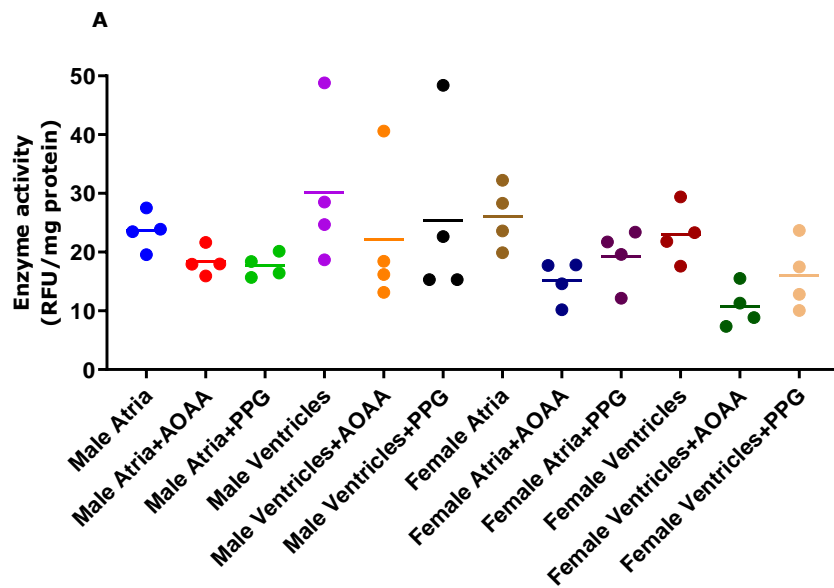


Figure 2.33: Comparison of CBS activity in (A) porcine heart cytosols (PHC) atria and ventricles (n=5) and in (B) pH 7.4 vs pH 9 in PHC (n=8). AOAA (aminooxyacetic acid, 100 μ M CBS inhibitor); PPG (propargylglycine, 100 μ M, CSE inhibitor). The y-axis represents relative fluorescence unit (RFU). Data presented as mean. * $p < 0.05$ vs control using one-way ANOVA followed by Sidak's multiple comparisons test, (n=5). ** $p < 0.01$ vs control using two-tailed paired Student's t-test, (n=8).

2.5.2.3.3. The influence of gender on CBS activity

Several pieces of clinical and epidemiological research have revealed that sexual dimorphism has a crucial effect on the cardiovascular system (Bucci et al., 2009; Brancaleone et al., 2015). Therefore, gender is an important factor which affects some biological functions and therefore, the gender may have an effect on CBS activity. For example, Brancaleone's study has reported that testosterone has a pivotal role in H₂S-mediated vasorelaxation in male rats (Brancaleone et al., 2015). Thus, it could be important to assess whether gender influences cardiac H₂S-generating enzyme activity (Abe and Kimura, 1996; Bucci et al., 2009; Brancaleone et al., 2015).

Therefore, the aim of these experiments was to assess gender effect on CBS enzyme activity in PHC atria and ventricles. The results of this study showed that there was no difference between male and female for CBS activity in PHC at both pH 7.4 and 9.0, respectively (figure 2.34: A and B).



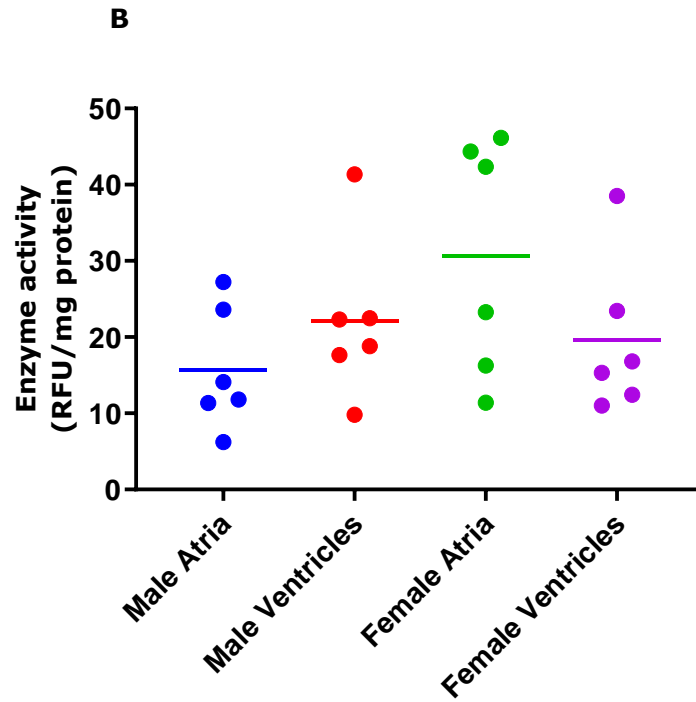


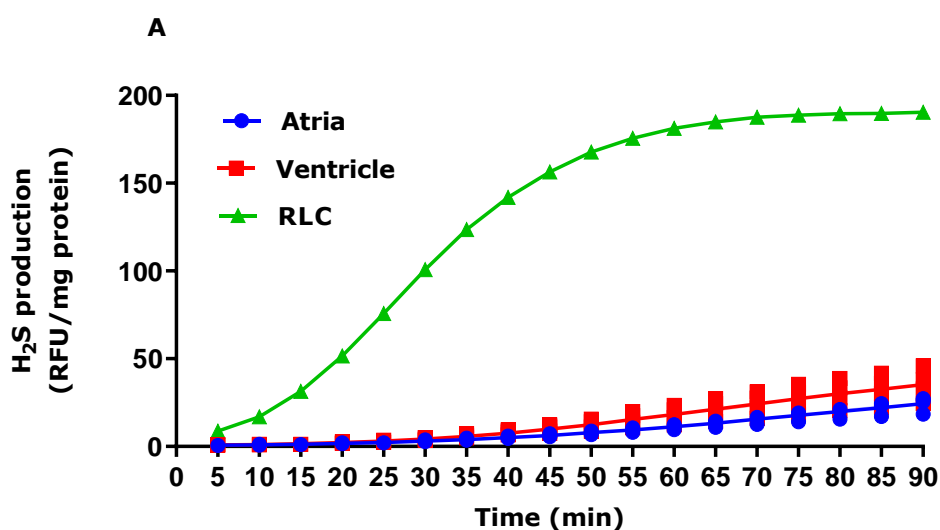
Figure 2.34: Comparison of CBS activity in porcine heart cytosols (PHC) atria and ventricles (A) at pH 7.4 and also (B) at pH 9 in males and females. AOAA (aminoxyacetic acid, 100 μ M CBS inhibitor); PPG (propargylglycine, 100 μ M, CSE inhibitor). The y-axis represents relative fluorescence unit (RFU) per mg of protein. Data presented as mean, (n=6).

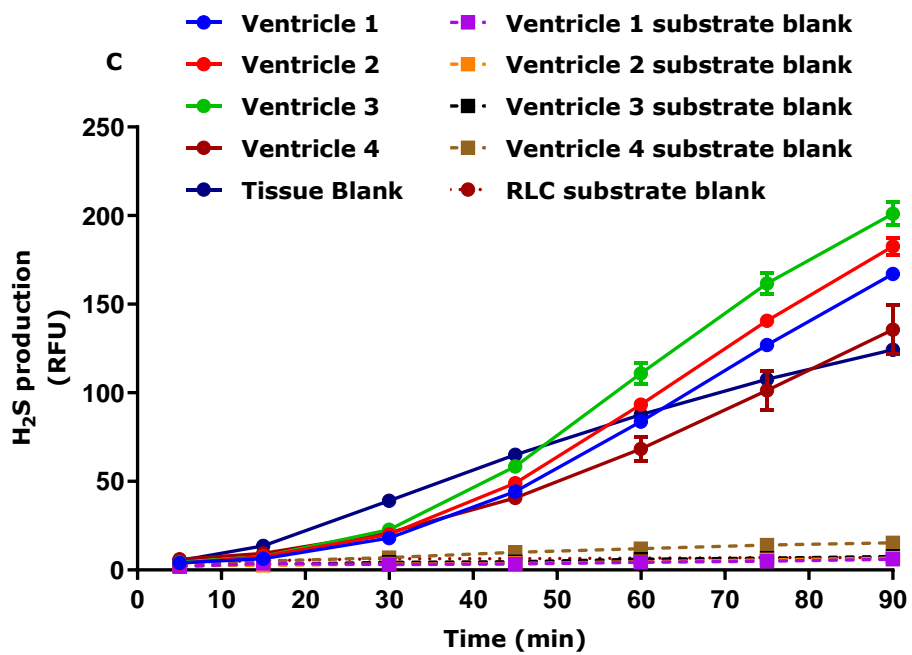
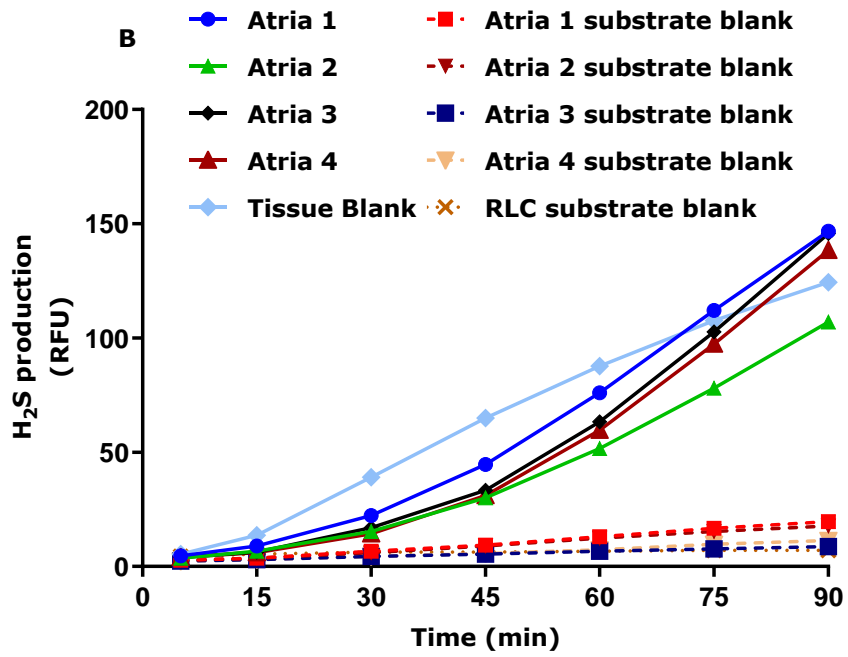
2.5.2.4. Measurement of MST enzyme activity using SF7-AM

2.5.2.4.1. MST activity in porcine atria and ventricles cytosols

The time profile for detection of H₂S through the MST pathway appeared to be 90 min for cytosolic fraction of porcine atria and ventricles and RLC (figure 2.35: A, B, C, and D). There was a low activity in PHC atria and ventricle compared to RLC (figure 2.35: A). Tissue blank (no tissue, no enzyme) was higher than the substrate blank (no mercaptopyruvate) (figure 35: B and C). After 90 minutes there was a plateau and a decrease of enzyme activity (figure 2.35: D).

Fluorescence in PHC atria and ventricles (figure 2.35: A). Fluorescence in PHC atria and ventricles and RLC all corrected with tissue blank and normalised per mg of protein (figure 2.35: A). Fluorescence in atria, substrate blank (no mercaptopyruvate) and tissue blank (no tissue) (figure 2.35: B). Ventricles substrate blank (no mercaptopyruvate) and tissue blank (no tissue) (figure 2.35: C). PHC atria and ventricles corrected with tissue blank and normalised per mg protein (figure 2.35: D).





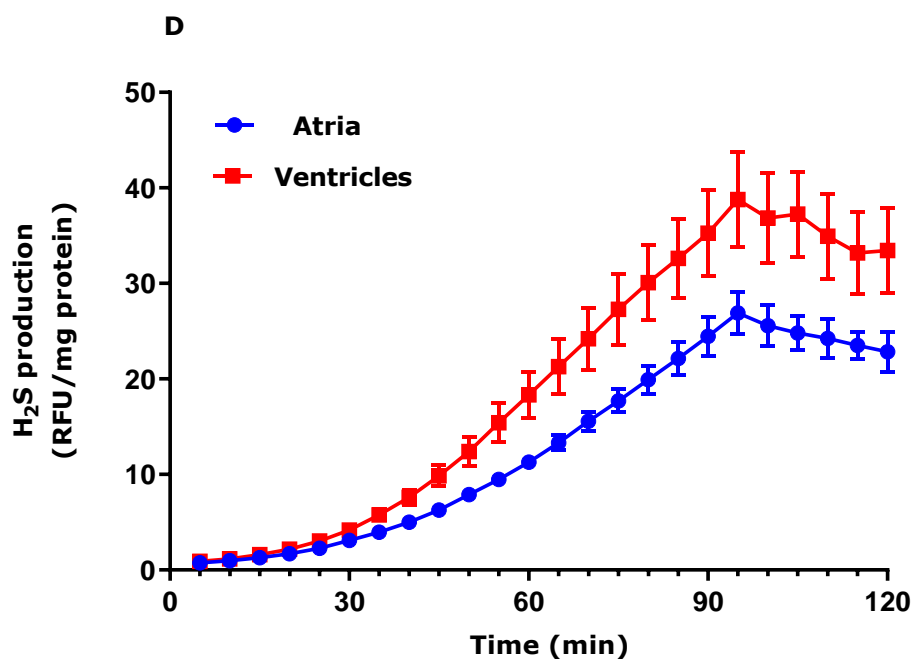
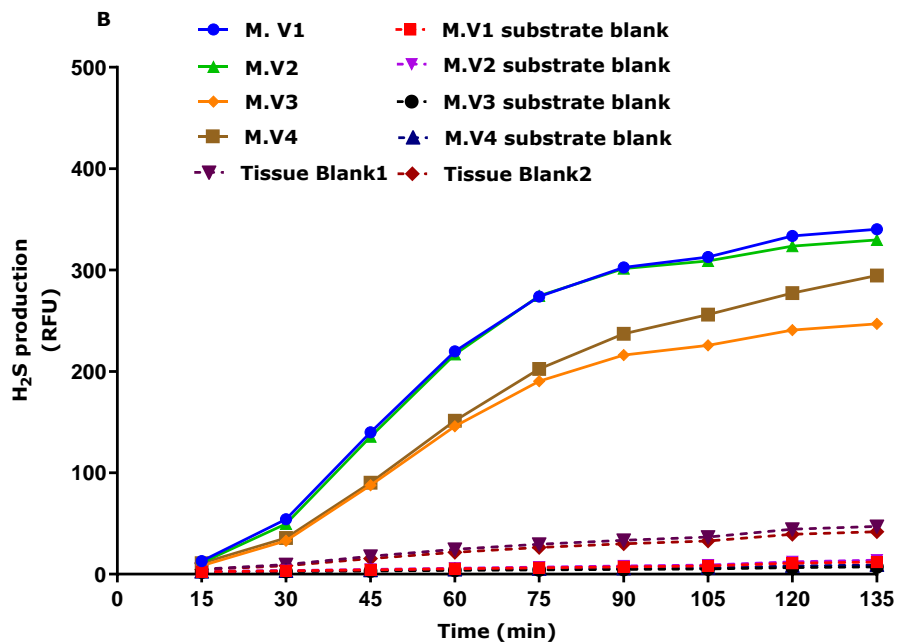
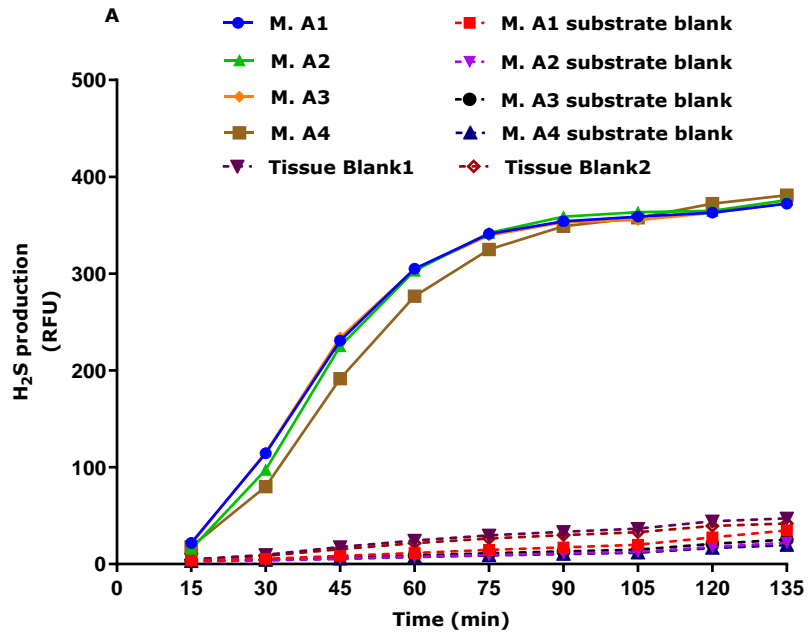


Figure 2.35: Incubation time-profile of H₂S production using SF7-AM: (A) PHC atria and ventricles and RLC corrected with tissue blank and normalised per mg protein; (B) atria, substrate blank (no mercaptopyruvate) and tissue blank (no tissue); (C) ventricles, substrate blank (no mercaptopyruvate) and tissue blank (no tissue); (D) PHC atria and ventricles corrected with tissue blank and normalised per mg protein. The y-axis represents relative fluorescence unit (RFU) per mg protein. (n=4).

2.5.2.4.2. Particulate fractions from atria and ventricles

There was a low MST activity level in PHC in comparison to RLC. Therefore, the aim of these experiments was to detect the optimal incubation time for detection of H₂S in particulate (including mitochondria) fractions from atria and ventricles. The results of this study demonstrated that the most appropriate incubation time appeared to be 90 min and after 90 minutes there was a plateau of enzyme activity (figure 2.36: A, B and C). There was a similar level of activity level in PHM (figure 2.36: A and B) compared to RLC (figure 2.36: C). Moreover, we observed that the tissue blank (no tissue, no enzyme) was higher than the substrate blank (no mercaptopyruvate) (figure 2.36: A, B and C).



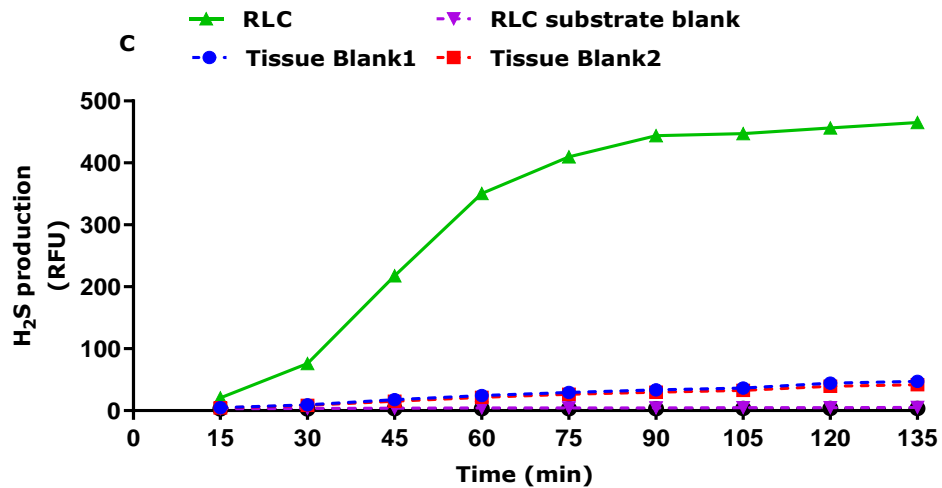


Figure 2.36: Incubation time-profile and fluorescence in PHM (porcine heart mitochondria/particulate, M) from (A) atria, substrate blank (no mercaptopyruvate) and tissue blank (no tissue); (B) ventricles (V), substrate blank (no mercaptopyruvate) and tissue blank (no tissue); (C) RLC (rat liver cytosol), substrate blank (no mercaptopyruvate) and tissue blank (no tissue). The y-axis represents relative fluorescence unit (RFU). (n=4).

2.5.2.4.2.1. Effect of PPA on MST activity

The results of this experiments showed that, there was a significant concentration-dependent inhibition of the H₂S production with difference with the addition of PPA (1, 3, and 10 mM) while it was non-significant with 0.1 and 0.3 mM in atria, ventricles and RLC (figure 2.37: A, B, and C).

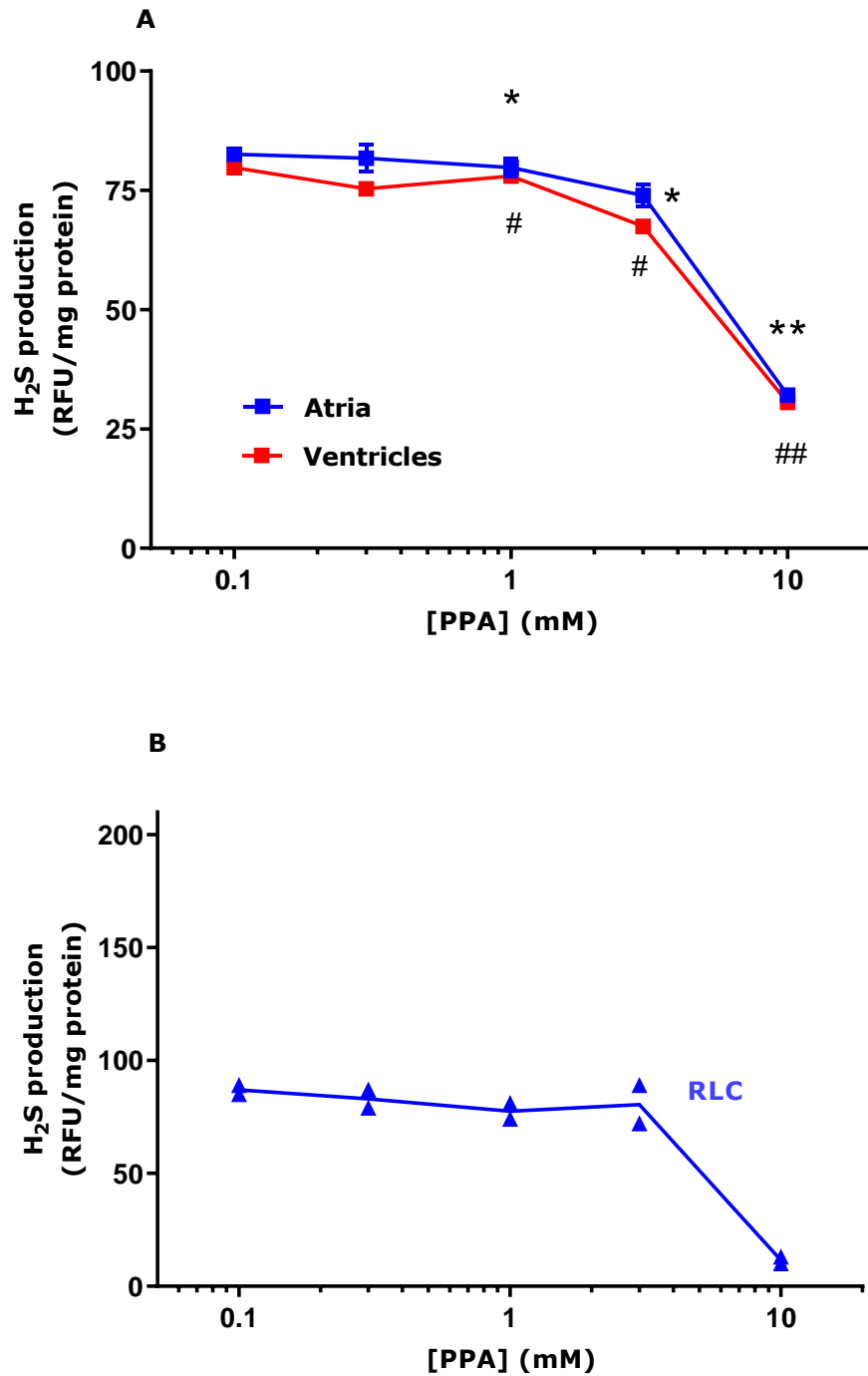


Figure 2.37: Comparison the effect of PPA (0.1, 0.3, 1, 3 and 10 mM, phenylpyruvic acid) on MST activity measured by SF7-AM in (A) particulate (PHM) atria and ventricles; (B) RLC (rat liver cytosol). The y-axis represents relative fluorescence unit (RFU). Data presented as mean. * (atria vs atria+ 1 mM PPA), * (atria vs atria+3 mM PPA), ** (atria vs atria+10 mM PPA); # (ventricles vs ventricles+1 mM PPA), # (ventricles vs ventricles+3 mM PPA), ## (ventricles vs ventricles+10 mM PPA). *, # p < 0.05, **, ## p< 0.01 using one-way ANOVA followed by Sidak's multiple comparisons test, (n=4).

2.5.2.4.3. pH sensitivity of MST activity

The aim of these experiments was to assess the differences between PHC atria and ventricles at pH 9 as H₂S in solution is more stable and detectable at alkaline pH (as it favours dissociation to hydrosulfide anion).

The comparison of PHC atria and ventricles enzyme activity at pH 9 indicated that there was a non-significant difference between PHC atria and ventricles at pH 9 compared to pH 7.4 (figure 2.38: A and B).

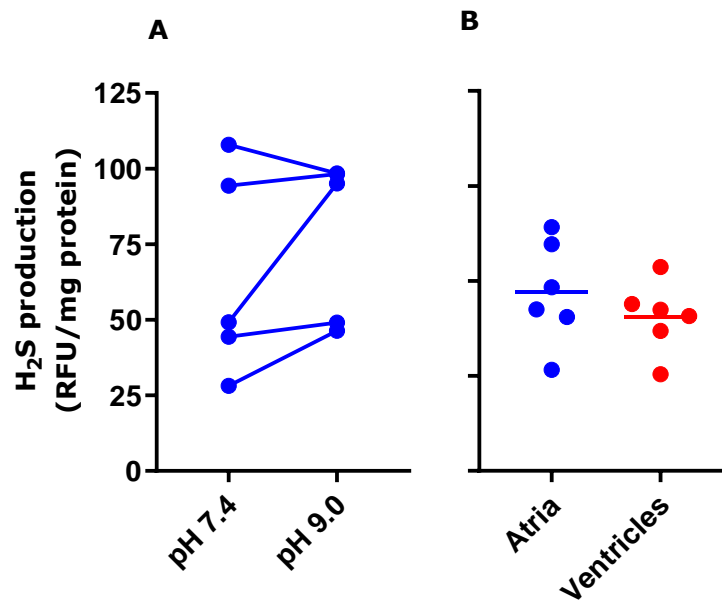


Figure 2.38: Comparison of MST activity in (A) PHC at pH 7.4 vs 9.0 (n=5) and (B) PHC atria vs ventricles at pH 9.0 (n=6). The y-axis represents relative fluorescence unit (RFU). Data presented as mean. Non-significant effects $p > 0.05$ using two-tailed unpaired Student's t-test.

2.5.2.4.4. The effects of dialysis

Given the relatively low activity in these tissues, there was the possibility that low molecular weight moieties might be interfering with the SF7-AM assay.

There was an increase in H₂S detected through both the CBS pathway (figure 2.39: A and B) and MST pathway (figure 2.40: A and B) in dialysed porcine heart cytosol (PHC) atria and ventricles at pH 7.4 in both males and females (figure 2.39: A and B; figure 2.40: A and B).

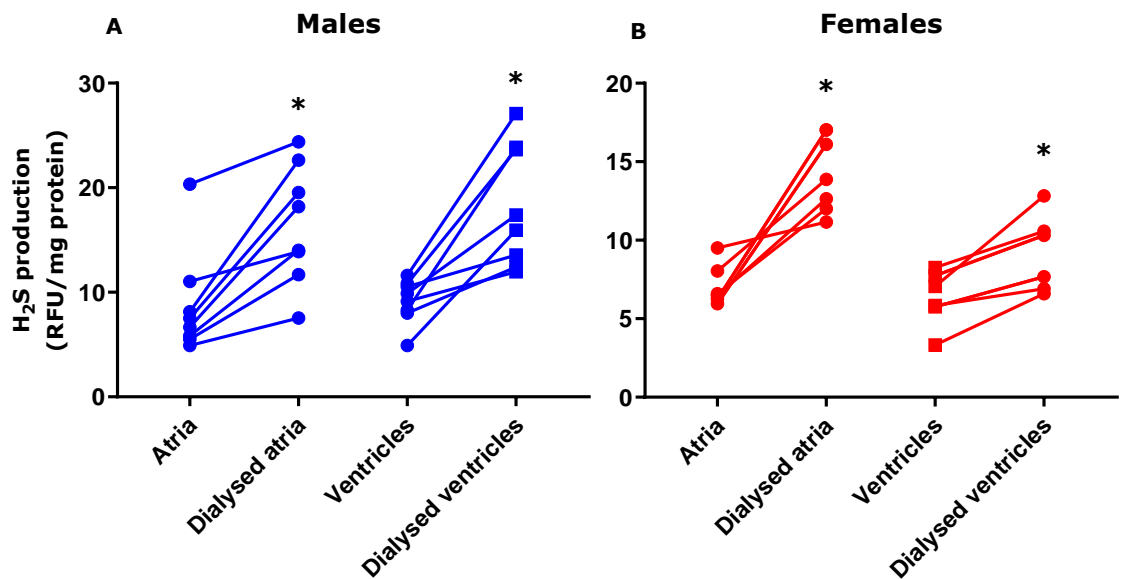


Figure 2.39: The comparison of CBS activity between non-dialysed and dialysed porcine heart cytosol (PHC) atria and ventricles at pH 7.4; (A) males and (B) females. The y-axis represents relative fluorescence unit (RFU). Data presented as mean. * $p < 0.05$ using two-tailed paired Student's t-test ($n=8$).

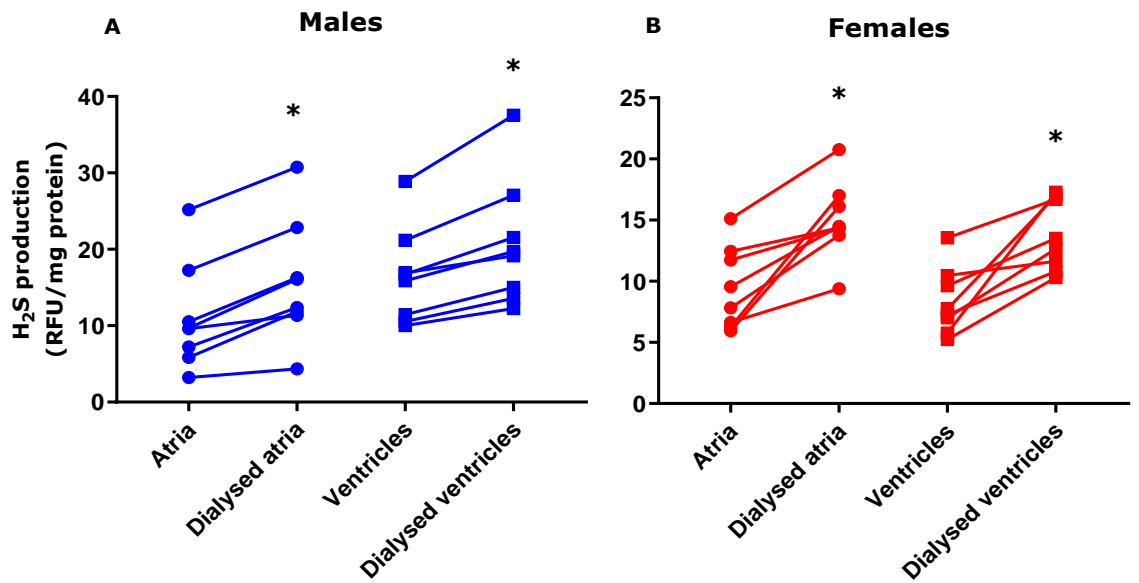


Figure 2.40: The comparison of MST activity between non-dialysed and dialysed porcine heart cytosol (PHC) atria and ventricles at pH 7.4; (A) males and (B) females. The y-axis represents relative fluorescence unit (RFU). Data presented as mean. * $p < 0.05$ using two-tailed paired Student's t-test, (n=8).

2.5.3. The effect of myocardium-derived H₂S on the relaxation responses in U46619-precontracted PCA

Data presented in this chapter demonstrated that H₂S could be endogenously synthesised in the myocardium. Therefore, in order to determine whether H₂S from myocardium could regulate coronary artery tone, the effect of myocardial tissue on the tone in U46619-precontracted PCA was determined.

2.5.3.1. Tissue Preparation

Hearts from large white hybrid pigs of either sex, 4-6 months of age, and weighing about 50 kg were obtained from a local abattoir and transported to the laboratory on ice cold Krebs'-Henseleit buffer ([mM]: NaCl 118, KCl 4.8, CaCl₂ 1.3, NaHCO₃ 25, KH₂PO₄ 1.2, MgSO₄ 1.2, glucose 11.1) (Fisher Scientific, Loughborough, UK). The Krebs'-Henseleit solution had been pre-gassed with 95% O₂/ 5% CO₂. The left circumflex porcine coronary artery (PCA) was then dissected out.

2.5.3.2. Isolated organ bath experiments

The PCA was maintained in Krebs'-Henseleit solution pre-gassed with 95% O₂ and 5% CO₂ for overnight storage at 4 °C. The next day, the myocardial tissue adherent/under/beneath the PCA was dissected and kept in Krebs'-Henseleit solution. Next the fat and connective tissue were removed from the left PCA, vessels finely dissected to prepare paired PCA rings from each artery ensuring vessels were not stretched/ or damaged and then cut into rings of ~5 mm in length (~2-3 mm internal diameter) and were suspended in a multichannel 5 or 20 ml tissue baths. Rings were attached to two metal hooks placed through the lumen, ensuring that the hooks were not overlapping. One hook was connected to a glass rod, and the other was connected to an isometric force transducer by a silk thread (unstretchable). Each bath was filled with Krebs'-Henseleit solution and maintained at 37 °C and constantly gassed with 95% O₂, 5% CO₂. After about 20 min equilibration period, the tension was applied to each segment, 8-10 g, and tissues allowed to equilibrate for about 60 min. Previous experiments have indicated that 8-10 g was the optimum level of tone for the PCA. This tension was measured using an isometric force transducer and recorded using a Power-Lab data acquisition system computer program (AD Instruments, Sydney, Australia). Transducers were calibrated with a 10 g weight on a daily basis.

When a stable baseline was reached, 60 mM KCl was added to allow normalization and confirm tissue viability. After about 10 min, the KCl was washed out with fresh Krebs'-Henseleit solution. Following return to a stable baseline and, after a further 20-30 min, exposure to KCl was repeated. Once again, the tissue was washed out

with Krebs'-Henseleit solution, to allow tone to re-stabilise to baseline. U46619, a thromboxane A₂ mimetic contractile agent (11 α ,9 α -epoxymethano-PGH₂) was used to induce contraction of PCA rings. PCA rings were exposed to U46619 (1 nM-300 nM) in a cumulative manner to about 40-60% of the final response to KCl contraction.

2.5.3.3. Effect of myocardial tissue on U46619-induced tone

These experiments were performed in order to assess the effect of the myocardial tissue (adherent/under/beneath the PCA) on the vascular tone, in particular, the relaxation response. In other words, these experiments aimed to investigate the functional consequences of H₂S production in myocardial tissue on vascular function as a bioassay. U46619 (thromboxane A₂ mimetic vasoconstrictor) was used to induce tone. PCA rings were exposed to U46619 (1-300 nM) in a cumulative manner to about 40-60% of the final KCl contraction response. After the contraction had reached a plateau, the myocardial tissue was added (about 0.25 g/ 5 ml-bath or about 1 g/ 20 ml-bath). Then, the tension was recorded for about 60 min (Rashid et al., 2013; Donovan et al., 2017; Donovan et al., 2018). Relaxation responses were expressed as a percentage of the response to U46619 response.

2.5.3.4. The effects of H₂S synthesising enzymes inhibitors on myocardial-induced relaxation response

The effects of the H₂S synthesising enzyme inhibitors AOAA (CBS inhibitor), PPG (CSE inhibitor) and CDS (1-(3,4-Dihydroxyphenyl)-2-[(4-hydroxy-6-methyl-2-pyrimidinyl) sulfanyl] ethanone, Compound 7, CDS00492, MST inhibitor) were investigated on the myocardial-induced relaxation response. U46619 was added to achieve 40-60 % of KCl response. Next, myocardial tissue was separately incubated for 30 min with either 100 μ M AOAA/100 μ M PPG, or with 100 μ M CDS, or with the combination of all 100 μ M AOAA/100 μ M PPG and 100 μ M CDS. Next, the separately incubated myocardial tissue with the H₂S synthesising enzymes inhibitors was added to the U46619-precontracted PCA. Changes in tone were expressed as a percentage of the response to U46619.

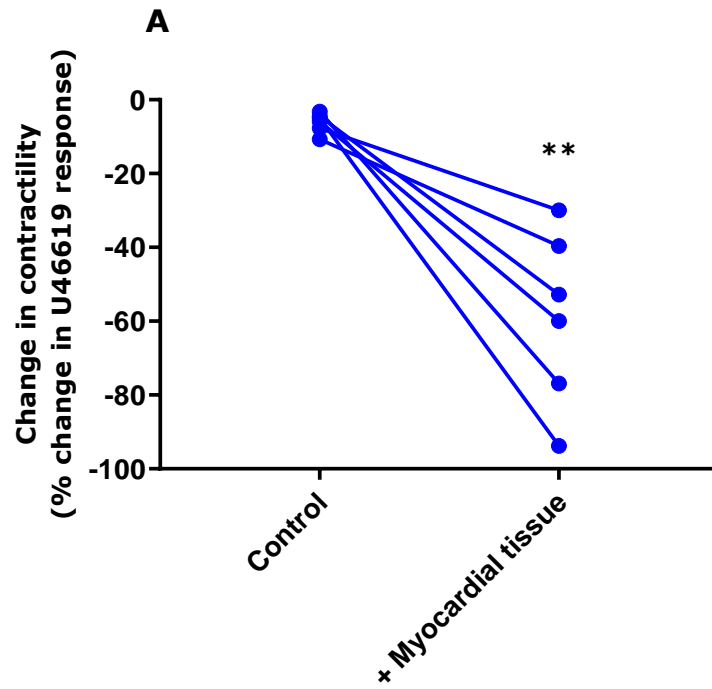
2.5.3.5. The effects of hypoxia on myocardial-induced relaxation response

After the dissection, the myocardial tissue (adherent/beneath/under the PCA) was divided in two parts, one part was continuously maintained oxygenated with 95% oxygen/5 % CO₂ in Krebs'-Henseleit buffer, while the other part continuously maintained non-oxygenated in Krebs'-Henseleit buffer. Next, the non-oxygenated and oxygenated myocardial tissues were added to the U46619-precontracted PCA. Changes in tone were expressed as a percentage of the response to U46619.

2.5.3.6. Results

2.5.3.6.1. Effect of myocardial tissue on U46619-induced tone

The previous experimental results demonstrated that H₂S could be endogenously synthesised in the myocardium. The presence of myocardial tissue led to significant relaxation responses in U46619-precontracted PCA (figure 2.41: A, B and C).



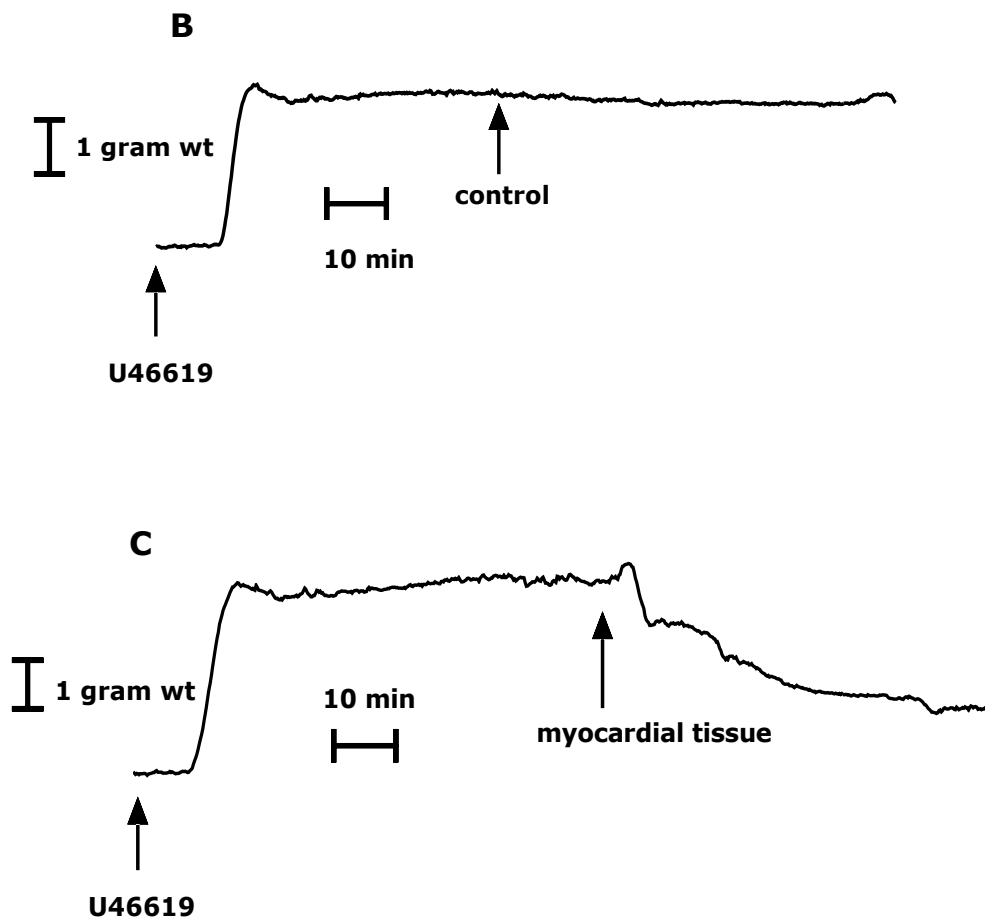
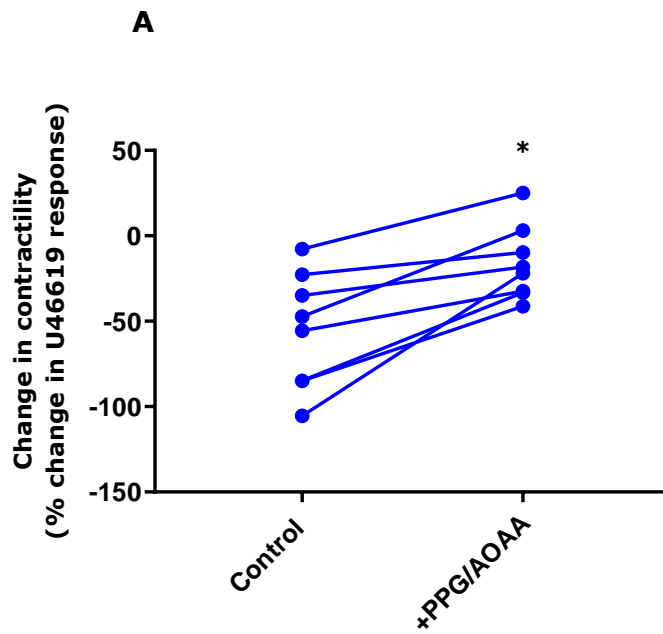


Figure 2.41: (A) The effects of myocardial tissue on the relaxation responses in U46619-precontracted porcine coronary artery (PCA). The Y-axis represents the change in contractility percentage to U46619 response. The representative traces of the relaxation response in the absence of the myocardial tissue (control, Krebs' solution) (B) and in the presence of the myocardial tissue (+ myocardial tissue) (C). ** $p < 0.01$ using two-tailed paired Student's t-test, (n=6).

2.5.3.6.2. The effect of H₂S synthesising enzyme inhibitors

To determine whether H₂S may contribute to the relaxation responses to myocardial tissue, the effects of PPG and AOAA on myocardium-derived relaxation responses in U46619-precontracted PCA was determined. Pre-incubation of myocardial tissue with 100 μM PPG and 100 μM AOAA for 30 min led to a significant decrease in the relaxation effects of myocardial tissue in U46619-precontracted PCA (figure 2.42: A, B and C).



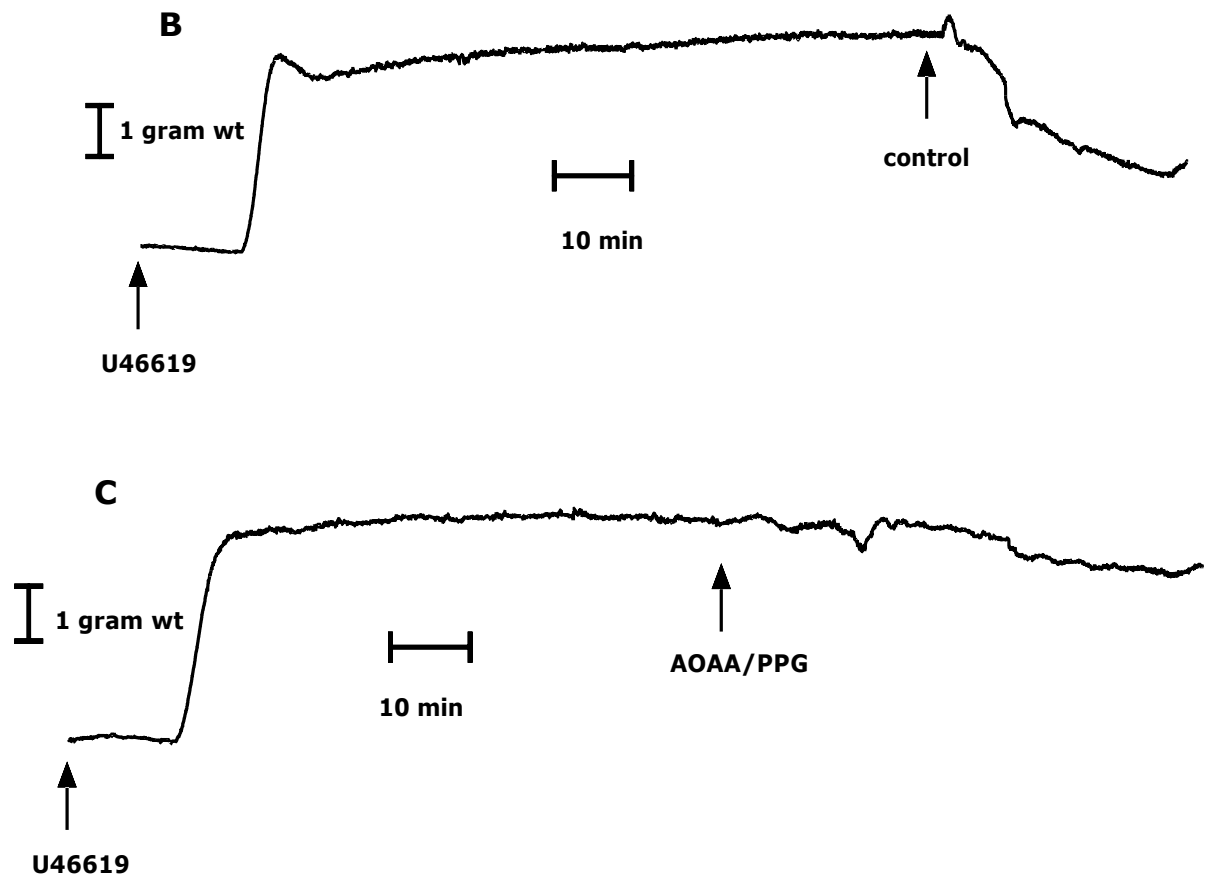


Figure 2.42: The effects of H₂S synthesising enzymes inhibitors, 100 μ M PPG (propargylglycine, CSE inhibitor) and 100 μ M AOAA (aminooxyacetic acid, CBS inhibitor) on myocardium-derived relaxation responses in U46619-precontracted porcine coronary artery (PCA). The Y-axis represents the change in contractility percentage of U46619 response. The representative traces of the relaxation response in the absence of AOAA/PPG in the myocardial tissue (control, myocardial tissue only) (B) and in the presence of the 100 μ M AOAA/ 100 μ M PPG in the myocardial tissue (myocardial tissue pre-incubated with 100 μ M PAOAA/100 μ M PPG) (C). * $p < 0.05$ using two-tailed paired Student's t-test, $n=8$.

2.5.3.6.3. The effect of H₂S synthesising enzyme candidate CDS on myocardium-derived relaxation responses in U46619-precontracted PCA

1-(3,4-Dihydroxyphenyl)-2-[(4-hydroxy-6-methyl-2-pyrimidinyl) sulfanyl] ethanone (CDS004892, identified by an MSc student, Maha Almehaize). Studies in the laboratory demonstrated that CDS inhibits MST production of H₂S (unpublished data). Pre-incubation of myocardial tissue with 100 μ M CDS for 30 min led to a significant increase in the relaxation of U46619-precontracted PCA (figure 2.43).

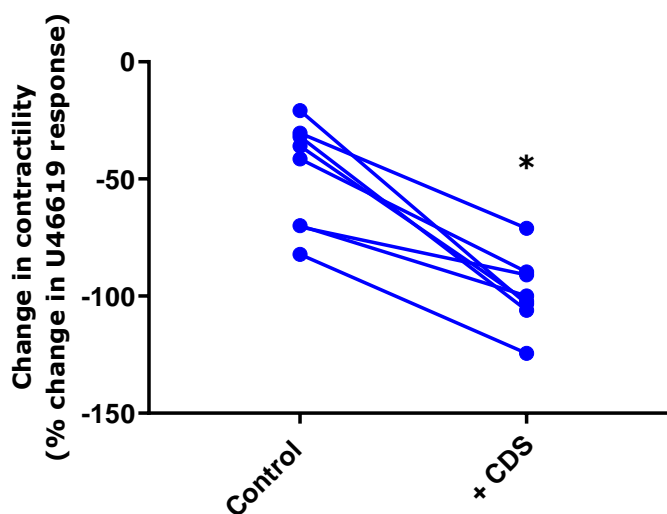


Figure 2.43: The effects of H₂S synthesising enzyme candidate MST inhibitor, 100 μ M CDS 1-(3,4-dihydroxyphenyl)-2-[(4-hydroxy-6-methyl-2-pyrimidinyl) sulfanyl] ethanone) on myocardium-derived relaxation responses in U46619-precontracted porcine coronary artery (PCA). The Y-axis represents the change in contractility percentage of U46619 response. * $p < 0.05$ using two-tailed paired Student's t-test, $n=8$.

The separate pre-incubation of the myocardial tissue with a combination of 100 μM PPG; 100 μM AOAA and 100 μM CDS for about 30 min and then its addition to U46619-precontracted PCA led to a significant decrease in the relaxation responses of myocardial tissue (figure 2.44).

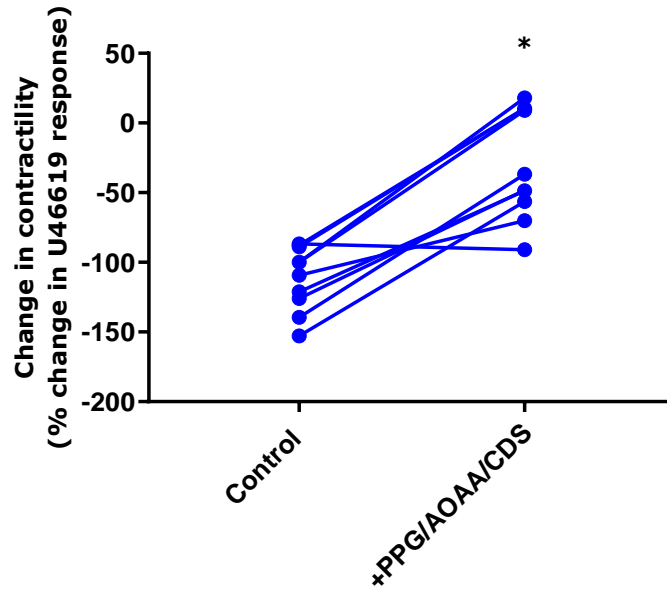


Figure 2.44: The effects of H₂S synthesising enzymes inhibitors in combination, 100 μM PPG (propargylglycine, CSE inhibitor), 100 μM AOAA (aminoxycetic acid, CBS inhibitor) and 100 μM CDS on myocardium-induced relaxation responses in U46619-precontracted porcine coronary artery (PCA). The Y-axis represents the change in contractility percentage of U46619 response. * $p < 0.05$ using two-tailed paired Student's t-test, $n=8$.

In order to determine whether the effect of 100 μM CDS was dependent on the myocardial tissue, 100 μM CDS was added to U46619-precontracted PCA in the absence of myocardium and elicited a significant relaxation, and this result may suggest a no specific and off-target effects of CDS (figure 2.45).

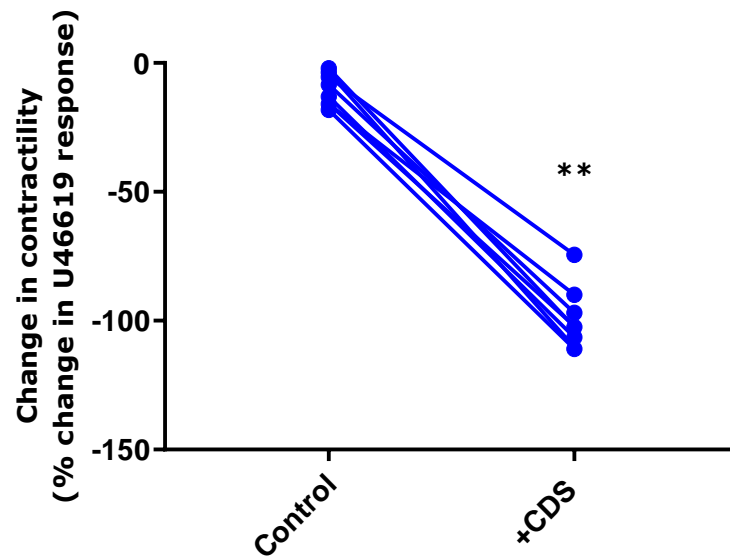


Figure 2.45: The effects of 100 μM CDS on U46619-precontracted porcine coronary artery (PCA). The Y-axis represents the change in contractility percentage of U46619 response. ** represents $p < 0.01$ using two-tailed paired Student's t-test, $n=8$.

2.5.3.6.4. The effect of hypoxia on myocardial-induced relaxation response

There are some studies reported that hypoxia may induce the release of H₂S and lead to vasorelaxation (Donovan et al., 2017; Donovan et al., 2018). Thus, these observations suggest a vasodilation evoked after oxygen is removed from coronary artery segments is mediated by H₂S. Therefore, the effects of hypoxia on myocardium-induced relaxation responses in U46619-precontracted PCA were investigated. After the dissection, the myocardial tissue (adherent/beneath/under the PCA) was divided in two parts, one part was continuously kept oxygenated with 95% oxygen/5 % CO₂ in Krebs'-Henseleit buffer, while the other part continuously kept non-oxygenated in Krebs'-Henseleit buffer. Next, the non-oxygenated and oxygenated myocardial tissues were added to the U46619-precontracted PCA. There were no significant differences between the non-oxygenated myocardial tissues and the oxygenated myocardial tissues in the relaxation responses in U46619-precontracted PCA (figure 2.46).

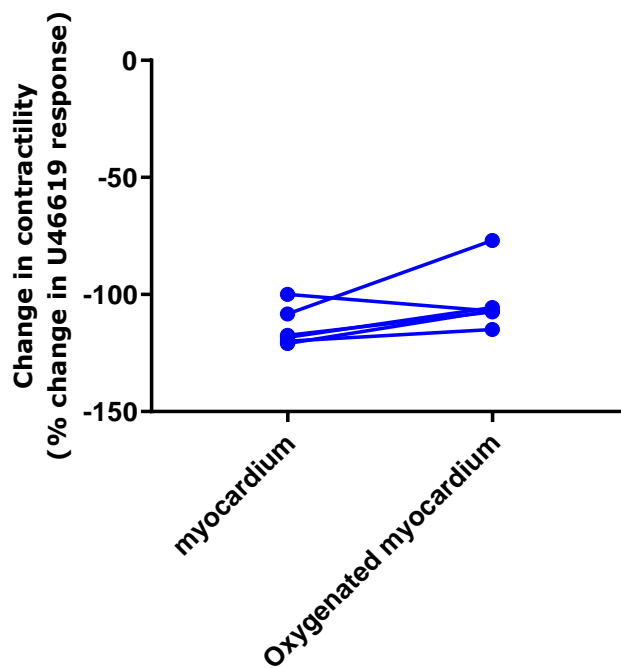


Figure 2.46: The effects of hypoxia and oxygenation on myocardium-induced relaxation responses in U46619-precontracted porcine coronary artery (PCA). The Y-axis represents the change in contractility percentage of U46619 response. Non-significant $p > 0.05$ using two-tailed paired Student's t-test, $n=6$.

2.6. Discussion

This chapter aimed to measure endogenous H₂S generation in the heart. This aim was carried out using Western blotting to measure H₂S synthesising enzyme expression levels and enzyme assays using the methylene blue and SF-7 AM to measure H₂S synthesising enzyme activities in different regions of the heart.

The main findings of this chapter are summarised as the following:

- CBS and MST were expressed in pig heart, while CSE was not detectable.
- Low MW CBS (active form) expressed in rat liver, while high MW CBS (less active form) expressed in pig heart.
- There was no difference in the expression levels of CBS and MST between atria and ventricles.
- There was low enzyme activities of CBS and MST in pig heart compared to rat liver.
- There was no difference in enzyme activities of CBS and MST between atria and ventricles, and also between males and females.
- The optimal incubation times are 60 min for the methylene blue assay and 90 min for the SF7-AM assay.
- DTT, alkaline pH and dialysis led to an increase of CBS and MST enzyme activities
- There was no significant effect of aspartic acid, degassing and SAM on enzyme activity.
- In rat liver there was more inhibition by PPG compared to AOAA, while in pig heart here was more inhibition by AOAA compared to PPG.
- Addition of myocardial tissue induced relaxation of PCA.

2.6.1. Pig heart tissues

There was no difference in the expression of H₂S synthesising enzymes CBS and MST between atria and ventricles of pig hearts. There was low CBS and MST activity in the heart compared to rat liver. MST is expressed at apparently low levels in the pig heart compared to the rat liver. Production of H₂S by MST pathway requires an alkaline pH and a reducing environment (Shibuya et al., 2009; Kimura, 2014). The biological importance of this enzyme remains poorly characterised (Mikami et al., 2011). H₂S production in the heart might be due to H₂S metabolising mitochondrial enzymes, such as rhodanese, SQOR (sulfide: quinone oxidoreductase) and MST (Beltowsky, 2015), or that H₂S production might be outside the myocardium by CSE of blood vessels, such as the coronary artery (vasculature) and then H₂S diffuse to the myocardium to produce potential H₂S cardioprotective effects. This process might be regulated acutely, and thus this might explain the low H₂S production via low enzyme activity observed in the myocardium.

Donovan's study reported that all H₂S-synthesising enzymes, CBS, CSE, and MST were detected in the pig myocardial tissue. H₂S generation was measured in the pig myocardial tissue using the methylene blue assay. CBS was played crucial role in control of vascular tone of PCA during hypoxia (Donovan et al., 2018). These results were in agreement with the results of the present study regarding CBS and MST detection, and H₂S measurement. Donovan's study was in disagreement with the results of the present study regarding CSE detection in the pig myocardium. This difference might be due to difference in methodology and myocardial tissue samples. For example, Donovan's study used 1.5:500 concentration of anti-CSE antibody, while we used 1:1000 concentration of anti-CSE antibody.

In the present study using the methylene blue assay H₂S production from L-cysteine was about 50 nmoles/ mg protein in rat liver, and 10 nmoles/ mg protein in pig and rat hearts, while H₂S production from mercaptopyruvate was about 300 nmoles/ mg protein in rat liver, 200 nmoles/ mg protein in rat heart cytosol and mitochondrial fraction and 50 nmoles/ mg protein in pig heart. Using the SF7-AM assay H₂S production from L-cysteine was about 200 RFU/ mg protein in rat liver and 80 RFU/ mg protein in pig heart, while H₂S production from mercaptopyruvate was about 200 RFU/ mg protein in rat liver and pig heart mitochondrial fraction and 50 RFU/ mg protein in pig heart.

There was low CBS and MST activity in the heart compared to rat liver. The simple explanation of low CBS and MST activity in the heart may be due to that the heart did not generate much H₂S as liver. However, the addition of myocardial tissue led to relaxation of PCA and this relaxation inhibited by AOAA/PPG. Another possible explanation that most of the synthesised H₂S in the heart could be stored until use/need.

In the present study, H₂S production via MST from mercaptopyruvate in the pig and rat heart mitochondrial fraction was higher than cytosol, because there was a decent MST activity in the mitochondrial fraction. The possible explanation that MST could be abundant in the mitochondria (about two-thirds) compared to cytosol (about one-third). Another possible interpretation that the delay in tissue storage and transport may lead to stress in tissue and migration of CBS and CSE to the mitochondria, although CBS was only detected in the pig heart cytosol. Therefore, more investigations about CBS and CSE expression in the mitochondria could explain the low H₂S production in pig heart cytosol compared to rat liver.

2.6.1.1. The effects of DTT

Dithiothreitol (DTT) is a reducing agent, used to decrease oxidation and potentially increase the liberation of H₂S from reductant-labile sulfur pools/stores (Chen et al., 2004; Kabil et al., 2011; Shen et al., 2012). Our results suggest that the addition of DTT could augment H₂S production capacity by increasing H₂S stability and release and appeared to decrease the variability of H₂S production. Our results showed that DTT effect was only significant at 30, 45 and 60 min of incubation by the methylene blue assay through the CBS pathway. Therefore, this effect might be time-dependent and only produce a significant increase of H₂S production at 30, 45 and 60 min of incubation. These results are in agreement with Chen et al. (2004) and Kabil et al. (2011) studies, which demonstrated that the presence of thiols, such as DTT increases H₂S synthesis. Moreover, DTT was useful for H₂S production via MST and increased H₂S release by MST pathway because the reducing agent is required for release of H₂S from the intermediate in the MST pathway, which was measured by the methylene blue and SF7-AM assays.

2.6.1.2. The effects of L-cysteine

L-cysteine is the main substrate for H₂S production and therefore, L-cysteine is an essential tool to study the enzyme activity of CBS to stimulate endogenous H₂S production, especially at saturating concentrations (Cheng et al., 2004; Al-Magableh and Hart, 2011; Bucci et al., 2012; Chitnis et al., 2013; Asimakopoulou et al., 2013; Rashid et al., 2013; Candela et al., 2016). Therefore, L-cysteine may be the rate-limiting factor for H₂S production and biological effect. L-cysteine, however, is not used solely for H₂S production, and it may be incorporated into protein, converted to glutathione (GSH, an important antioxidant inside the cells) or taurine to prevent intracellular oxidation of cysteine (Beltowski, 2015). L-cysteine has also been reported to react with PLP and produce H₂S via a non-enzymatic decomposition (Wang, 2012), and it might be that some of the background optical density is likely due to the interaction between cysteine and PLP in no tissue blank, which contains L-cysteine, PLP and no tissue.

CSE was not detected in pig hearts in our results. It has been proposed that the use of L-cysteine compared to homocysteine in combination with L-cysteine might underestimate CBS activity and bias H₂S production towards CSE (Singh et al., 2009; Kabil et al., 2011). This is because CBS is reported to be more efficient in producing H₂S from a combination of L-cysteine with homocysteine than L-cysteine alone, but this would not be a problem in the present study because CSE was not detected in pig heart. CBS has another role, which is responsible for homocysteine metabolism (detoxification).

CBS and CSE, as well as CAT are PLP-dependent enzymes. Therefore, in the absence of PLP, there would be no conversion of L-cysteine to H₂S via CBS/CSE and mercaptopyruvate via CAT. Therefore, in situations associated with low or restriction of vitamin B6 and L-cysteine nutrition. This would lead to decrease of endogenous H₂S production via these enzymes in tissues and these situations were reported to be associated with increase of cardiovascular pathologies (DeRatt et al., 2014; Mys et al., 2017). Therefore, use of PLP (vitamin B6 derivative) and L-cysteine leads to augmentation of endogenous H₂S production via CBS and CSE as well as CAT, and cardiovascular protective effects.

2.6.1.3. The effects of aspartic acid

Aspartic acid (AA, aspartate) is a structural analogue of cysteine and might be a competitive inhibitor of CAT (figure 1.1: A) (Miyamoto et al., 2014; Shibuya et al., 2009; Madden et al., 2012; Donovan et al., 2017; Donovan et al., 2018). CAT is PLP dependent and so some of the H₂S production in the presence of L-cysteine could be mediated through the CAT/MST pathway. Therefore, in the absence of PLP, there would not be conversion of L-cysteine to mercaptopyruvate through CAT. Our study demonstrated that aspartic acid had no significant effect on H₂S production in the presence of PLP.

CBS and CSE is a PLP-dependent enzyme being the major enzymes for H₂S generation in the liver using L-cysteine as a substrate, because in rat and mouse tissues the liver was reported to have 50-fold CSE expression than any other tissue (Ishii et al., 2004). The absence of PLP might allow to visualise whether aspartic acid has an inhibitory action on L-cysteine derived H₂S. In the absence of PLP (and without DTT), there was a small inhibition in the presence of aspartic acid. This finding might be due to aspartic acid and might lead to decrease of CBS/CSE activity and H₂S detection.

This interpretation could be problematic because the conversion of L-cysteine to mercaptopyruvate via CAT activity also requires PLP because CAT is PLP-dependent. Aspartic acid led to a significant decrease of H₂S production from L-cysteine in rat aorta endothelial cells and human embryonic kidney 293 cells (HEK 293 cells) using gas chromatography for H₂S detection (Shibuya et al., 2009). They also reported that H₂S produced from L-cysteine in the absence of PLP. Similarly, aspartic acid reduced H₂S production via MST in rat lung tissues (Madden et al., 2012). They used SISE for measurement of H₂S levels. These variations in aspartic acid effects between our results and these results may be attributed to differences in species, tissues/cells, H₂S measurement and methodology.

DTT increased H₂S formation might be via decreased oxidation of H₂S or increased release of H₂S from reductant-labile sulfur pools in a non-enzymatic way and therefore, DTT might decrease aspartic acid effect, and DTT is also required to release H₂S through the MST pathway. Thus, investigation of this pathway is problematic. Moreover, it has been reported that there are no selective and potent inhibitors for either CAT or MST, which could help to confirm or exclude the contribution of CAT or MST in H₂S production (Asimakopoulou et al., 2013; Papapetropoulos et al., 2015).

2.6.1.4. The effects of change of pH

A change of pH may appear to increase the stability of hydrosulfide anion and may affect solute protonation and enzyme interaction with the substrate, and thus, pH could alter H₂S measurement and detection (Kolluru et al., 2013). Our results indicated that changing pH from 7.4 to 9 increased H₂S detection by the methylene blue assay in pig and rat hearts. Moreover, an increase of pH in the pig heart and rat liver led to a significant increase in H₂S detection. This result could be due to increase of H₂S stability. It has been claimed that alkaline pH is more related to MST activity, especially in mitochondria, which favours an alkaline and reducing environment (Shibuya et al., 2009; Wang, 2012; Kolluru et al., 2013).

Although the alkaline pH may make H₂S more stable and detectable. In the methylene blue assay increase of pH to pH 9 led to the increase H₂S detection in both CBS and MST pathways in the heart, while in the SF7-AM assay at pH 9 there was an increase in H₂S detection produced through CBS pathway. At pH 9, there was no significant increase in H₂S detection produced through MST pathway using SF7-AM assay. The possible explanation might be due to instability of SF7-AM with mercaptopyruvate, or SF7-AM at pH 9, or that SF7-AM with L-cysteine might be more stable at high pH and several authors recommended pH around 7.4 (Lin et al., 2013).

2.6.1.5. Time profile for detection of H₂S (incubation time)

We observed that in the methylene blue assay, the optimal incubation time for detection of H₂S production capacity is 60 min at both pH 7.4 and 9 by the methylene blue assay. This observation is in agreement with the study of Asimakopoulou's study using a recombinant CBS which suggests that this time is optimal for H₂S formation in the linear phase with a little noise/signal ratio (Asimakopoulou et al., 2013). After this time, there was saturation, loss of H₂S and this may lead to a decrease of H₂S detection. This decrease of H₂S production might be a homeostatic mechanism to control H₂S biological activity.

In order to confirm the data with the methylene blue assay, we developed the use of SF7-AM assay to measure H₂S production in tissue samples. We observed that the optimal time for detection of H₂S production by SF7-AM is 90 min at pH 7.4. This time is optimal for H₂S formation in the linear phase with a little noise/signal ratio, and after this time, it looks that there was a plateau or decrease in the measurement of H₂S.

2.6.1.6. The effects of H₂S synthesising enzymes inhibitors

H₂S synthesising enzymes inhibitors are pharmacological tools which are commonly used to determine contribution of each enzyme in H₂S production. It was reported that PPG could be selective at 100 µM. In the present study, since CSE was non detectable in the heart, then it would be expected that there was no inhibition with PPG. The results of our study suggested favourable inhibition (trending) by AOAA compared to PPG at 100 µM in the heart. This is because at this concentration, AOAA is useful for inhibition for CBS, which was detected by immunoblotting in the porcine heart. Moreover, Asimakopoulou's study reported that AOAA causes more potent inhibition on CSE than CBS because the IC₅₀ for AOAA against CBS is 8.6 µM and against CSE is 1.2 µM. Therefore, there is less than 10 fold difference, so IC₅₀ are very similar and no difference in potency between CBS and CSE. At 100 µM, AOAA will inhibit both CBS and CSE. Additionally, there was no effect of PPG on CBS activity (Whiteman et al., 2011; Asimakopoulou et al., 2013).

It seems that PPG led to inhibition of H₂S production in the liver, which had a high expression level of CSE, while in the heart CSE was non-detectable. It has been reported that PPG could lose its selectivity at high concentrations, which may be necessary to get the maximal inhibitory action on CSE and PPG may inhibit other PLP-dependent enzymes including aspartate aminotransferase (Tanase and Morino, 1976) as well as alanine aminotransferase (Burnett et al., 1992). These results suggested a non-selective action for AOAA and PPG.

Although we used 100 µM AOAA in the present study, it was reported that AOAA could lose its blocking effect for CBS at low concentrations because the IC₅₀ for AOAA against CBS is 8.6 µM (Asimakopoulou et al., 2013) and could inhibit CSE as well as CBS. AOAA could have a dual-action, and therefore, AOAA is non-selective. In the present study, CSE was non detectable and only CBS was detected in the heart and therefore, this dual-action was not important. It has been reported that there is no selective and potent inhibitor for CBS.

Although, phenylpyruvic acid (PPA) has been used to decrease MST activity (Wing and Baskin, 1992) we found that PPA was not useful as a MST inhibitor because PPA had a non-selective/off-target effect and interacted with H₂S in the methylene blue assay of the test, blank and standard. This observation might be attributed to acidic pH dependent nature of the methylene blue assay. Therefore, it has been reported that there is no selective and potent inhibitor for CBS and MST (Asimakopoulou et al., 2013; Rashid et al., 2013). Therefore, it could be difficult to confirm or exclude the biological contribution of each of these enzymes in H₂S production in the heart,

although the results of the present study suggested CDS a new candidate as selective MST inhibitor.

The present study reported that there was no additive effect of PPG in combination with AOAA in the heart and liver. This observation might mean that only CBS is present in the heart and CSE is present in liver. Therefore, AOAA was useful for inhibition of H₂S production in the heart, while PPG was useful for inhibition of H₂S production in the liver.

2.6.1.7. The effects degassing

Oxidation of H₂S is the major pathway for rapid clearance of H₂S and it may be the reason for the lag phase during H₂S synthesis (Sharma and Yuan 2010; Paul and Snyder, 2012). Therefore, degassing (removal of oxygen by vacuum chamber) and a hypoxic environment may increase the H₂S availability, stability and release, which may facilitate H₂S measurement (Kolluru et al., 2013; Olson, 2013).

Olson's study reported that H₂S generation is increased by hypoxic condition and decreased on the return of oxygen (reoxygenation) in vascular tissues, such as lamprey dorsal aorta, rat aorta, rat pulmonary and bovine pulmonary arteries. Moreover, the hypoxic response (contraction and relaxation responses) to low oxygen level could be inhibited by PPG and AOAA (Olson et al., 2006). Donovan's study reported that hypoxia relaxation response was inhibited by AOAA in PCA (Donovan et al., 2017).

Our observations reported that degassing produced no significant increase in H₂S detection in the heart. This finding could be because degassing doesn't have an effect on H₂S detection in the heart and liver, or it might be related to that issues with methylene blue technique, such as prolonged procedure with multi-steps. Monobromobimane fluorimetric assay and previous studies have shown that the low oxygen level and degassing increased H₂S detection (Shen et al., 2011; Shen et al., 2015). Moreover, measuring of oxygen levels/tension after degassing/hypoxia may useful to confirm/validate the effect of degassing/hypoxia.

2.6.1.8. The effects sample dialysis

Our results demonstrated that dialysis of pig heart samples led to improved enzyme detection of H₂S using the SF7-AM method in both CBS and MST pathways. Therefore, this observation might be due to the removal of an endogenous inhibitor, which interferes with the SF7-AM assay regardless of whether it is CBS or MST assays.

2.6.2. Heart tissues of different species

Our results demonstrated that CBS was expressed, MST was detectable and there was no detectable expression of CSE in the left atria and ventricle of the porcine heart. The expression of CBS and MST immunoreactivity in the pig heart might not necessarily mean that the expressed proteins of CBS and MST are active, although H₂S synthesising enzyme activity was low in both pig and rat hearts compared to liver.

In agreement with the present study, CSE was not detected in cardiac tissues of rat and mouse and rat cardiomyocytes, although in this study they used five different antibodies (Fu et al., 2012). In line with our study, CSE was detected in rat liver tissues by Fu's study. Interestingly, in Fu's study mRNA of CSE was detected in rat cardiomyocytes and this result may suggest translational issue (Fu et al, 2012). CSE and CBS were not detected in mice heart, while CSE was detected in mice aorta (Al-Magableh and Hart, 2011). Nrf2 could lead to an elevation of CSE expression in mouse hearts (Calvert et al., 2009). Therefore, H₂S may directly activate Nrf2, which might indirectly control H₂S production by enhancing CSE expression as a positive feedback loop and this process likely needs hours to occur.

Quantification of enzymatic production of H₂S in the pig heart indicated low levels of activity compared to the liver, which was also observed in the rat heart. Although enzyme activity in rat heart was higher than pig heart, both pig and rat hearts were lower low compare to rat liver. The significance and reason for this apparently low production of H₂S in heart tissue remain to be identified. In agreement with our results, Ishii's study reported that there was no CSE protein detected in the heart of murine under physiological conditions (Ishii et al., 2004). The present study demonstrated that there was low CBS activity and undetectable CSE in the heart. In line with this idea, in mice with induced heart failure there was low expression level of CSE and low H₂S production in mouse heart, while CBS and MST were detected in mouse heart (Kondo et al., 2013). Kondo's study did not measure CBS/CSE/MST activity using L-cysteine or mercaptopyruvate and also did not use AOAA/PPG/AA to inhibit these enzymes in order to confirm the contribution of each enzyme in H₂S generation. In contrast to our results, CSE was expressed and involved in endogenous production of H₂S and inhibited by PPG in the heart tissue of rats (Geng et al., 2004). This difference could be attributed to different antibody, methodology and experimental conditions used. For example, Kondo's study used the gas chromatography (GC) technique for measuring H₂S production in the cardiac tissues of mice with heart failure, while Geng's study used the methylene blue assay for measuring H₂S production in the cardiac tissues of rats.

Zhu and his group pointed out that during ischaemic states in rat hearts, there was an increase in CSE expression (Zhu et al., 2007). Although it is plausible, this observation might be attributed to that endogenous H₂S could be low in the heart under physiological situations and endogenous H₂S production might be increased acutely (transiently) as sparks during periods of stress. During ischaemia, hypoxia and stress there is a potential increase in H₂S synthesising enzymes expression (Wang et al., 2014). In the cardiac tissue of mice with chronic heart failure there was low level of H₂S generation and CSE expression (Sen et al., 2008). In another study, H₂S generation using SISE in the mice heart was higher than mice aorta in both the wild-type and CSE-KO mice, although this study did not parallelly measure the enzyme activity and expression of CBS and MST (Yang et al., 2008) because CBS and MST may be compensatively increased in CSE-KO mice.

H₂S has cardiovascular protective effects. For instance, Kuo et al's study reported that MST, but not CSE, plays the principal role of H₂S effects on vascular tone and blood flow in rat, mouse and human coronary arteries, which supply blood and oxygen/nutrients to the myocardium and therefore this might result in potential cardioprotective effects during IRI and coronary artery diseases (Kuo et al., 2015). These potential cardiovascular protective effects of H₂S could be mediated via antioxidant, prevention of calcium-overload and mitochondrial protection effects, especially during reperfusion injury. These results were paralleled with high H₂S production using mercaptopyruvate as a substrate compared to low H₂S production using L-cysteine as a substrate (Kuo et al., 2015). In our results there was more H₂S production from mercaptopyruvate compared to L-cysteine and this observation suggests the importance of MST in H₂S production in the heart.

Interestingly, Peleli's study found that knocking down of MST in mice results in cardiac pathologies for older mice more than young mice, as an age-related pattern (Peleli et al., 2020). Taken together, the results of the present study suggest that H₂S production is low in the heart compared to the liver.

The detection of which H₂S synthesising enzymes (CBS, CSE, and MST) are present in human heart has not looked yet. The presence of CBS and CSE was detected in many human tissues, such as liver, brain, aorta, internal mammary artery, mesenteric artery, umbilical vein, placenta, lung, eye, joint, colon, ovary and uterus has been demonstrated (Levonen et al., 2000; Sidhu et al., 2001; Webb et al., 2008; Whiteman et al., 2011; Yang et al., 2011; Wang, 2012; di Villa Bianca et al., 2015; Leucker et al., 2017; Materazzi et al., 2017). Less extent of MST was reported in human brain and red blood cells (Whiteman et al., 2011). Therefore, future studies about the responsible enzymes for H₂S generation in human heart are needed in

order to confirm the roles of H₂S pathways in the physiology and treatment of CVD in humans, because CVD are one of the common causes of death in humans.

2.6.3. Comparison of different tissues of the pig

The results of the present study demonstrated that CBS was detected, MST was detected and no detectable expression of CSE in the left atria and ventricles of the pig heart. It could be useful to compare CBS, CSE, and MST expression in and activity in pig heart tissue with different tissues of the pig, such as liver.

Donovan's study indicated that all H₂S-synthesising enzymes CBS, CSE and MST were expressed in PCA, which supply blood and oxygen to the cardiac tissue. Therefore, this result might result in potential cardiovascular protective effect. Furthermore, PPG and aspartic acid led to no significant effect on enzyme activity, while only AOAA resulted in a significant decrease in enzyme activity using 1 mM PLP in the methylene blue assay (Donovan et al., 2017). They reported that PPG and AA (aspartic acid) led to additive inhibition effect only in the presence of AOAA. These results suggested that the CBS is the main enzyme involved in the endogenous production of H₂S in the PCA, in particular during hypoxia (Donovan et al., 2017). Using the methylene blue assay in the present study in pig heart and Donovan's study (Donovan et al., 2017) in PCA H₂S production from L-cysteine was similar as well as similar inhibition by AOAA, while H₂S production from mercaptopyruvate in pig heart was higher than PCA. This difference might be attributed to variation in MST expression and H₂S effects between the heart and coronary artery.

In another study, Donovan's study indicated that only CBS and MST were expressed in the pig PVAT, while all three H₂S-synthesising enzymes, CBS, CSE, and MST were reported in the pig myocardial tissue and PCA. The relative abundance of CBS was the highest in the myocardial tissue compared to PVAT and PCA. The relative abundance of CSE was higher in the myocardial tissue compared to PCA. The relative abundance of MST was higher in the myocardial tissue compared to PVAT and PCA. They indicated that CBS, especially in PVAT was played crucial role in control of vascular tone of PCA during hypoxia via increase of hypoxia response and increase of blood flow in coronary artery. This role was inhibited by AOAA, but not by PPG and AA (Donovan et al., 2018).

H₂S generation was measured in the myocardial tissue, pig PVAT, and PCA. H₂S generation in the PVAT was higher than the myocardial tissue and PCA, although Donovan's study did not differentiate the contribution of each of CBS, CSE and MST to H₂S generation in each tissue and not compared to positive controls, such as rat liver. This might be due to the absence of selective and potent CBS/CSE/MST inhibitors, using high concentration of L-cysteine (100 mM, which is about 10-fold higher than of L-cysteine concentration used in the present study) and different aim of Donovan's study (Donovan et al., 2018). AOAA was inhibited H₂S generation in PVAT relaxing effect during hypoxia, but not PPG and AA. This results suggest the significance of H₂S generated via CBS in the PVAT in control of vascular tone of PCA during hypoxia (Donovan et al., 2018).

Therefore, endogenous production of H₂S in the PVAT might diffuse to myocardium and PCA to produce cardiovascular protective effects. Therefore, H₂S synthesised via CBS activity in PVAT led to an increase of hypoxia response and increase of blood flow in coronary artery. Thus, H₂S could act as diffusible agent (Donovan et al., 2018).

Rashid et al's study reported that CBS, CSE and MST were expressed in the pig lung tissues. They found that CBS were not detected at 63 kDa (high MW) in rat liver (Rashid et al., 2013). They found that CSE was detected at about 45 kDa and also there was additional band of CSE at about 50 kDa in pig liver, pulmonary artery and lung tissues, such as bronchioles and trachea. MST was detected about 30 kDa in pig lung tissues and rat liver tissues and also there were additional bands of MST at about 50 kDa in pig lung tissues and at about 43 kDa in rat liver tissues. These different results in pig heart and lung tissues may be due to different level of posttranslational modifications, proteolysis and H₂S physiological roles between the heart, liver and lung. Using the methylene blue assay in the present study in pig heart and Rashid's study in pig bronchioles and trachea, H₂S production from L-cysteine was similar as well as similar inhibition by AOAA, while H₂S production from mercaptopyruvate in pig heart was higher than bronchioles and trachea. This difference may be due to variation in MST expression level between the heart and lung tissues. Rashid's study suggested that H₂S generation in lung tissues may lead to relaxation of airways.

These results are in agreement with results of Pong and Shibuya studies, which reported that there were additional bands of CBS, CSE and MST in mouse and rat tissues (Pong et al., 2007; Shibuya et al., 2009). The reason for these additional bands is unclear but it could be the presence of other versions of the enzymes. Another possible interpretation is cross-reactivity with enzymes similar to CBS, CSE and MST (Rashid et al., 2013). Rashid et al's study reported that there was detectable H₂S enzyme activity in the lung tissues using 1 mM PLP in the methylene blue assay, this H₂S enzyme activity was only significantly inhibited by AOAA and this result suggested that CBS is the important enzyme responsible for endogenous production of H₂S in lung tissues.

It has been reported that there was no selective and potent inhibitor for CBS and MST in the heart. Therefore, it would be difficult to confirm or exclude the biological contribution of each of these enzymes in H₂S generation in the heart. Thus, the development of selective inhibitors for CBS and MST would be important in future studies. Our results indicated that with use of mercaptopyruvate as a substrate for MST enzyme, there was detectable MST enzyme activity in the pig heart and this observation with MST expression suggested the involvement of MST in endogenous production of H₂S in the heart. This result was in the agreement with Rashid's study results in pig lung tissues, Donovan's studies in PCA and PVAT. Therefore, MST is suggested to participate in endogenous H₂S production and biological effects in different pig tissues, such as heart, PCA and lung tissues.

2.6.4. Pig heart tissues compared to different tissues and species

It could be useful to compare CBS, CSE, and MST expression and activity of the pig heart tissues with different tissues and species. Therefore, this might be the reason for different expression and activity in the heart in our data in comparison with the results of different tissues and species in previous studies.

2.6.4.1. Presence of low and high MW bands of CBS

In the results of the present study, there was a difference in the apparent MW of CBS between the pig heart and the rat liver. In the heart there was higher expression of the high MW CBS (less active form) and lower expression of the low MW CBS (active form, truncated form) and this may be the reason for low activity in the heart. On the other hand, in the liver, there was lower expression of the high MW CBS (less active form) and higher expression of the low MW CBS (active form), and this may be the explanation for high activity in the liver.

These observations might be due to high metabolic rate, proteolysis/post-translational modifications (PTMs) and protein content in the liver, which may facilitate cleavage to the low MW CBS (Skovby et al., 1984; Zou and Banerjee, 2003). According to Skovby's study, two polypeptides were found in the fresh cytosol of rat liver, one-week incubation of rat liver cytosol at 4 °C, led to an increase in CBS activity by about two or three-fold in parallel with a relative increase in the level of the smaller polypeptide and disappearance of the bigger polypeptide (high MW) of CBS. Furthermore, they demonstrated that the specific activity of the smaller polypeptide was equal to about sixty times more than the larger polypeptide. They inferred that CBS conversion from less active form to a more active form might depend on physiological factors, such as the rate of proteolysis in the cells and tissue.

2.6.4.2. CBS, CSE and MST expressions

The expression of CBS and MST immunoreactivity in the pig heart might not necessarily mean that the expressed proteins of CBS and MST are active, although H₂S synthesising enzymes activity was low in both pig and rat hearts compared to rat liver. Although there was no detectable CSE and detectable MST expression in the pig heart, relative to the rat liver expression of CBS immunoreactivity appeared high. Ishii et al's research reported that CSE was about 50 times more expressed in rat and mouse liver tissues than other tissues and also CSE activity is reported to be the highest in liver tissues (Ishii et al., 2004). H₂S production in mouse hepatocytes was via CSE (Untereiner et al., 2016). In rats, CSE expression in liver was about 100-fold higher than brain (Swaroop et al., 1992).

Moreover, in different tissues, such as rat mesenteric, pulmonary and aortic arteries (Cheng et al., 2004) and human internal mammary arteries (Webb et al., 2008) CSE was expressed and principally participated in endogenous H₂S production and this endogenous H₂S production was inhibited to about 50 % by PPG. Therefore, about 50 % production in human internal mammary artery may be via CBS or MST because Webb's study did not measure CBS or MST expression or their enzyme activity (Webb et al., 2008). These differences in the tissue expression level of CBS, CSE and MST among different tissues and species were attributable to specific function of each of these tissues. Using the methylene blue assay in the present study in pig heart and rat liver and Webb's study (Webb et al., 2008) in human internal mammary artery and rat liver, H₂S production from L-cysteine was similar, but inhibition of H₂S production was by PPG. This difference might be attributed to variation in CSE expression level between the heart and internal mammary artery or species variation. Webb's study suggested the involvement of H₂S in the control of vascular tone and blood pressure, which may be important in control of blood flow.

2.6.4.3. Quantification of enzymatic production of H₂S

Quantification of enzymatic production of H₂S in the pig heart by the methylene blue and SF7-AM assays indicated low levels of activity compared to rat liver. The significance and reason of this apparently low production of H₂S in this tissue remain to be clarified. H₂S at low concentrations may be more physiological (beneficial). For example, in the mitochondria which are abundant in the heart, at low H₂S level the H₂S acts as an electron donor (nucleophile) for the respiratory chain and maintains the cellular bioenergetics (Wang, 2012; Módis et al., 2014). Our study indicated that H₂S measurement by the methylene blue and SF7-AM assays was high in the rat liver. Similarly, H₂S generation using SISE was the highest in mice liver compared to mice aorta, kidney, heart, lung and brain (Al-Magableh and Hart, 2011).

2.6.4.4. Possible explanations of low enzymatic production of H₂S

Low enzymatic production of H₂S may lead to low H₂S level. In the present study was low H₂S generation in the heart compared to rat liver. The simple explanation of this observation may be due to that the heart just not generate much H₂S as liver. However, the addition of the myocardial tissue led to relaxation of PCA and this relaxation inhibited by AOAA/PPG. Another possible explanation that most of the synthesised H₂S in the heart could be stored until use/need.

Wang (2012) reported that there are many possible explanations of the low H₂S level. First, fast decomposition of H₂S level from micromolar to unmeasurable level within about 30 minutes *in vitro*. These 30 minutes may be enough to modulate some

physiological roles and also it may reflex a rapid homeostatic process of start and end of H₂S signalling effects. Second, H₂S may be transformed into other forms than free H₂S such as bound form (acid-labile, sulfane sulfur, and sulfhydrated protein). Therefore, it might reflect the presence of various H₂S forms.

In the line with this idea, Whitfield's study reported that there was low H₂S level (< 100 nM) using PHSS (polarographic hydrogen sulfide sensor) in blood of mice, rats, cows, pigs and trout. Moreover, they found that exogenously applied H₂S (via NaHS) rapidly disappeared with the addition of 5 % bovine serum albumin (BSA) (Whitfield et al., 2008). Thus, this result suggested rapid clearance of H₂S within the body might be via storage as bound form. Similarly, low H₂S level was reported in mice and rat brains under basal circumstances. Moreover, the addition of reducing agents, such as DTT led to increase of H₂S release from reductant-labile stores (sulfane sulfur) (Ishigami et al., 2009). Therefore, most of biological H₂S may be stored as a bound form, which may be releasable under specific conditions. Thus, it could be important to detect and consider these stored forms of H₂S during H₂S measurement. These storage forms of H₂S could tell us the amount of H₂S which is stored, but not enzyme activity.

In our study, we measured the trapped sulfide (as a free H₂S) using the methylene blue assay and free H₂S by the SF7-AM assay. Also, the detection of H₂S levels could be influenced by H₂S production and transport that are still debatable. For example, the detected H₂S level is the net of H₂S production minus oxidation.

Our results showed that CBS and MST are expressed in the heart. Additionally, there was H₂S synthesising enzymes activity in the heart and therefore, H₂S is endogenously produced in the heart. Although the enzyme activity was lower than the liver, the heart is an important source of H₂S because myocardial tissue induced relaxation of PCA, which was reduced by inhibitors of H₂S synthesis. The reason for the low activity in the heart compared to liver remains to be clarified.

2.6.4.5. The effects of SAM

SAM is a positive CBS allosteric modulator. Abe and Kimura (1996) demonstrated that SAM (2 mM) led to a significant increase in CBS activity in the hippocampus with high PLP concentration (2 mM). Surprisingly, in our study SAM failed to elicit a significant increase in the CBS activity in the liver, heart and brain. This result could be due to the observation that the liver expressed a truncated form of CBS and it has been suggested that the truncated form cannot be activated by SAM (Ereño-Orbea et al., 2014). Moreover, this observation could be due to differences in concentration of PLP cofactor 2 mM PLP or SAM used, or it could be attributed to the use of different

brain regions. Abe and Kimura's study demonstrated that SAM led to a significant increase in CBS activity in the hippocampus of the rat with a high PLP concentration in the experimental approach (Abe and Kimura, 1996).

Abe and Kimura's study reported that L-cysteine and SAM concentrations are important factors for H₂S production in rat brain. In the present study, the liver, heart and brain were preincubated with SAM for 15 min. This is because it has been suggested that SAM effect needs pre-incubation for 15 minutes, and this might suggest that SAM activates CBS with time. Furthermore, Manteuffel-Cymborowska et al. (1992) reported that testosterone may increase SAM activity. Therefore, a gender difference may be involved in differential effects of SAM (Eto and Kimura, 2002). In agreement with the present study, SAM led to no significant difference in the relaxation response to GYY4137 in bovine ciliary artery muscle (Chitnis et al., 2013). They attributed this result to possible variations in tissue and species.

2.6.4.6. The effects of gender

Manteuffel-Cymborowska et al. (1992) found that testosterone administration in female mice could increase H₂S production in the kidney and therefore, H₂S levels are normally lower in females. Furthermore, in mice with deleted CSE, the presence of hyperhomocysteinemia and hypertension was about 9 times more in females than males (Yang et al., 2008). Therefore, a gender difference may be involved in H₂S production (Eto and Kimura, 2002) and it may be a tissue-specific effect. Moreover, H₂S levels were lower in female mice compared to male mice brains (Eto and Kimura, 2002). H₂S levels measured in healthy human blood was lower in females compared to males (Brancaleone et al., 2015). This difference may be due to a testosterone augmenting effect on H₂S endogenous production possibly via increasing enzymatic transformation of cysteine to H₂S potentially by stimulation of the enzymatic activity of CBS in mouse brain (Eto and Kimura, 2002), CBS/CSE in rat aorta (Bucci et al., 2009), and CSE in rat aorta (Brancaleone et al., 2015). Therefore, testosterone and oestrogen may have influence on H₂S production. In contrast, our study showed that there was no significant difference in H₂S production between male and female pig hearts, though H₂S production in male pig hearts was higher than females using the methylene blue and SF7-AM assays. This difference between our study and other studies, such as Eto and Kimura's, Bucci's and Brancaleone's studies might be due to species difference; pigs vs rats and mice; or tissue variation; heart vs kidney, brain, and aorta.

2.6.4.7. Methylene blue assay compared to SF7-AM

The methylene blue assay has many disadvantages, such as it needs strong acidic pH, which may be aggressive and lead to the release of other forms of H₂S such as acid-labile sulfur pools/stores. Moreover, the methylene blue assay leads to irreversible sample destruction and therefore, it is a single-point method and unsuitable for measuring H₂S level in a real-time pattern. Furthermore, methylene blue assay measures the H₂S production capacity of samples. It was designed for industrial use rather than biological and medical application.

Additionally, the H₂S detection range of the methylene blue assay is reported to be from 8 µM (minimum) to 301 µM (maximum) and therefore it is non-linear with high concentrations of H₂S, such as > 301 µM H₂S (Wen et al., 2018). Therefore, it lacks specificity and sensitivity, especially in tissue with low H₂S concentration. Thus, it is unsuitable for detection of low H₂S concentration < 8 µM H₂S. Furthermore, the methylene blue assay is complicated and needs multiple steps, long incubation and procedure time and aggressive acidic pH, which could be non-physiologically relevant, and it seems to be efficient in rat liver more than in pig and rat hearts. The methylene blue assay might be more suitable for the liver rather than the heart since this technique is less sensitive at low H₂S levels (Kolluru et al., 2013) and moreover, the methylene blue assay may overestimate H₂S production by releasing of acid-labile pools, which might be more available in the liver, which might have more acid-labile sulfur pools stores of H₂S than the heart, which could have more of reductant-labile pools and these pools are releasable by reducing agents, such DTT *in vitro* and thioredoxin and DHLA *in vivo*. Therefore, further investigations about the contribution of these forms in the heart are needed.

The methylene blue assay has some additional drawbacks, which may lead to inconsistent results, such as time-dependency for colour development/interference and poor response to low oxygen condition (Kolluru et al., 2013).

In contrast, the SF7-AM assay is simple and does not need extensive steps for sample preparation (no sample destruction) and requires physiologically relevant pH conditions. Thus, the SF7-AM assay is compatible with living cells and tissues and has a real-time and physiological concentrations of H₂S detection properties. Furthermore, it has high specificity to H₂S and the detection range is reported to be from about 0.5 µM (minimum) to about 100 µM (maximum) (Lin et al., 2013; Guo et al., 2015; Wen et al., 2018). Furthermore, SF7-AM assay could be linear to about 1 mM H₂S concentration (Guo et al., 2015). SF7-AM assay seems more efficient in rat liver and pig hearts by detection of higher enzyme activity level than the methylene

blue assay. Therefore, the SF7-AM assay efficiency is apparent and it is a promising alternative method over traditional methylene blue assay for reliable measurement for H₂S generation. Therefore, the SF7 assay is a robust, easy, and reliable assay for measuring H₂S production in living tissues/cells with a real-time pattern. Therefore, SF7 assay may have more opportunities for development and application.

2.6.5. Myocardial tissue effect on PCA vascular tone

Interestingly, the results of the present study showed that the addition of myocardial tissue led to a relaxation in PCA, and this relaxation was inhibited by AOAA/PPG but not by CDS. Therefore, measurement of enzyme activity only indicates the potential to produce H₂S not the amount that the tissue actually produces. Thus, H₂S production by myocardial tissue may act in a paracrine manner, rather than directly on atrial or ventricular contractility. In line with this idea, Donovan et al's study reported that the application of myocardial tissue to PCA led to a decrease in the transient contraction phase level of hypoxia condition (Donovan et al., 2018). These results suggest that H₂S is endogenously produced in the heart and may be considered as a diffusible factor (paracrine) and this H₂S could lead to a relaxation response in PCA and therefore, it could be important in maintenance of blood flow. These results emphasise the importance of the H₂S pathway in cardiac and vascular physiology. Moreover, these results might suggest that CBS and CSE are involved rather than MST in the relaxation response in PCA. A recent study in mouse liver adipocytes suggested that MST inhibited H₂S production through either CBS or CSE likely via a direct interaction or negative feedback interference (Li et al., 2017). So inhibition of MST by CDS may enhance the H₂S production via CBS, and hence the increase in PCA relaxation. Moreover, CDS might be an endogenous substrate for CBS/CSE, or CDS has a non-specific and off-target effects, or that MST activity could be present in the PCA, because Kuo's study reported that MST is the principal enzyme responsible for H₂S generation in the coronary artery (Kuo et al., 2015), or that MST enzyme might responsible for production of H₂S and a vasoconstrictor.

Peleli's study reported that MST knock out in mice led to some cardio-protection against IRI by a decrease of the infarction area size in adult mice possibly via a mild increase in ROS, which might cause vasorelaxation at physiological concentrations. This is because high ROS concentrations are associated with oxidative stress and vascular pathologies, such as hypertension and atherosclerosis. In contrast, MST knock out led to more cardiac pathologies in the older mice than adult mice in an age-dependent pattern (Peleli et al., 2020) and therefore, MST inhibition might lead to decrease in the contraction and increase in the relaxation response, which may need further investigations to be clarified.

2.6.6. Summary

There was detectable enzyme activity in the heart, but it was lower than the liver by both the methylene blue assay and SF7-AM assay. The detection level was higher in the SF-AM assay, which is a simple, efficient and promising method for H₂S measurement.

The development of a reliable assay for measuring the H₂S could be a useful tool to understand H₂S generation and regulation in biological systems, in particular, the heart, which could provide an enhanced understanding of the biological role of H₂S. The heart is an important source of H₂S because myocardium causes relaxation of the coronary artery through a H₂S dependent pathway, suggesting that the H₂S produced in the cardiac muscle may help regulate vascular tone and cause coronary artery relaxation, which could play a beneficial role to decrease ischaemia/hypoxia injury. Therefore, the development of a reliable assay for measurement of H₂S production could be useful to measure any changes in cardiac and vascular dysfunction.

Chapter Three

Development of an Assay to Measure Sulfhydration in the Pig Heart

3.1. Introduction

Based on Western blotting and enzyme assays (methylene blue and SF7-AM assays), there appeared to be a low enzyme activity level in the pig heart compared to liver. Hydrogen sulfide exerts its signalling effects, in part, through post-translational modifications of different proteins in biological systems, a process known as sulfhydration. This occurs through the reaction of H₂S with free reactive cysteine residues of the targeted protein (Paul and Snyder, 2012). Thus, sulfhydrated proteins (persulfides, reductant-labile) can be considered as signalling pathways and stores of H₂S, which can release H₂S under specific conditions, such as reducing condition and sulfhydration may explain low H₂S detection in the heart (Ubuka, 2002). Sulfhydration leads to conformational changes in the targeted protein, which can alter the localisation and the activity of the protein under diverse physiological and pathophysiological conditions. For example, sulfhydration of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) causes an increase in catalytic activity by about seven-fold, while sulfhydration of β-actin causes polymerisation (Paul and Snyder, 2012). The importance of the sulfhydration process may be similar to phosphorylation (Paul and Snyder, 2015; Meng et al., 2017). H₂O₂ is an oxidising agent, and it may oxidise cysteine-SH and increase the level of sulfhydration of GAPDH protein, and therefore, it may increase the detection of sulfhydrated GAPDH (Wedmann et al., 2014; Zhang et al., 2014; Moore et al., 2015).

The specific cellular and subcellular targets of H₂S are unclear. They have been suggested to include ion channels, such as voltage-gated calcium channels, K_{ATP} (ATP sensitive potassium) and TRP (transient receptor potential) channels, and transcription factors (Paul and Snyder, 2012; Filipovic, 2015; Meng et al., 2017).

Therefore, the development of an assay to measure sulfhydration could be important to understand H₂S signalling and also it could be used as an index for H₂S production in different tissues and pathologies, such as cardiovascular dysfunctions. Measurement of sulfhydration is not easy and it is challenging in biological systems. The biotin-switch assay (BSA), was firstly used by Mustafa and co-workers to assess the level of sulfhydration of proteins (Mustafa et al., 2009). In the BSA, the persulfides are labelled with the biotin-HPDP (N-[6-(biotinamido) hexyl]-3-(2-pyridyldithio) propionamide). Biotin-labelled (tagged) proteins are then extracted and detected (measured) by Western blotting (Mustafa et al., 2009).

Sen's study suggested the red maleimide assay (RMA) as an alternative method for the detection of sulfhydration by blocking sulfhydrated protein with red maleimide, which, on exposure to DTT, leads to a decrease in the fluorescence signal, which indicates the presence of sulfhydration (Sen et al., 2012). Therefore, the aim of this chapter was to investigate and develop these assays to measure the sulfhydration process in pig heart preparations.

3.2. Materials and chemicals:

The following compounds were dissolved in deionised water: HEPES (H3375, Sigma, UK); S-methyl methanethiosulfonate (MMTS, 208795, Sigma-Aldrich, UK); Neocuproine (N1501, Sigma-Aldrich, UK); Deferoxamine (D9533, Sigma-Aldrich, UK); Sodium dodecyl sulfate (SDS, 151-21-3, ACROS ORGANICS, Germany); Ethylenediaminetetraacetic acid disodium salt (EDTA, 100935V, BDH, UK); Sodium sulfide nonahydrate (Na₂S, 208043, Sigma-Aldrich, UK); Nonidet-P40 (IPEGAL CA-630, I3021, Sigma, UK); 30 % Hydrogen peroxide (H₂O₂, H1009, Sigma, UK); Acetone (32201, Sigma, UK).

Biotin hexyl pyridyldithio propionamide (Biotin-HPDP, 207359, Santa Cruz, USA) was dissolved in DMSO. Coomassie Brilliant Blue (B0770, Sigma-Aldrich, UK) was dissolved in glacial acetic acid (537020, Sigma-Aldrich, UK) and methanol (322415, Sigma-Aldrich, UK). High capacity streptavidin agarose beads (20357, Thermo Fisher Scientific, USA). 2x Laemmli Sample Buffer (S3401, Sigma, UK).

3.2.1. Antibodies used in Western blotting:

The properties of the primary and secondary antibodies, which were used in Western blotting, are based on the producer's datasheets as in the following table:

Primary antibodies	Catalogue number	Company	Species Raised in	Type	Anticipated MW (kDa)	Dilution
Anti-GAPDH	G8795	Sigma, UK	mouse	monoclonal	37	1:5,000
Anti-MEK1	Ab32091	Abcam, UK	rabbit	monoclonal	45	1:1,000
Secondary antibodies						
Infrared dye, IR680CW(red)/IR800CW (green)		Licor, Cambridge UK	Goat			1:10,000

The antibodies were prepared according to the producers' data sheets, as illustrated in the following table:

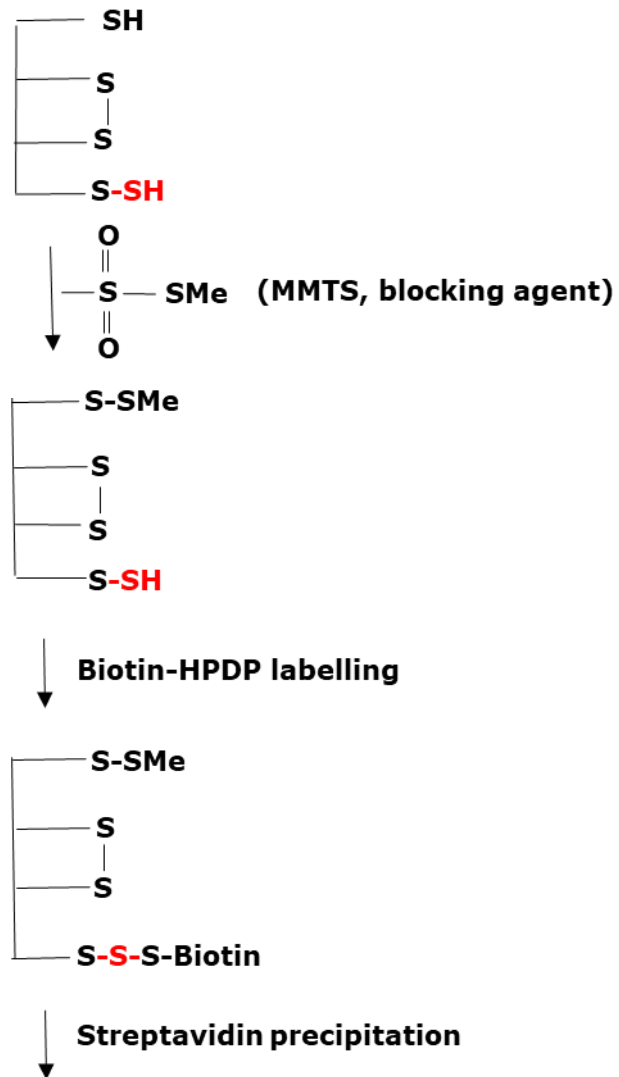
The primary antibody (AB)	Notes	Dilution
Anti-GAPDH	3 µl of antibody stock+15 ml of 5% fat-free milk in TBST	1:5000
Anti-MEK1	10 µl of antibody stock+10 ml of 5% bovine serum albumin in TBST	1:1000
The secondary antibody		
Goat anti-rabbit, IR800 CW, Green	3 µl of antibody stock+ 30 ml of 5% TBST fat-free -fat milk	1:10,000
Goat anti-mouse IR680 CW, Red	3 µl of antibody stock+ 30 ml of 5% TBST fat-free fat milk	1:10,000

3.3. Methodology

In this chapter, three techniques were applied. First, the biotin switch assay (BSA) was used to detect the level of sulfhydrylation. Second, the red maleimide assay (RMA) was used to detect the level of sulfhydrylation. Third, Western blotting was carried out to detect the sulfhydrylation/expression levels of GAPDH and MEK1 after treatment with Na₂S (Sodium sulfide, H₂S salt).

3.3.1. Biotin Switch Assay (BSA)

Sulfhydrylation can be detected by a modified version of the BSA (Mustafa et al., 2009) in which unmodified (free) cysteines of proteins are blocked by the methiolating (alkylating) agent methyl methanethiosulfonate (MMTS). Subsequently, sulfhydrated cysteines are labelled (tagged) with biotin which can be readily purified by streptavidin-agarose. The biotinylated proteins (sulfhydrated) are then identified by SDS-PAGE/Western blotting (figure 3.1) (Filipovic, 2015; Zhang et al., 2017).



Western blotting using GAPDH or MEK1 antibody

Figure 3.1: Illustrative diagram of the biotin switch assay (BSA) steps. Protein cysteines are shown as sulfhydrated (-SSH), with disulfide bonds (S-S) and with free SH groups (-SH). Unmodified (free) cysteine in the protein is blocked with methyl methanethiosulfonate (MMTS). Unmodified (free) MMTS is then eliminated by acetone, and the reduced thiols are treated with biotin-HPDP. The biotinylated protein is extracted using streptavidin beads and analysed using Western blotting.

3.3.1.1. Methodology

The biotin switch assay (BSA) was conducted according to protocols outlined by Mustafa et al., 2009; Mustafa et al., 2011; Paul and Snyder, 2015 and Módis et al., 2016 with some modifications.

3.3.1.2. Sample preparation

Pig heart tissues were isolated at the abattoir and transported to the laboratory on ice. Sections of the left ventricle or rat liver tissues were dissected. The tissue was weighed and homogenised in tris-EDTA buffer (0.1 M tris, 1 mM EDTA, pH 7.4) on ice using a hand-held Ultra-Turrax homogeniser on full speed for 30 s in order to generate fractions termed pig heart homogenate (PHH) or rat liver homogenate (RLH). Homogenates were centrifuged at 1000 x g for 20 min at 4 °C (Sigma 3-18K refrigerated centrifuge). The pellets and supernatant layers were subsequently collected and the latter centrifuged at 30,000 x g for 1 h at 4 °C in order to obtain fractions termed pig heart cytosol (PHC) and rat liver cytosol (RLC).

Tissue samples were incubated with 1 mM Na₂S for 30 min at 37 °C. In some experiments, the incubation was carried out in the presence of 1 mM H₂O₂ in order to determine the effect of oxidation on sulfhydrylation detection level. The resultant mixture was diluted 1:1 with HEN buffer (250 mM HEPES, 1 mM EDTA, 0.1 mM neocuproine, 0.1 mM deferoxamine and 1% Nonidet-P40 (NP-40), pH 7.7) and then sonicated for 15 s in an ultrasonic water-bath. The mixture was then centrifuged at 13,000 xg for 30 min at 4°C (Sigma 3-18K refrigerated centrifuge). The supernatant layer was removed, and a Lowry protein assay was carried out to determine protein concentration in each sample. Following that, the sample was diluted to 5.5 mg/ml (to equalise protein concentration in each sample) in HEN buffer supplemented with 2.5% SDS and 22 mM MMTS. Next, the samples were incubated at 50 °C for 20 min with vortex mixing every 5 min.

Next the resultant mixtures were placed on ice (4 °C) for 20 min with vortex mixing every 5 min. In one experiment, 5 mM DTT was added at this step. Then, the mixtures were diluted 1:10 with pre-chilled acetone and placed at -20 °C for 20 min in order to precipitate protein. The acetone was then removed by centrifugation at 13,000 x g for 5 min at room temperature (RT). Next, the resultant pellets were suspended in HEN buffer containing 1% SDS and 4 mM biotin-HPDP. The mixtures were incubated for 3 h at 25 °C. After incubation, streptavidin-agarose beads (30 µl) were added for purification of biotinylated proteins and samples incubated for 30 min at 4 °C with vortexing every 5 min. Samples were then centrifuged at 13,000 rpm for 10 minutes at RT. The buffer was removed, and the pellet was retained. Fresh HEN buffer was

added to the pellet and the samples centrifuged at 13,000 rpm for 10 min at RT again, and then the buffer was removed. Laemmli sample buffer (2 x: 25 % Tris HCl, 20 % glycerol, 20 % SDS, 0.0004 % Bromophenol blue, 35 % deionised water) was added and the samples boiled at 95 °C for 5 min. Samples were run on SDS-polyacrylamide gels using a pre-cast 4-20 % gradient gel (Bio-Rad, UK) and transferred to nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) by Western blotting. The nitrocellulose membrane was quickly washed with milli-Q water and then in Tris-buffered saline-Tween 20 (TBST: 25 mM Tris, 125 mM NaCl and 0.1 % v/v Tween 20). The nitrocellulose membrane was then blocked with 5% w/v fat-free milk in TBST at RT for 60 min on a shaker platform (as in chapter two: Western blotting methodology).

Some of the polyacrylamide gels were stained using Coomassie Brilliant Blue instead of Western blotting. The gel was fixed in 50% methanol and 10% glacial acetic acid overnight with gentle agitation. Next, the gel was stained in staining solution (0.1% Coomassie Brilliant Blue, 50% methanol and 10% glacial acetic acid) for 20 min with gentle agitation. Then, the gel was destained with destaining solution (40% methanol and 10% glacial acetic acid), the destaining solution was replenished several times until the background of the gel was fully destained. Finally, the gel was stored in the storage solution (5% glacial acetic acid) (Mustafa et al., 2009).

3.3.1.3. Antibody incubation in Western blotting

After the nitrocellulose had been blocked in blocking buffer as above, the nitrocellulose membrane was incubated with the appropriate primary antibody: mouse anti-GAPDH (1:5000 dilution) in 5% fat-free milk in TBST or rabbit anti-MEK1 (1:1000 dilution) in 5% BSA in TBST. The nitrocellulose membrane was then incubated overnight at 4 °C with shaking.

Next day, the primary antibody was removed, and the nitrocellulose membrane was washed with TBST as follows: three times for 5 min and three times for 15 min at room temperature. Subsequently, the nitrocellulose membranes were incubated with secondary antibodies; goat anti-rabbit IR800CW (emitting a green fluorescence) and anti-mouse IR680CW (emitting a red fluorescence) (LICOR, Cambridge, UK) both 1:10,000 dilution in 5 % fat-free milk. The nitrocellulose membranes were then incubated for 60 min at room temperature, with shaking. The secondary antibodies were then discarded, and membranes were washed with TBST before proceeding with the next washing with TBST, three times for 5 min and three times for 15 min. Finally, nitrocellulose membranes were washed with Milli-Q water and visualised using LICOR Odyssey Infrared Imaging Scanner using the following settings: Image studio

software programme version 3.1.4., 700/800 channels, intensity 4, mu 84, and Q lowest. Quantification of the bands was performed by densitometric analysis of the fluorescent signals using Image Studio (Application software application version 3.1. LI-COR Biosciences, Cambridge, UK).

3.4. Statistical analysis

Data and statistical analyses were performed using Graph-Pad PRISM 6 (Software version 6, USA). All values were expressed as means \pm standard error of the mean (SEM). Comparisons between more than two data sets were made using one-way ANOVA (analysis of variance) followed by Sidak's post-hoc test. For comparisons between two data sets, a two-tailed unpaired Student's t-test was used, or, for paired data, a two-tailed paired Student's t-test. A p-value of less than 0.05 indicated a significant difference between the data sets, where n= number of experiments from separate heart, liver and coronary artery samples.

3.5. Results

3.5.1. Biotin switch assay (BSA)

The aim of these experiments was to detect the level of sulfhydrylation in PHH left ventricles. RLH was used as a positive control. 1 mM Na₂S was used as a H₂S releasing salt (drug).

3.5.1.1. Detection of the presence of sulfhydrated proteins

Coomassie Brilliant Blue stain was used to detect the presence of sulfhydrated proteins in RLH and PHH. The results of this study demonstrated that there were some sulfhydrated proteins with no specific dense band in RLH (figure 3.2: A and B) and PHH (from left ventricles) (figure 3.2: A). There was less protein in samples exposed to Na₂S; in other words, there was no obvious increase of sulfhydrylation level on exposure to Na₂S. Also, there were no defined proteins and variable results in rat liver homogenate (RLH) and rat liver cytosol (RLC) (figure 3.2). There were variable results, in particular from RLC (figure 3.2: A and B).

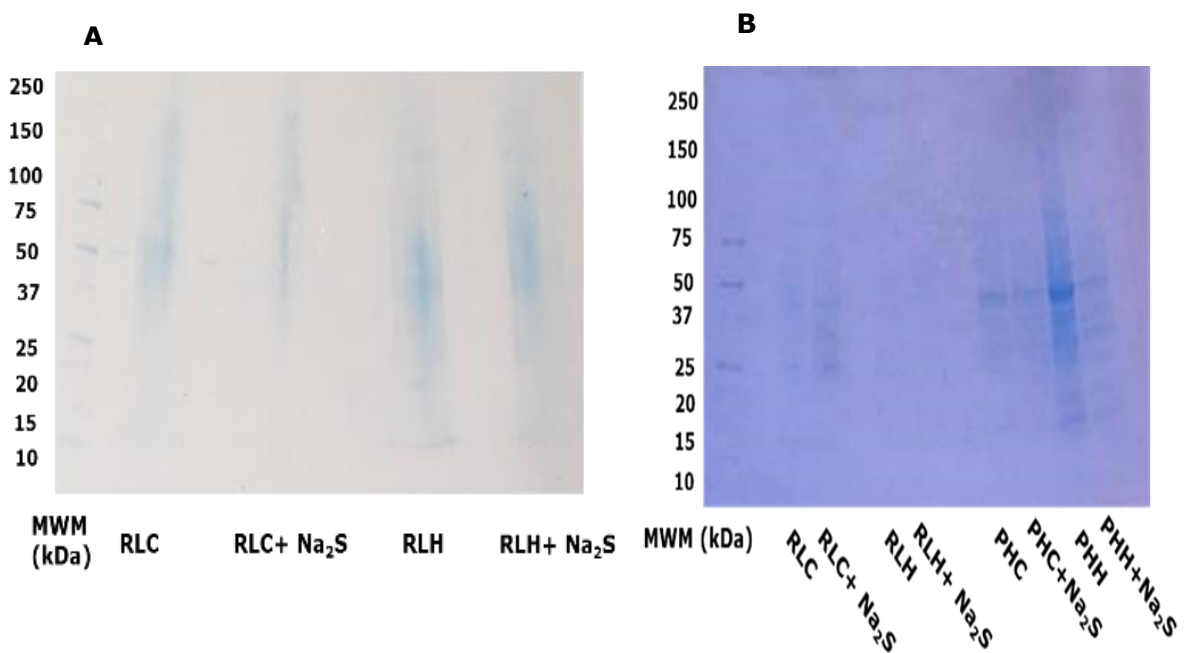


Figure 3.2: Examples of gel stained by Coomassie brilliant blue stain for sulfhydrated proteins of rat liver cytosol (RLC), rat liver homogenate (RLH) (A and B), porcine heart cytosol (PHC) and porcine heart homogenate (PHH) (B) with the addition of 1 mM Na₂S (sodium sulfide, H₂S releasing salt); MWM (molecular weight marker). Experiment repeated in 3 different rat liver homogenates.

3.5.1.2. GAPDH for detection of sulfhydrated proteins

GAPDH is a protein that can be sulfhydrated and plays important roles in glucose metabolism, energy production during stress/hypoxia and cell proliferation (Hara et al., 2005; Paul and Snyder, 2015). In figure 3.2, a number of protein bands were observed in Coomassie-stained gels. The blot shown in figure 3.3 shows bands at about 37 kDa corresponding to GAPDH immunoreactivity in PHH (ventricles), PHC, RLC and RLH (figure 3.3: A and B). However, there was a slight increase in the levels of GAPDH immunoreactivity with the addition of 1 mM Na₂S (figure 3.3: A and B). The results were more variable in PHC than PHH with the addition of 1 mM Na₂S (figure 3.3: A and B).

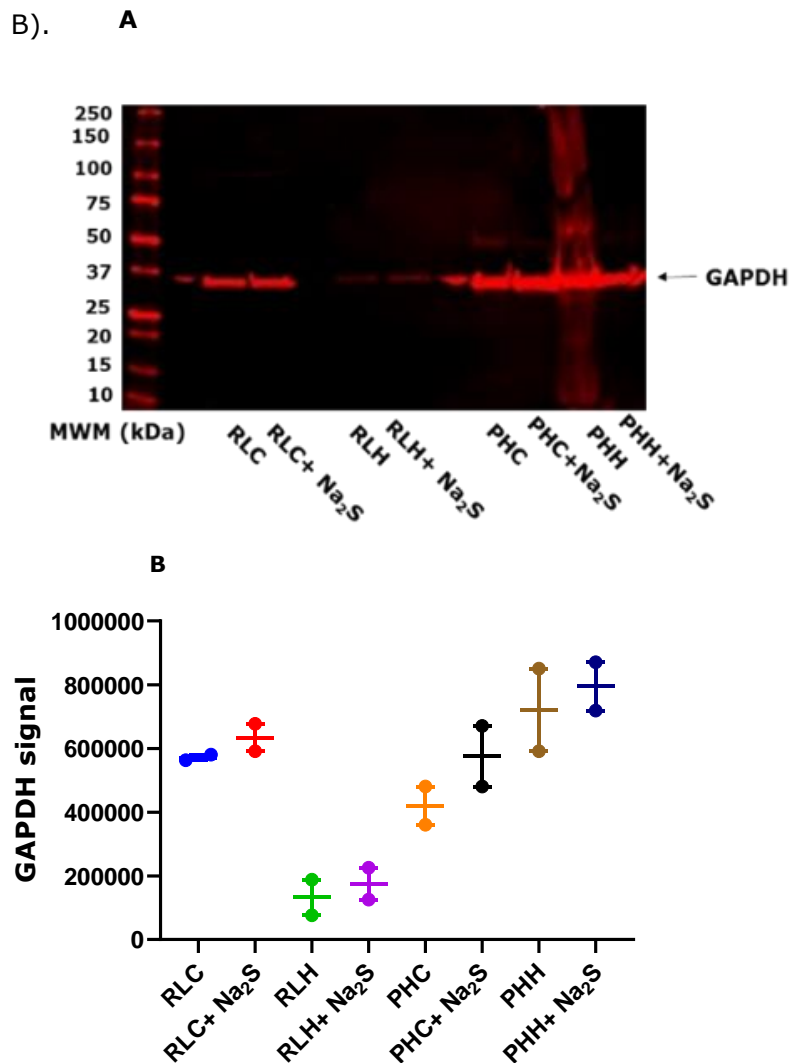


Figure 3.3: (A) Western blot example showing bands at 37 kDa after probing with the anti-GAPDH antibody. (B) Quantifications of the GAPDH band densities from samples of rat liver cytosol (RLC), rat liver homogenate (RLH), porcine heart cytosol (PHC) and porcine heart homogenate (PHH) in the absence and presence of 1 mM Na₂S. The Y-axis represents the GAPDH band signal quantification (densitometric

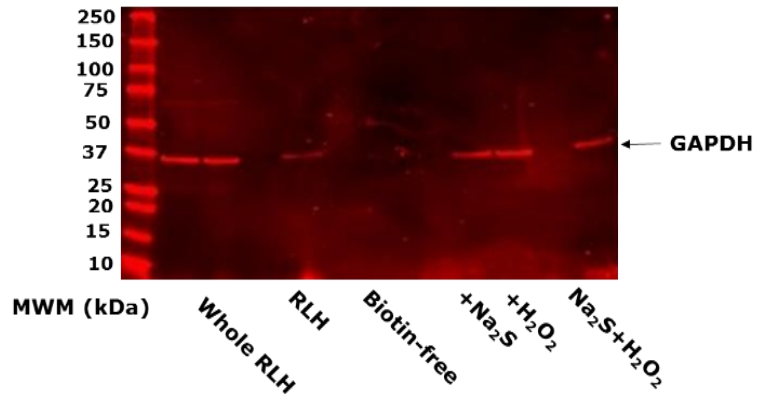
analysis). Data presented as individual points and the central line represents the mean. MWM (molecular weight marker), (n=2)

3.5.1.3. Effect of hydrogen peroxide (H₂O₂) on the detection of sulfhydrated proteins

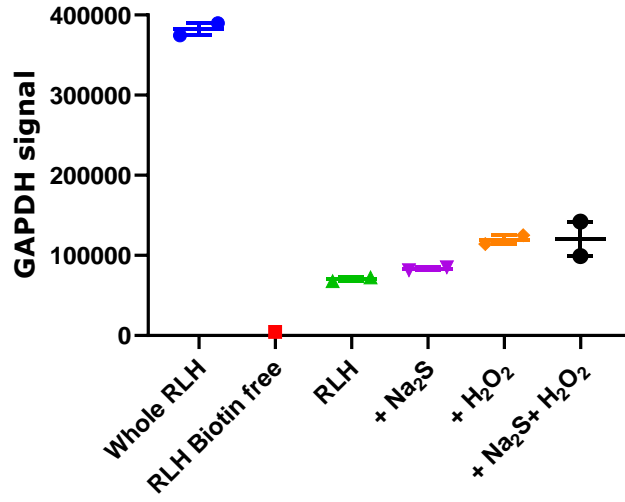
H₂O₂ is an oxidising agent, and it may oxidise cysteine-SH and increase the level of sulfhydration of GAPDH protein, and therefore, H₂O₂ may increase the detection of sulfhydrated GAPDH (Wedmann et al., 2014; Zhang et al., 2014; Filipovic, 2015). Therefore, the aim of these experiments was to assess the effect of 1 mM H₂O₂ (preincubated for 30 min) on GAPDH sulfhydration levels in RLH and PHH. The results of this study suggest that there was a slight increase of the sulfhydrated GAPDH in RLH with exposure to 1 mM Na₂S and 1 mM H₂O₂ compared to the absence of H₂O₂ in RLH (figure 3.4: A and B) and PHH (figure 3.4: C and D). Apparently, there was a variable increase with Na₂S but not with H₂O₂ in both RLH (figure 3.4: A and B) and PHH (figure 3.4: C and D). The results were more variable in RLH in the presence of Na₂S with H₂O₂ (figure 3.4: A and B).

In some experiments, whole RLH and PHH (homogenised and mixed with sample buffer and subjected to Western blotting, without biotin tagging and immunoprecipitation) were used to determine the total amount of GAPDH in the sample. RLH and PHH biotin-free samples were subjected to all BSA steps except for the addition of biotin. They were used to determine the basal levels of proteins in the samples. There was high GAPDH expression level in RLH (figure 3.4: A and B) and PHH (figure 3.4: C and D) whole homogenates compared to samples that had been put through the biotin-switch assay. There was a very low, but detectable GAPDH signal in the absence of biotin (biotin-free), indicating low endogenous levels of biotinylation of GAPDH. Therefore, any increase in biotinylated GAPDH should be due to the process of the biotin-switch assay.

A



B



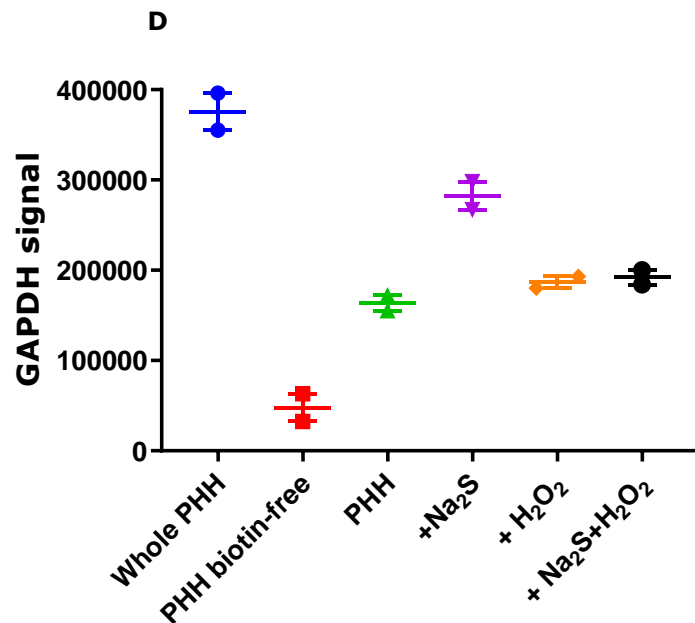
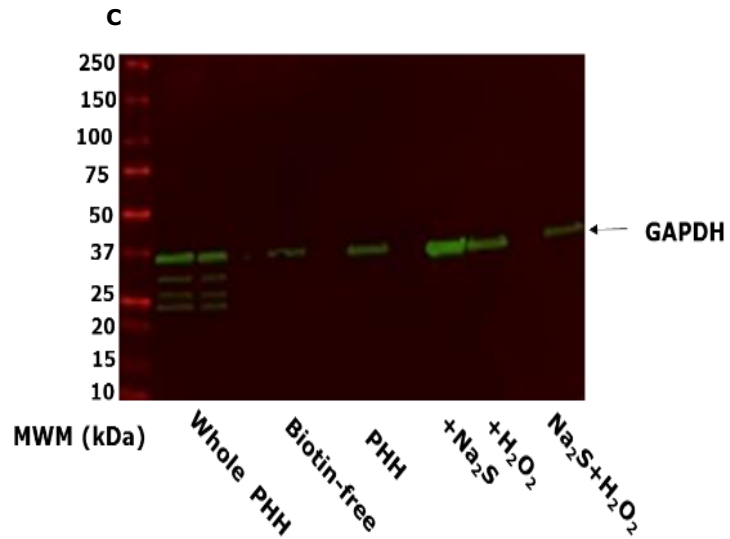


Figure 3.4: Example blots of GAPDH in (A) RLH and (C) PHH. (B and D) Quantification (densitometric analysis) of GAPDH band signal at about 37 kDa of rat liver homogenate (RLH); PPH (porcine heart homogenate) with pre-exposure to 1 mM Na₂S and 1 mM H₂O₂ for thirty min; MWM (molecular weight marker). The Y-axis represents the GAPDH band signal quantification (densitometric analysis). Data presented as individual points and the central line represents the mean. Whole RLH or whole PHH (rat liver or porcine heart homogenised and added sample buffer and subjected to Western blotting). RLH biotin-free and PHH biotin-free (subjected to all BSA steps except the biotin addition), (n=2).

3.5.1.4. Effect of biotin concentrations on the detection of sulfhydrated proteins

The results from the experiments above failed to show major changes in GAPDH sulfhydration using the biotin switch assay. The aim of these experiments was to assess the effect of increased biotin concentrations (4 vs 1 mM). However, detected levels of GAPDH using 1 mM biotin appeared to be the same as that seen with 4 mM in RLH (figure 3.5: A and B) and PHH (figure 3.6: A and B). The results were more variable in RLH using 1 mM biotin concentration in the presence of H₂O₂ (figure 3.5: A and B).

Although, there was no obvious increase in the GAPDH signal in RLH with the modified conditions (figure 3.5: A and B). There was a significant increase in the GAPDH signal in PHH with the addition of 1 mM Na₂S using 1 mM biotin (figure 3.6: A and B). The data were more variable with the addition of 1 mM Na₂S (figure 3.6: A and B).

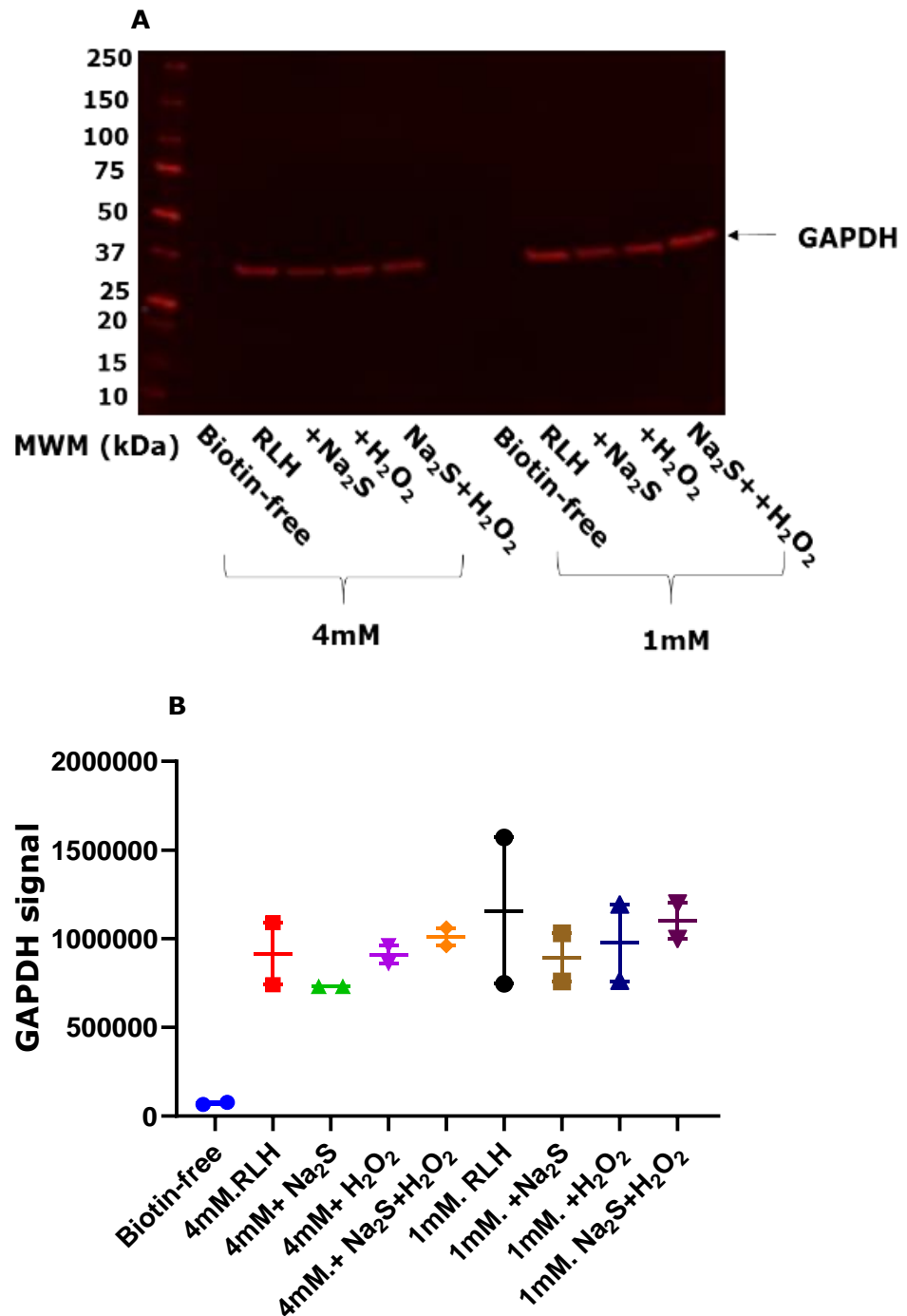


Figure 3.5: (A) Comparison of the effect of incubating the samples with 4 mM biotin compared to 1 mM biotin on levels of GAPDH detected by Western blots in rat liver homogenate (RLH) example blot; after exposure to 1 mM Na₂S in the presence or absence of 1 mM H₂O₂ for thirty min. (B) Quantification of the GAPDH band signal. MWM (molecular weight marker). The Y-axis represents GAPDH band signal quantification at about 37 kDa (kilo Dalton) (densitometric analysis). Data presented as individual points and the central line represents the mean. RLH biotin-free (RLH sample subjected to all BSA steps except the biotin addition); (n=2).

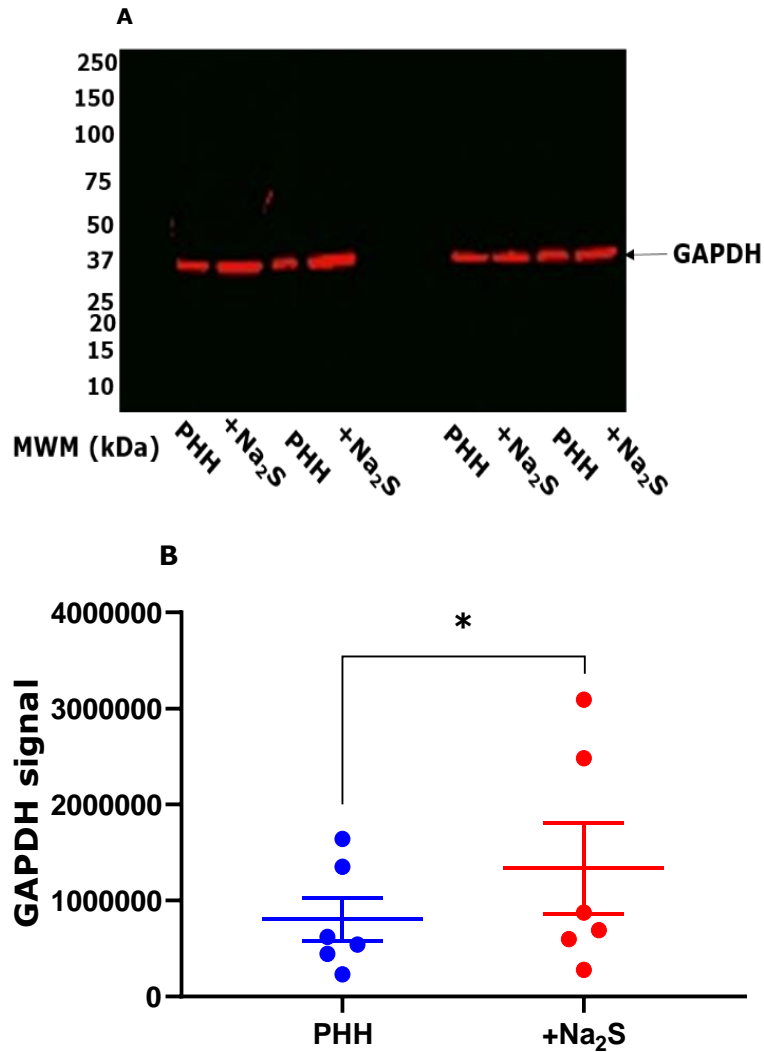


Figure 3.6: (A) Example blot showing the effect of 1 mM Na₂S on sulfhydrylation of GAPDH measuring using 1 mM biotin concentration and in the absence of H₂O₂ in PHH (porcine heart homogenate) using the biotin switch assay. (B) Quantifications of the GAPDH band signals in PPH after exposure to 1 mM Na₂S for thirty min. The Y-axis represents GAPDH signal quantification (densitometric analysis). Data presented as individual points and the central line represents the mean ± SEM. MWM (molecular weight marker); * p < 0.05 using two-tailed paired Student's t-test; (n=6).

3.5.1.5. Effect of dithiothreitol (DTT) on the detection of sulfhydrated proteins

DTT is a strong reducing agent and therefore, DTT may reduce disulfide bond formation, and it may also increase the availability of free sulfhydrated groups (-S-S-H) (Filipovic, 2015; Paul and Snyder, 2015) and therefore, to improve the detection of sulfhydrated proteins. The aim of these experiments was to test the effect of 1 mM DTT (pre-incubated for 30 min before the biotin addition step) on the level of GAPDH sulfhydration in RLH. There was no noticeable increase in the GAPDH signal (sulfhydrated protein) with exposure to DTT (figure 3.7: A and B). There was relatively less variation and more consistency in the results with exposure to DTT (figure 3.7: A and B).

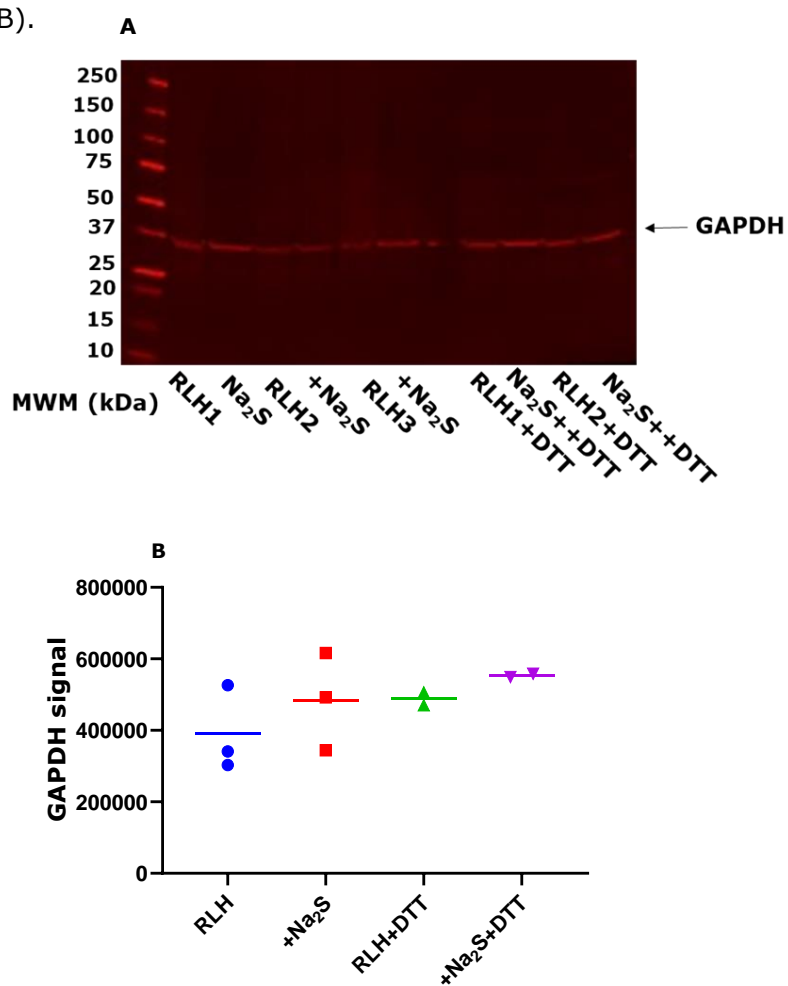
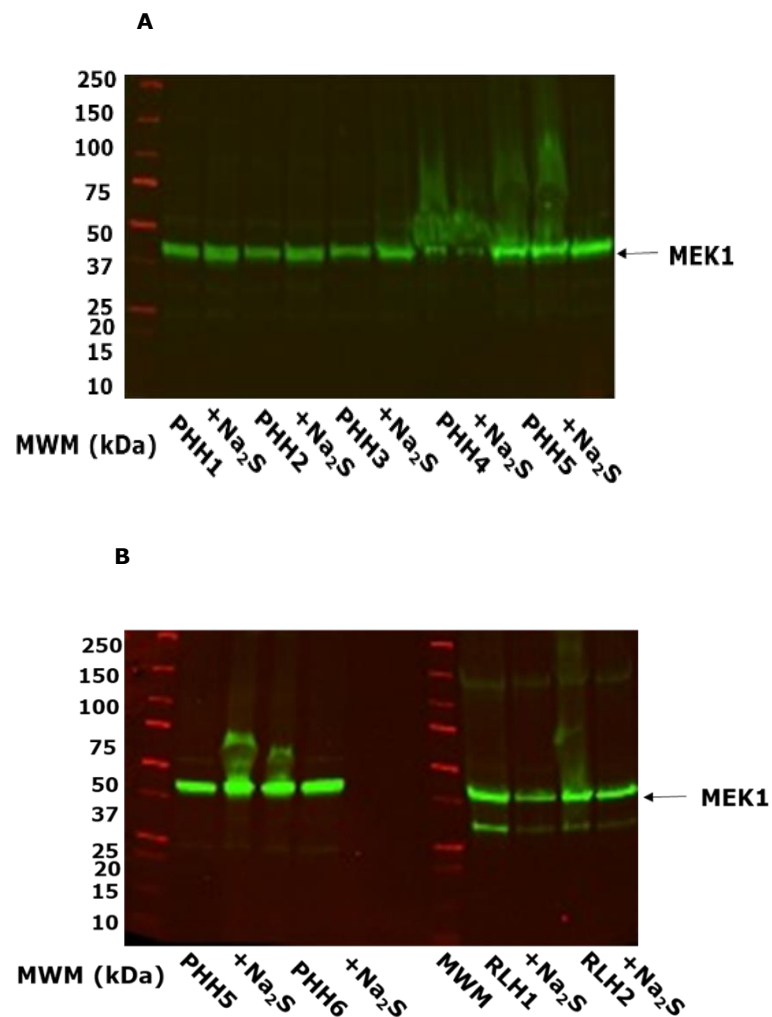


Figure 3.7: (A) Example blot showing sulfhydrated GAPDH levels detected by the biotin switch assay in the presence or absence of 1 mM DTT (dithiothreitol) in rat liver homogenate (RLH). (B) Quantification of the GAPDH band signals with exposure to 1 mM Na₂S and 1 mM DTT for thirty min. The Y-axis represents the GAPDH band signal quantification (densitometric analysis). Data presented as individual points and the central line represents the mean. MWM (molecular weight marker); (n=2-3).

3.5.1.6. Mitogen-activated extracellular signal-regulated kinase 1 (MEK1) sulfhydration

MEK1 is a protein that can be sulfhydrated and play important roles in cell proliferation and apoptosis (Paul and Snyder, 2015; Meng et al., 2017). There was an observable, but variable difference in the level of sulfhydrated GAPDH detected in porcine hearts. Therefore, we determined whether MEK1 could be used as an indicator of protein sulfhydration. GAPDH is reported to have a basal sulfhydration level of about 25 %. In contrast, MEK1 has a basal sulfhydration level of about 10 %, which should allow easier detection of increased levels of sulfhydration than GAPDH (Mustafa et al., 2009; Zhao et al., 2014; Meng et al., 2017). The aim of these experiments was to investigate the sulfhydration of MEK1 in PHH and RLH. There was a significant increase of MEK1 sulfhydration at about 43 kDa in PHH (figure 3.8: A, B and C) and RLH (figure 3.8: B and D) after exposure to 1 mM Na₂S.



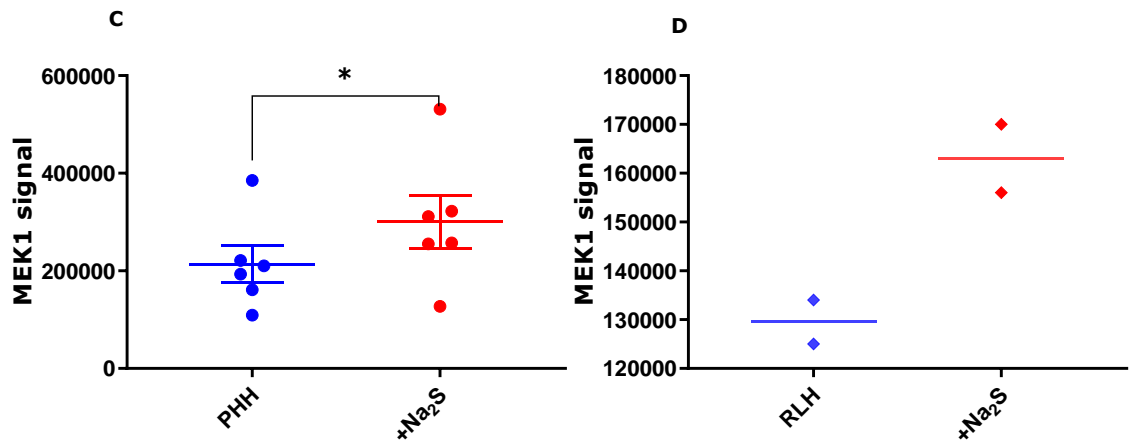
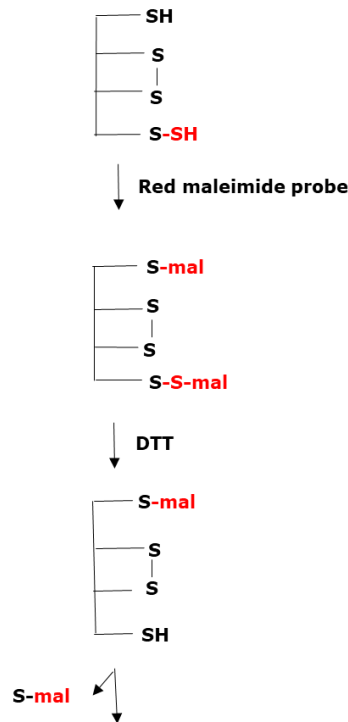


Figure 3.8: (A and B) Immunoblots showing levels of sulfhydrated MEK1 detected using the biotin switch assay in PHH and RLH. (C and D) Quantification of the MEK1 band signals obtained with the anti-MEK1 antibody at about 43 kDa in porcine heart homogenate (PHH) (n=6) and rat liver homogenate (RLH) (n=2) with exposure to 1 mM Na₂S for thirty min. Data presented as individual points with the central line represents the mean. The Y-axis represents MEK1 band signal quantification (densitometric analysis). MWM (molecular weight marker). * p < 0.05 using two-tailed paired Student's t-test; (n=6).

3.5.2. Red Maleimide assay (RMA)

Although there was some evidence that the biotin switch assay could detect changes in protein sulfhydrylation, particularly in porcine heart homogenates, the number of steps involved could lead to loss of sample and increase variation in responses. Therefore, the red maleimide assay (RMA) was investigated as an alternative method.

The principle of RMA is that proteins are labelled with fluorescent red maleimide (RM) probe, which interacts with sulfhydryl groups (Sen et al., 2012). DTT breaks the disulfide bond and releases the red maleimide (RM) probe from the sulfhydrated cysteine but not from the thiol cysteine. Therefore, if there is a decrease in fluorescence signal after exposure to DTT, this indicates the presence of sulfhydrylation (figure 3.9) (Sen et al., 2012; Paul and Snyder, 2015; Meng et al., 2017; Zhang et al., 2017).



**SDS-PAGE decrease of the fluorescence indicates sulfhydrylation
and Western blotting using GAPDH or MEK1 antibody**

Figure 3.9: Illustrative diagram of the red maleimide assay (RMA) steps. Proteins are labelled with red maleimide probe. Then, part of the reaction mixture exposed to DTT, which will lead to the release of red maleimide from the sulfhydrated cysteine and lead to a decrease in the fluorescence. Next, the samples subjected to SDS-PAGE/Western blotting and probed by GAPDH (1:5000) or MEK1 (1:1000) antibodies.

3.5.2.1. Methodology

Samples were prepared as before.

The red maleimide method was according to Sen et al., (2012), Paul and Snyder, (2015) and Sen (2017) with some modifications. Pig heart homogenate (PHH) was incubated with 1 mM Na₂S for 30 min at 37 °C, and then PHH was incubated with 2 µM red maleimide for 2 h at 4 °C with mixing. After the incubation period, one portion of the PHH sample was exposed to 1 mM DTT for 1 h at 4 °C with mixing. Next, the PHH was diluted 1:1 with a non-reducing solubilising buffer (2x SB: 25 % Tris-HCl, 20 % glycerol, 20 % SDS, 35 % distilled water, 0.0004 % Bromophenol Blue) and the PHH samples boiled at 95 °C for 5 min. Then, PHH samples were run on polyacrylamide gels (pre-cast 4-20 % gradient gel, Bio-Rad gels, UK; as in chapter one: Western blotting methodology). Next, the gels were scanned for red maleimide fluorescence signal using Li-COR Odyssey imaging infrared scanner (visualised using the following settings: Image studio software programme version 3.1.4., 684 nm wavelength for excitation and 700 nm wavelength [red] for emission red maleimide signal). Then, the gel was transferred to nitrocellulose membrane paper (GE Healthcare, Little Chalfont, Buckinghamshire, UK) by Western blotting and probed by incubation with mouse anti-GAPDH (1:5000) antibody in 5% fat-free milk in TBST or rabbit anti-MEK1 (1:1000) antibody in 5% BSA in TBST. Finally, nitrocellulose membranes were washed with Milli-Q water and visualised using LI-COR Odyssey Infrared Imaging as described before.

3.5.2.2. Materials

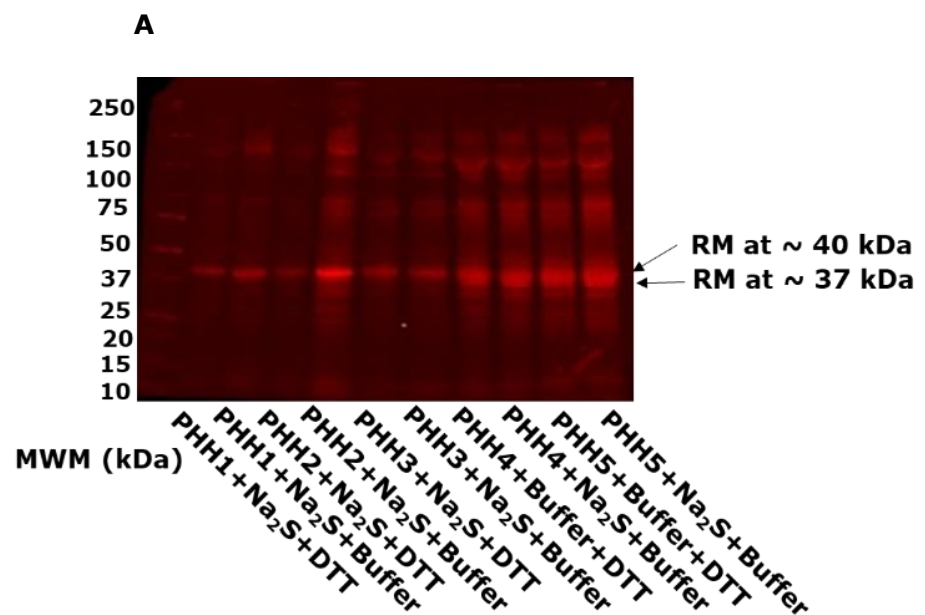
Red maleimide: Alexa fluor 680 C-maleimide (A20344) was obtained from Invitrogen by Thermo Fisher Scientific. All other materials were as mentioned in chapter 2 and 3.

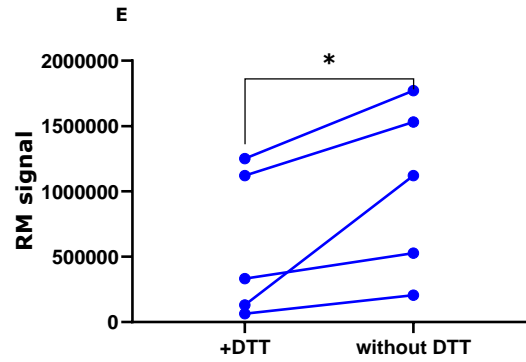
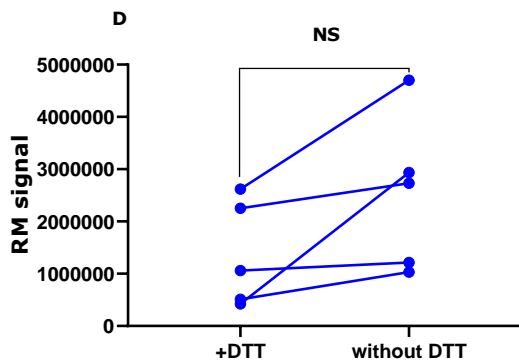
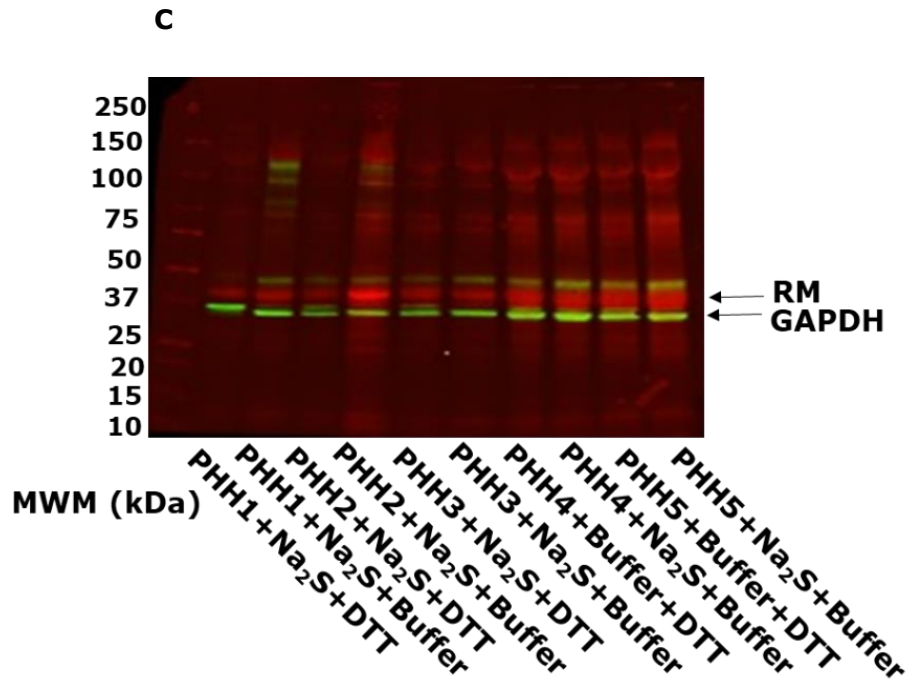
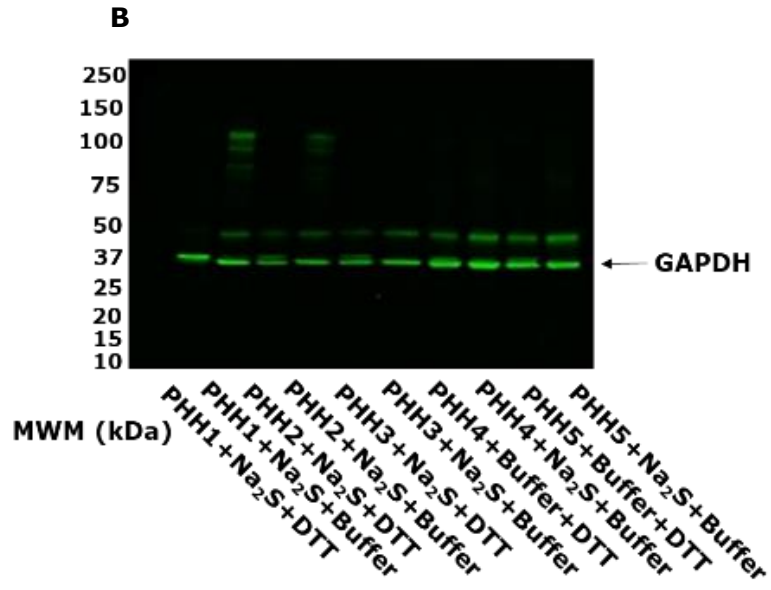
3.5.2.3. Results

3.5.2.3.1. GAPDH and Red Maleimide detection

There appeared to be a decrease in the RM (red maleimide) signal at around 40 kDa in PHH after exposure to 1 mM DTT (figure 3.10: A, C, D and E). However, analysis of the intensity of the bands failed to demonstrate a significant reduction. The anti-GAPDH antibody detected a band at ~37 kDa (figure 3.10: B), which correlated to some of the bands seen with red maleimide (figure 3.10: A, B and E) which were observed during quantification of red maleimide bands at ~40 kDa RM band (figure 3.10: A and D) and at ~37 kDa RM band (figure 3.10: A and E).

Interestingly, there was an apparent shift in the molecular weight of the band obtained with the anti-GAPDH antibody in samples not exposed to DTT, which was not present in DTT exposed samples (figure 3.10: B and C). Moreover, there was no significant difference in the GAPDH signal in the presence of 1 mM Na₂S and absence of 1 mM DTT (figure 3.10: B and F).





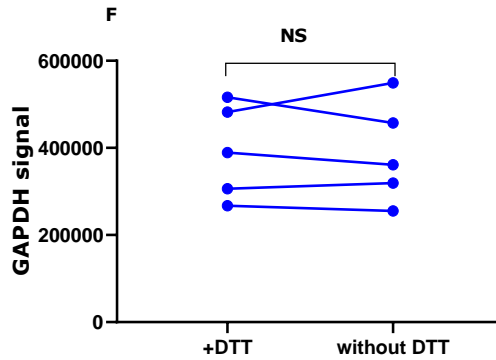
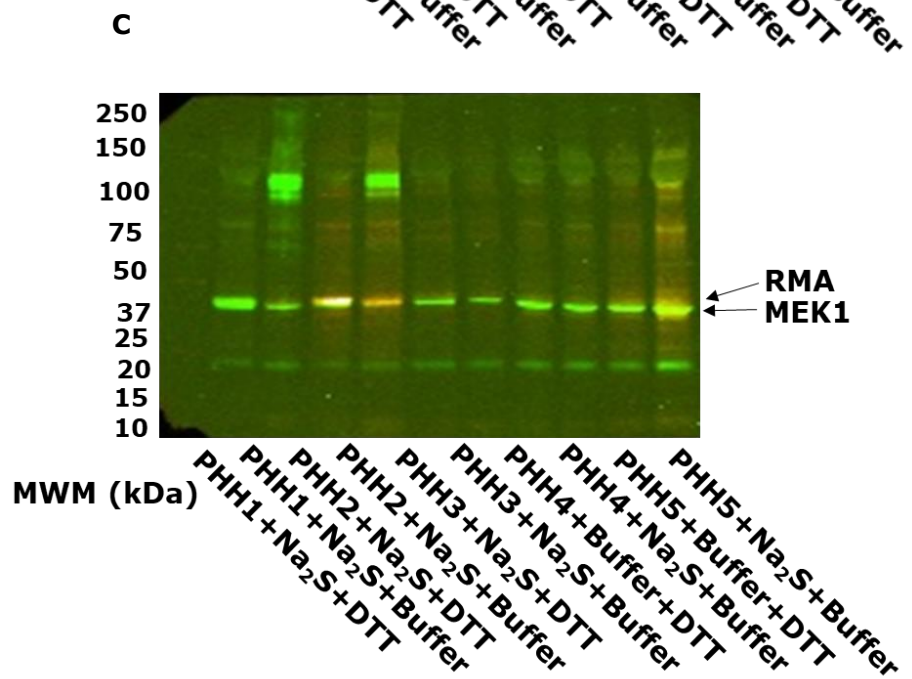
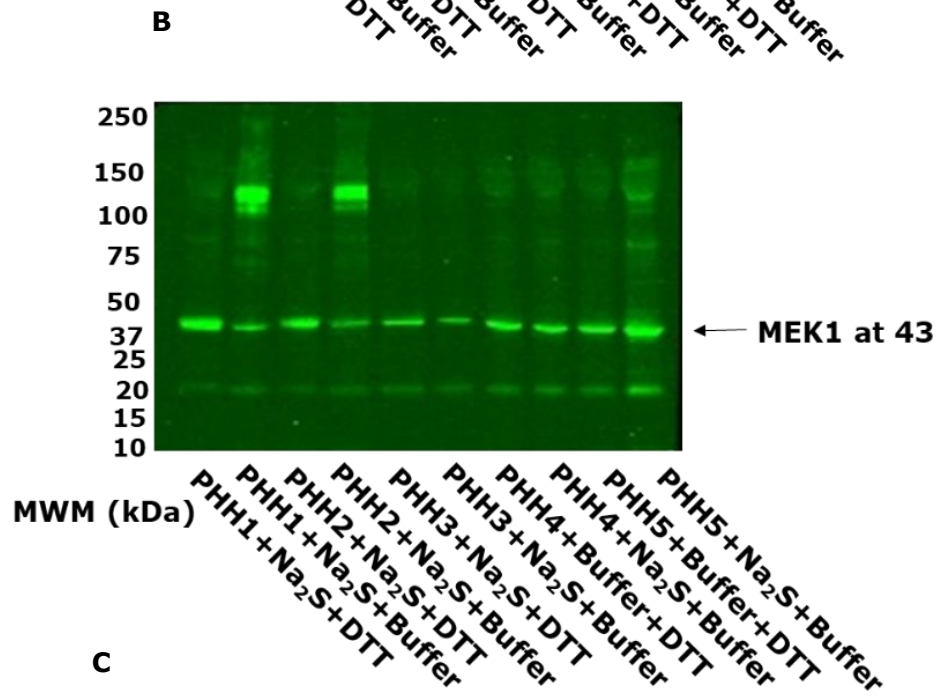
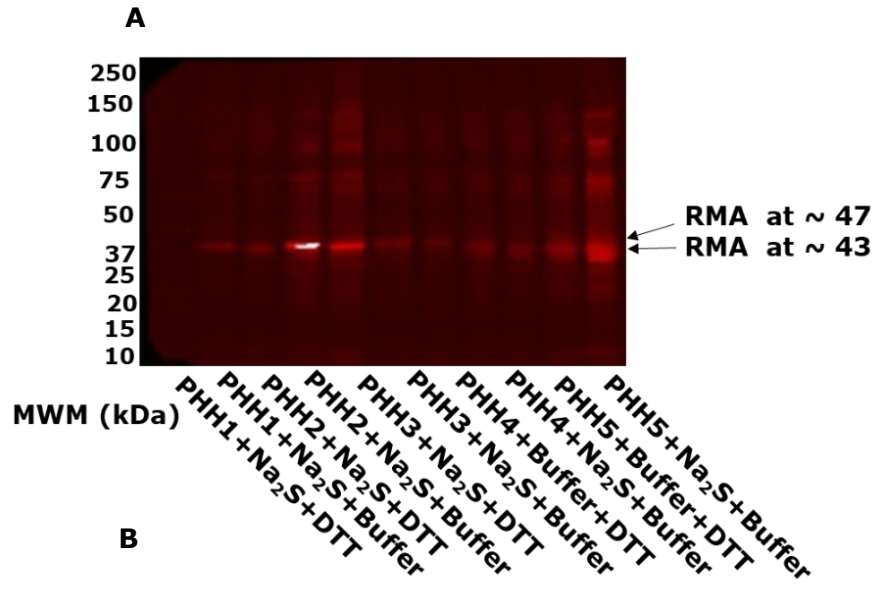


Figure 3.10: Western blot showing RM (red maleimide) bands at ~ 37 kDa and ~ 40 kDa (A) and GAPDH band expression at ~ 37 kDa (B) and both RM band and GAPDH band (C). (D) Quantification of the RM bands of ~ 40 kDa. (E) Quantification of the RM bands of ~ 37 kDa. (F) Quantification of GAPDH band at ~ 37 kDa. Na_2S (sodium sulfide, H_2S releasing salt); DTT (dithiothreitol); PHH (pig heart homogenate) of five different tissues ($n=5$). The Y-axis represents RM or GAPDH signal quantification (densitometric analysis). Data presented as individual points. MWM (molecular weight marker); NS (non significant) $p > 0.5$, * $p < 0.05$ using two-tailed paired Student's t-test; ($n=5$).

3.5.2.3.2. MEK1 and Red Maleimide detection

The aim of this experiment was to test whether this shift in GAPDH immunoreactivity (outlined above) to a lower MW was specific to GAPDH protein, by investigating MEK1. There was a decrease in RM signals in some of the PHH samples after exposure to 1 mM DTT at 43 kDa, which corresponds to the predicted MW of MEK1 (figure 3.11: A, C and D) and at ~ 47 kDa (figure 3.11: A, C and E). Moreover, there were some consistent shifts to low molecular weight (MW) bands in the presence of 1 mM Na_2S and absence of 1 mM DTT in the band obtained with the anti-MEK1 antibody (figure 3.11: B). There was no significant difference in the MEK1 signal in the presence of 1 mM Na_2S and absence of 1 mM DTT (figure 3.11: B and F) at either 43 or 47 kDa (figure 3.11: D).



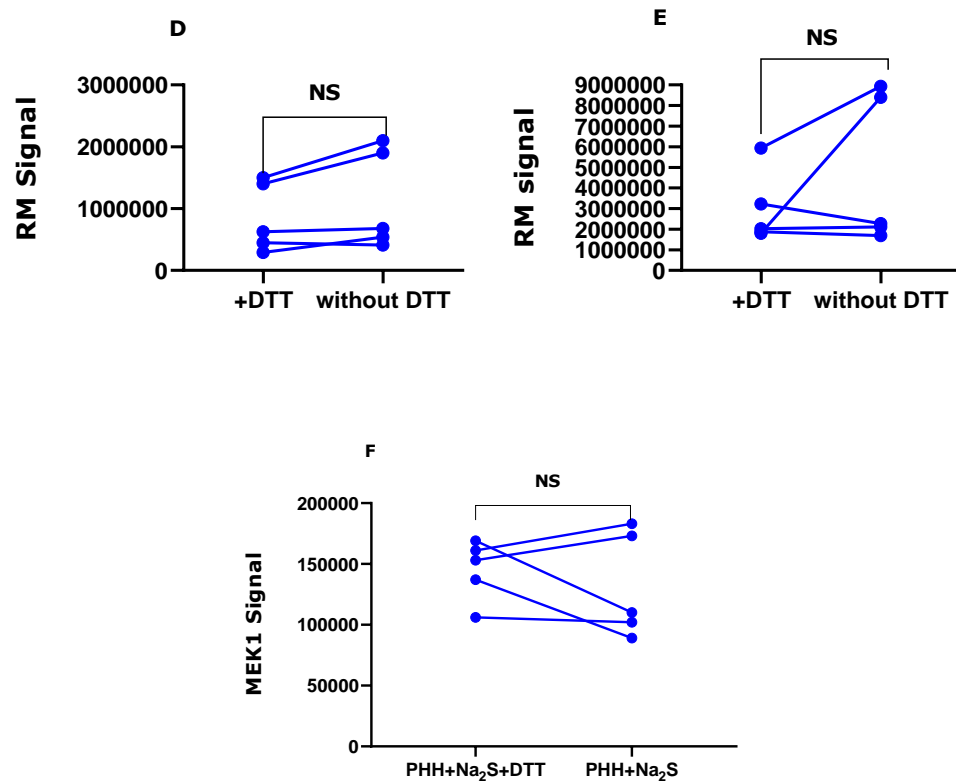


Figure 3.11: Western blot showing (A) RM (red maleimide) bands signal at ~43 kDa and at ~47 kDa; (B) MEK1 (mitogen-activated protein kinase) expressions at 43 kDa; (C) both RM band and MEK1 band. (D) Quantification of the RM band at ~43 kDa. (E) Quantification of the RM band at ~47 kDa. (F) Quantification of the MEK1 band at ~43 kDa. The Y-axis represents band signal quantification (densitometric analysis) (D, E and F). Data presented as individual points. DTT (dithiothreitol, 1 mM); Na₂S (Sodium sulfide, H₂S releasing salt, 1 mM); PHH (pig heart homogenate) of five different tissues (n=5). NS (non significant) $p > 0.5$ using two-tailed paired Student's t-test; (n=5).

3.5.2.3.3. Molecular weight shifts after non-exposure to DTT

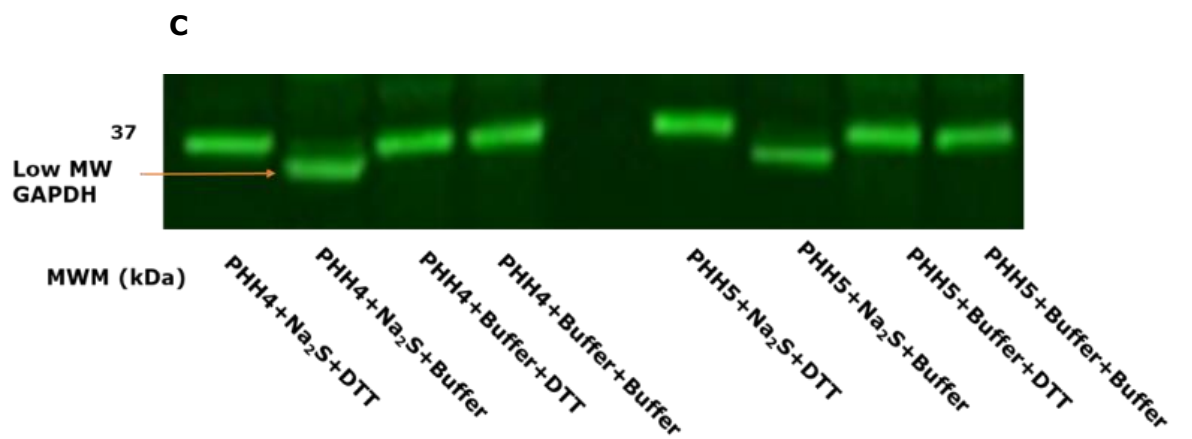
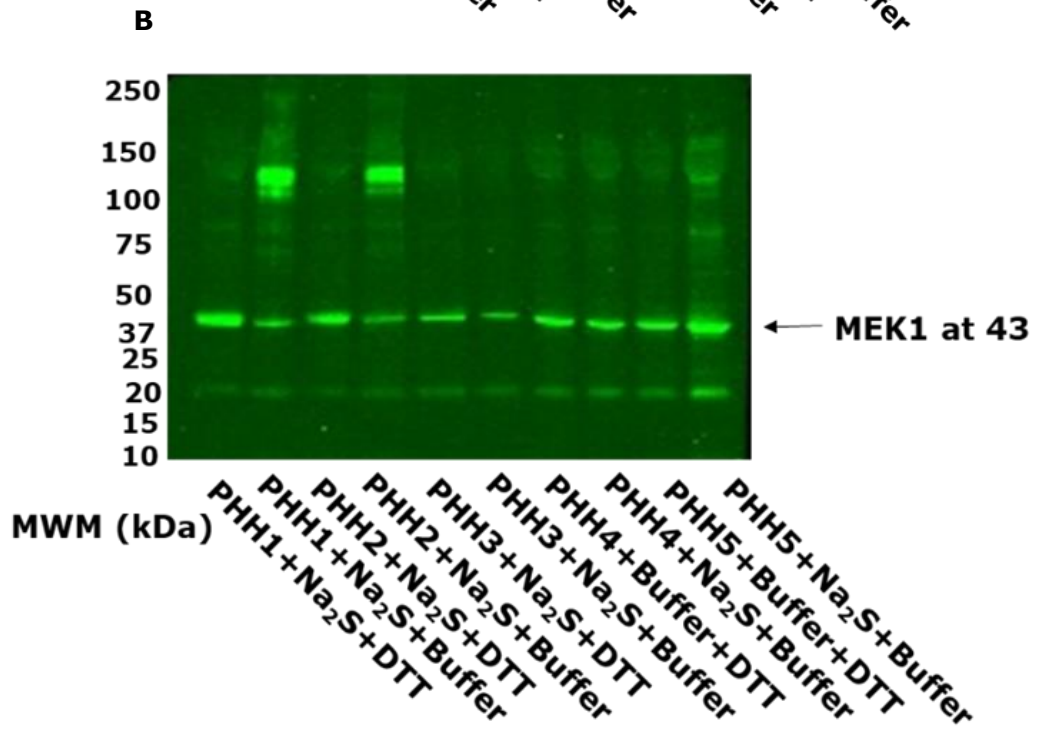
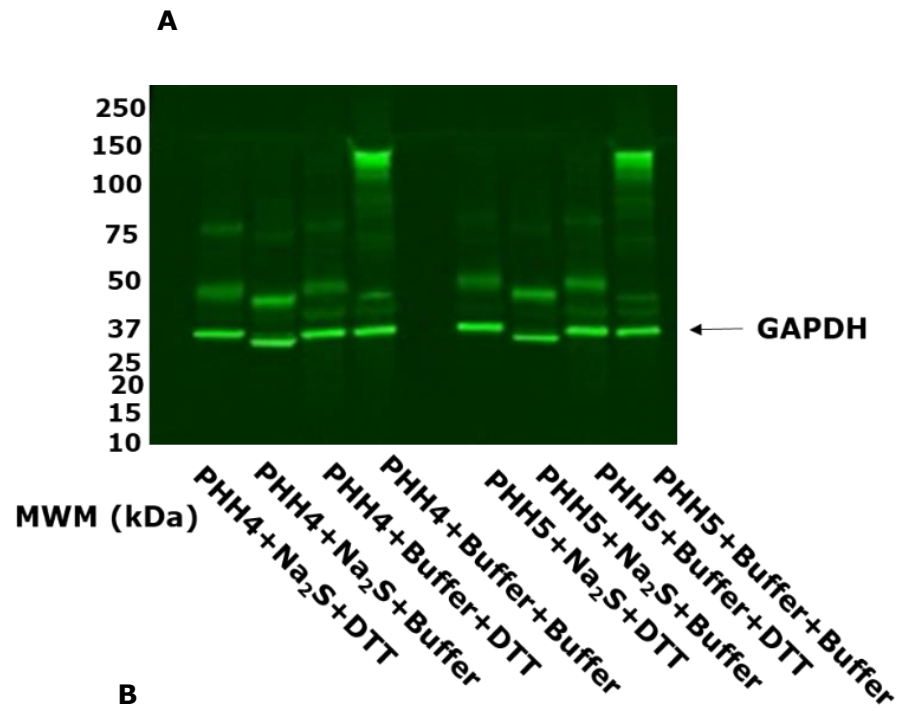
As the studies with red maleimide above indicated that SDS-PAGE under non-reducing conditions might uncover changes in the migration of GAPDH and MEK1, we repeated the experiment in the absence of red maleimide. We tested the effect of DTT on the potential sulfhydration of GAPDH or MEK1 by Na₂S.

There were consistent shifts to low molecular weight bands in the presence of 1 mM Na₂S and absence of DTT compared to samples incubated with DTT, with both anti-GAPDH and anti-MEK1 antibodies (figure 3.12: A, B, C and D). There was no shift in the apparent molecular weight in samples exposed to buffer instead of Na₂S, in the absence or presence of DTT (figure 3.12: C and D). Quantification of the ratio of the low MW to high MW band intensity are shown for GAPDH (figure 3.12: E) and MEK1 (figure 3.12: F)

There was a significant increase in ratio of the low/high MW GAPDH intensity on exposure to Na₂S and absence of DTT condition (figure 3.12: E) in comparison to other conditions, such as exposure to Na₂S and DTT, DTT and buffer, and buffer only (figure 3.12: E). The results were relatively variable on exposure to Na₂S and absence of DTT condition (figure 3.12: E).

There was no significant increase in ratio of the low/high MW MEK1 intensity on exposure to Na₂S and absence of DTT condition in comparison to exposure to Na₂S and DTT condition (figure 3.12: E). The results of MEK1 were more variable than GAPDH on exposure to Na₂S and absence of DTT condition (figure 3.12: E and F).

There was no significant difference in the total GAPDH (figure 3.12: G) or total MEK1 band signal quantification (figure 3.12: H) after exposure to 1 mM Na₂S in the presence or absence of 1 mM DTT, indicating that there was the same amount of GAPDH or MEK1 present and this means equal loading, albeit running at a different MW (high and low MW).



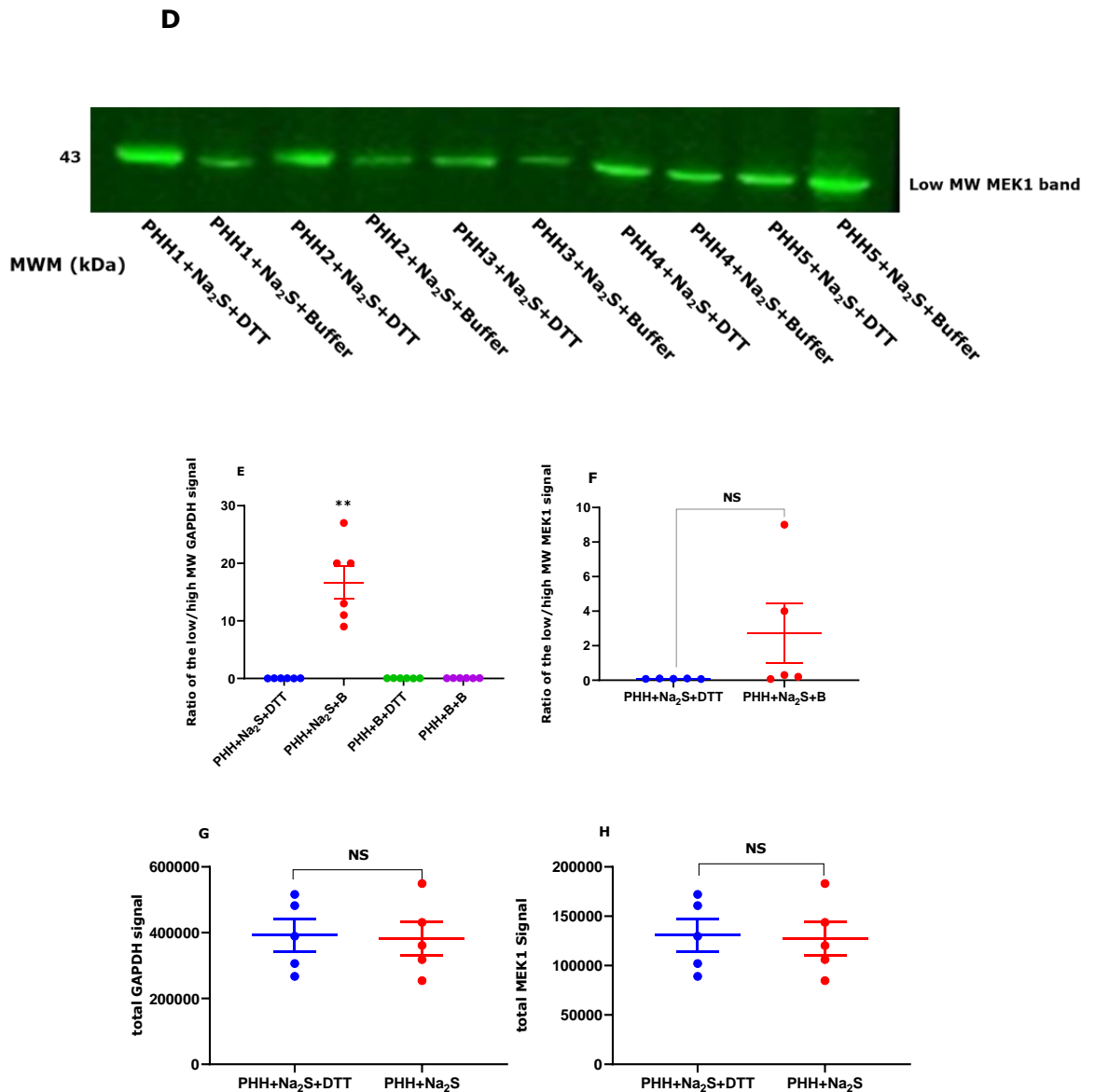


Figure 3.12: Western blot images of porcine heart homogenate (PHH) under different conditions by exposure of PHH to 1 mM Na₂S (Sodium sulfide, H₂S salt) in the presence or absence of 1 mM DTT (dithiothreitol) or B (buffer, Tris-EDTA buffer 0.1 M, 1 mM, pH 7.4). (A) Blot showing bands of GAPDH of low MW at 37 kDa and ~30-33 kDa. (B) Blot showing bands of MEK1 antibody bands at 43 kDa and ~40 kDa (B). (C and D) The expanded version of the images showing the high and low MW bands of GAPDH and MEK1. (E) Quantification of ratio of the low MW to high MW GAPDH band signal. (F) Quantification of ratio of the low MW to high MW MEK1 bands signal. (G) Quantification of the total GAPDH bands signal (low MW+ high MW GAPDH bands signal). (H) Quantification of the total MEK1 bands signal (low MW+ high MW MEK1 bands signal). The Y-axis represents band signal quantification (densitometric analysis) (E, F, G and H). Data presented as individual points and central line represents mean data \pm SEM. PHH (pig heart homogenate); B (buffer, Tris-EDTA buffer 0.1 M, 1 mM, pH 7.4). NS (non significant) $p > 0.5$ using two-tailed paired Student's t-test; ** $p < 0.01$ PHH+Na₂S+B vs PHH+Na₂S+ DTT, PHH+B+ DTT and PHH+B+B using one-way ANOVA followed by Sidak's multiple comparison test, (n=5).

3.5.3. Immunoprecipitation (IP) of GAPDH in PHH

3.5.3.1. Principle and aim

Enrichment of GAPDH was conducted by immunoprecipitation with an appropriate antibody as recommended by Sen et al. (2017).

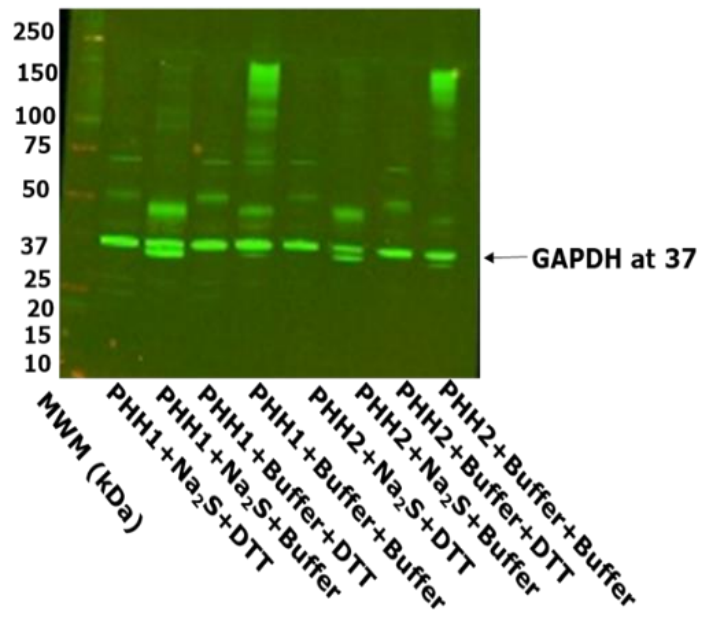
3.5.3.2. Methodology

After exposure to Na₂S, the PHH sample was incubated with a high concentration of anti-GAPDH antibody (1 µl of anti-GAPDH antibody per 60 µl of PHH, 1:60) for 8-12 h (overnight) at 4 °C on a shaker. Subsequently, 20 µl protein A/G plus-agarose beads (Santa Cruz Biotechnology, USA, SC-2003) was added to the supernatant layer and mixed for 4-6 h at 4 °C on a shaker. Then, the mixture was centrifuged at 13,000 rpm for 5 min at room temperature. The supernatant layer was then removed. The pellet was washed by mixing with two cycles of 50 µl Tris-EDTA buffer (0.1 M, 1 mM, pH 7.4). The mixture was centrifuged at 13,000 rpm for 10 min at room temperature. The supernatant layer was removed and 2x non-reducing solubilising buffer was added to the pellet. The sample was boiled at 95 °C, and then SDS-PAGE/Western blotting and probed with anti-GAPDH antibody (1:5000).

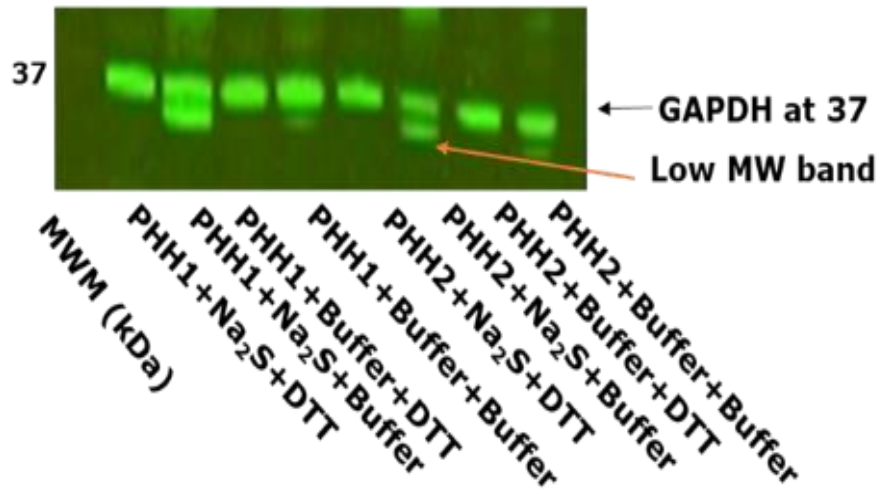
3.5.3.3. Results of immunoprecipitation: GAPDH signal after exposure to DTT

Following quantification of ratio of the low/high MW GAPDH signal (figure 3.13: C), there was a significant increase in ratio of the low MW/high MW GAPDH signal on exposure to Na₂S and absence of DTT condition (figure 3.13: A, B and C) in comparison to other conditions, such as exposure to Na₂S and DTT, DTT and buffer, and buffer only. The results were variable on exposure to Na₂S and in the absence of DTT (figure 3.13: A, B and C). Therefore, shifts to low MW bands were observable in the presence of 1 mM Na₂S and absence of DTT (figure 3.13: A, B and C). In some samples, only the low MW band was present following exposure to Na₂S, but in some samples, both the higher and lower bands were detected (figure 3.13: A and B). There was no significant difference in the total GAPDH signals between all conditions (figure 3.13: D).

A



B



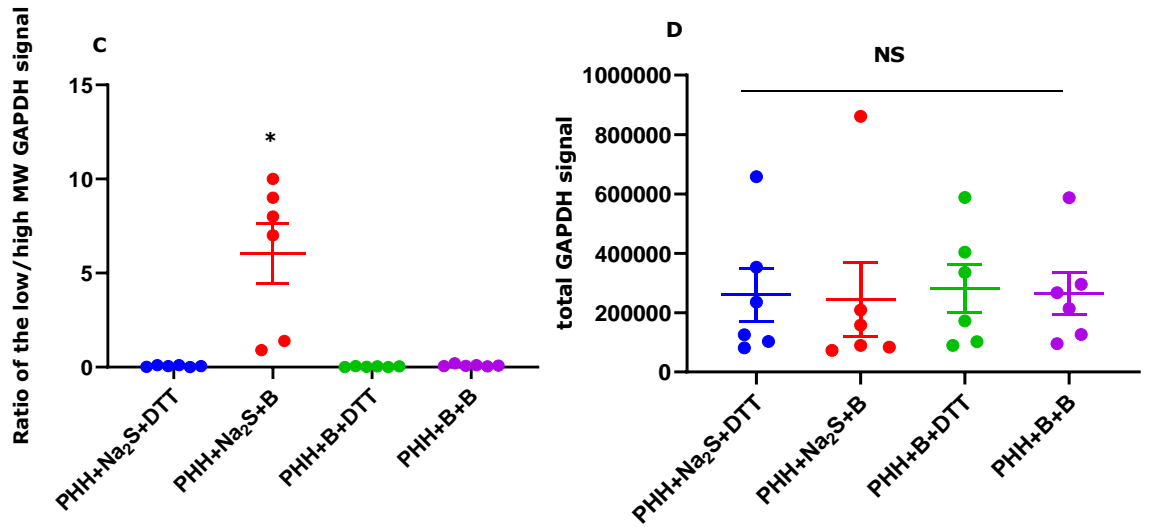


Figure 3.13: (A) Western blot image showing bands to anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibody at about 37 kDa after immunoprecipitation with anti-GAPDH in the presence or absence of 1 mM Na₂S (Sodium sulfide, H₂S salt) and with or without 1 mM DTT (dithiothreitol). (B) The expanded version of the images showing the high and low MW GAPDH bands. (C) Quantification of ratio of the low/high MW GAPDH signal. (D) Quantification of total GAPDH signals (low MW + high MW GAPDH signal). The Y-axis represents the GAPDH band signal quantification (densitometric analysis). Data presented as individual points and central line represents mean data \pm SEM. PHH (pig heart homogenate); B (buffer, Tris-EDTA buffer 0.1 M, 1 mM, pH 7.4). NS (non-significant) $p > 0.05$ using One-way ANOVA following by Sidak's multiple comparison test. * $p < 0.05$ PHH+Na₂S+B vs PHH+Na₂S+ DTT, PHH+B+ DTT and PHH+B+B using One-way ANOVA followed by Sidak's multiple comparison test, (n=5).

3.5.4. GAPDH sulfhydration in the PCA

3.5.4.1. Methodology:

The experiments outlined above indicate that electrophoresis under non-reducing conditions may uncover a change in the observed molecular weight with both GAPDH and MEK1 in samples exposed to Na₂S. This observation indicates that this may be a simpler method for detecting changes in sulfhydration. In order to determine whether this method could be used to detect changes in sulfhydration in intact blood vessels, 5 mm of proximal PCA segments were set up and mounted for isometric tension recording system in organ baths filled with 20 ml Krebs'-Hensleit buffer at 37 °C and aerated with 95 % O₂/5 % CO₂ and then incubated with 1 mM Na₂S for 15 min at 37 °C and then rapidly removed and finely homogenised in Tris-EDTA buffer (0.1 M, 1 mM, pH 7.4) on ice with a glass: glass homogeniser. Subsequently, 2x non-reducing solubilising buffer was added to the sample and boiled at 95 °C for 5 min and centrifuged at 13,000 xg for one min. Western blotting was conducted using a 4-20 % gel as above and probed with anti-GAPDH antibody (1:5000).

3.5.4.2. Results

There was no significant increase in ratio of the low/high MW GAPDH intensity on exposure to Na₂S (figure 3.14: A, B and C). The results were relatively variable on exposure to 1 mM Na₂S (figure 3.14: C). There were some observable shifts to the low molecular weight (MW) bands. This shifting was associated with the presence of double bands of GAPDH in some PCA samples in the presence of 1 mM Na₂S compared to the absence of Na₂S (buffer) (figure 3.14: A, B and C). There was no significant difference in the total GAPDH signals (figure 3.14: D).

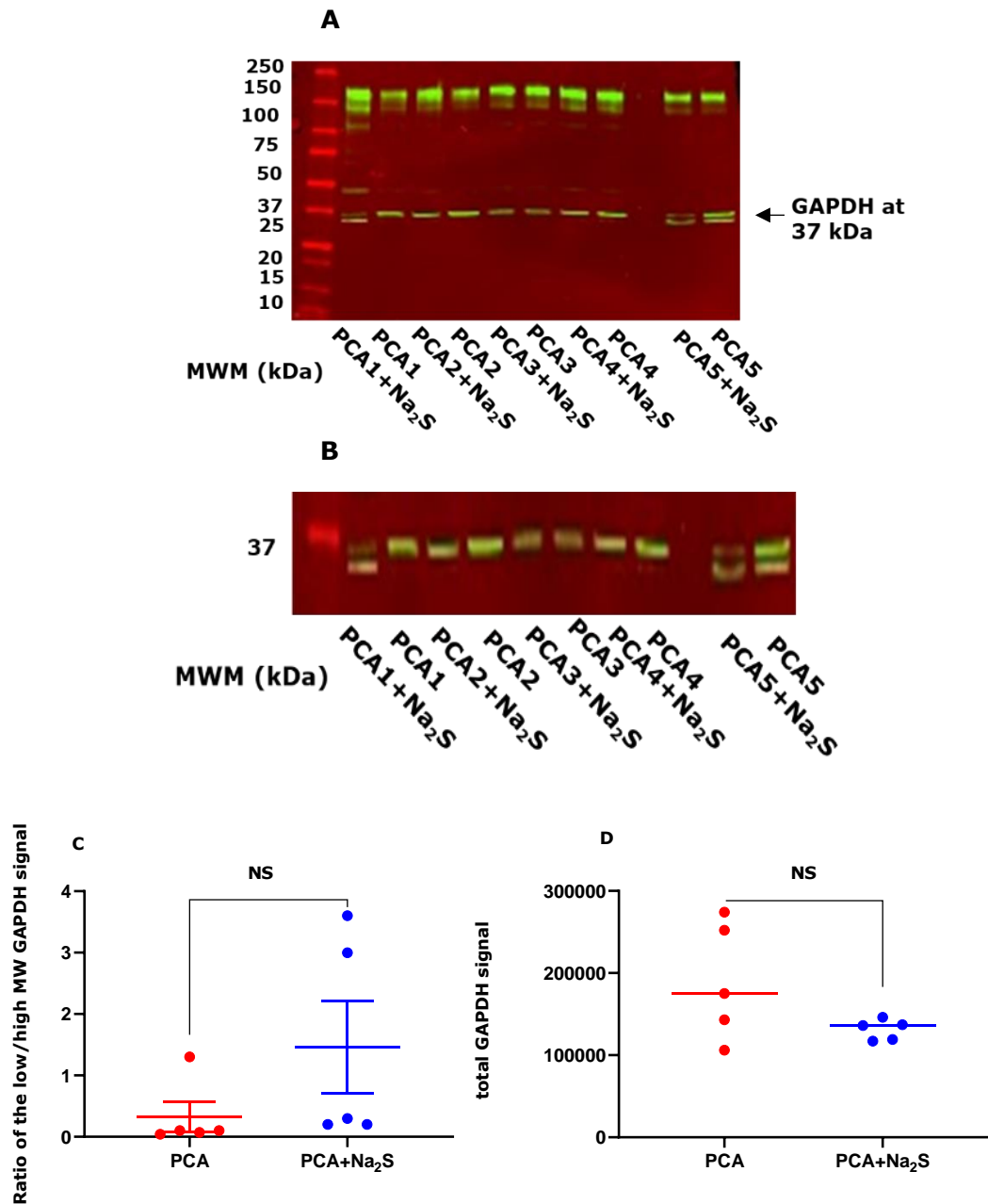


Figure 3.14: (A) Western blot images of anti-GAPDH antibody bands in the presence or absence of 1 mM Na₂S (Sodium sulfide, H₂S salt) in PCA (porcine coronary artery). The blots showing bands to the anti-GAPDH antibody of low MW (molecular weight) bands at 37 kDa and ~30-33 kDa). (B) The expanded version of the image showing the high and low bands of GAPDH. (C) Quantification of ratio of the low/high MW GAPDH signal. (D) Quantification of total GAPDH signals (low MW+ high MW GAPDH bands signal). The Y-axis represents GAPDH bands signal quantification (densitometric analysis). Data presents as individual points and the central line represented mean± SEM. NS (non significant) $p > 0.5$ using two-tailed paired Student's t-test, (n=5).

3.6. Discussion

Based on Western blotting and enzyme assays (methylene blue and SF7-AM assays), there appeared to be a low enzyme activity level in the pig heart compared to liver. Sulfhydrylation has emerged as a potential novel signalling pathway for H₂S in biological systems. Therefore, measuring sulfhydrylation levels of target proteins, such as GAPDH and MEK1 may help us to understand the signalling pathways and could also be used as an index of H₂S production. Sulfhydrylated proteins (persulfides, reductant-labile) can be considered as signalling pathways and stores of H₂S, which can release H₂S under specific conditions, such as reducing condition (Ubuka, 2002). Therefore, the development of a reliable assay for measuring sulfhydrylation could be a useful tool to study and understand signalling pathways of H₂S in biological systems, in particular, the heart. Therefore, it is important to develop a reliable assay for measurement sulfhydrylation, which may regulate cardiac function as discussed below and could be used as index for any changes in cardiac dysfunction.

It has been reported that sulfhydrylation of proteins results in conformational alterations in the targeted proteins, which lead to the alteration of protein activity. For example, sulfhydrylation of some proteins, such as GAPDH by NaHS could lead to an increase in the enzyme activity of GAPDH by about seven-fold (Mustafa et al., 2009). Conversely, sulfhydrylation of some proteins, such as calcium channels could lead to decreasing of protein activity and closing of ion channels. For example, Zhang's study suggested that sulfhydrylation is the responsible mechanism for the blockade of calcium channels and negative inotropic effects induced by H₂S. Diamide is an oxidant sulfhydryl modifier, which decreases the availability of free sulfhydryl and decreases sulfhydrylation (Zhang et al., 2012; Dai et al., 2019). In rat cardiomyocytes, diamide decreases the sulfhydrylation and therefore, decreases the calcium channels blockade induced by NaHS (Zhang et al., 2012). In another study, sulfhydrylation of phospholamban (PLN) by NaHS led to relaxation of the myocardium of rats and frogs possibly via decrease of intracellular calcium level (Mazza et al., 2013). Similarly, sulfhydrylation could be responsible for NaHS mediated calcium channel blockade in rat vascular smooth muscle cells (Dai et al., 2019). In another study, sulfhydrylation of TRPV channels by Na₂S leads to activation of TPV channels and plays important role in H₂S-mediated relaxation in aorta (Naik et al., 2016). Sulfhydrylation of Keap1 (Kelch-like ECH-associated protein 1) via GYY4137 led to augmentation of Nrf2 signalling and decrease of oxidative stress and inflammation in the aorta of mice with diabetes and atherosclerosis (Xie et al., 2016). Similarly, sulfhydrylation of mouse and human platelet proteins via GYY4137 led to an attenuation of platelet activation and thrombus formation in microvessels (Grambow

et al., 2014). Therefore, sulfhydrylation of proteins and ion channels, such as calcium and TRPV channels is an important signalling pathway of H₂S in the cardiovascular system.

3.6.1 The biotin switch assay results

The biotin switch assay (BSA) was the first assay developed for measuring sulfhydrylation in tissue homogenates (Mustafa et al., 2009). The data presented in this chapter indicate that there was an increase in the amount of biotinylated GAPDH present in the samples after incubation with Na₂S, indicating an increase in the sulfhydrylation of GAPDH. However, the data are extremely variable. Therefore, there is a question mark about whether the biotin-switch assay is a reliable method to measure sulfhydrylation level in tissues, such as the heart. Moreover, in some of the biotin switch studies there was no clarity in some aspects, such as the variability of results, protein concentration, incubation period with H₂S donor, blot image, and few number of experiment repeats. For example, Zhao's study incubated the HUVECs with NaHS for 2 h (Zhao et al., 2014) compared to 30 min incubation (Mustafa et al., 2009).

In the biotin switch assay, there are some concerns about the selectivity of the methiolating (alkylating) reagent methyl methanethiosulfonate (MMTS). However, MMTS is selective for reversible blocking thiol because thiol and sulfhydrylated cysteine residues (persulfides) have different chemical properties. Moreover, MMTS does not interact with oxidative and nitrosylated thiols (Mustafa et al., 2009). Therefore, the development of more selective thiol blocking agents could be a useful factor to improve measurement of sulfhydrylation using the biotin switch assay.

The stability of sulfhydrylated protein in the biotin switch assay is questionable because the biotin switch assay needs a long time using multiple steps of mixings and precipitations, which may lead to loss of sulfhydrylated protein and variability in the results. Our results demonstrated that the variability was relatively more in the liver than the heart. This result could be attributed to species or tissue variation. Therefore, the level of sulfhydrylated protein detected by the biotin switch assay might not reflect the actual sulfhydrylation level. Moreover, there are several proteins which may be sulfhydrylated. Therefore, it might be not easy to quantify and confirm which protein band is specifically sulfhydrylated.

H₂O₂ might alter SH level in rat liver and pig heart since some reports have claimed that oxidation of cysteine-persulfides led to more sulfhydrylation susceptibility by H₂S (Filipovic, 2015; Meng et al., 2017). However, our results showed that oxidation of

RLH and PHH by H₂O₂ produced no difference in the GAPDH signal by the biotin switch assay. Similar result was observed in mouse liver (Mustafa et al., 2009).

We observed that more GAPDH signal was detected by BSA in PHH than RLH and this result suggests a greater level of sulfhydration in the heart. This difference might be attributed to variation in tissue or species properties or different posttranslational modifications between the liver and heart, or it might be due to the antibody used, or it could be due to prevalence of the sulfhydration in the heart rather than the liver. The role of biotin in the BSA is labelling the sulfhydrated cysteines, which can be readily extracted using streptavidin-agarose. In several studies in the literature, they have commonly used 4 mM biotin in the BSA (Mustafa et al., 2009; Módis et al., 2016). Our results demonstrated the use of low concentration biotin (1 mM) vs commonly used (traditional) (4 mM) produced similar results and it might be useful to demonstrate biotin dependency for the detection of sulfhydrated proteins. The absence of biotin (biotin-free) led to absence or very low, but detectable band signal. Therefore, these results might suggest that biotin's role in the biotin switch assay is qualitative rather than quantitative, or that 4 mM biotin concentration may be in excess and less than 4 mM biotin may safely be used in biotin switch assay.

Our results of the biotin switch assay, demonstrated that the absence of biotin (biotin-free) resulted in a very low, but detectable band signal. This result is in agreement with Mustafa et al's study, which reported that there was no signal in the absence of biotin (Mustafa et al., 2009). This result indicated that the basal levels of biotinylated proteins are low. Therefore, the signal obtained through the biotin switch assay did not reflect the endogenously biotinylated protein.

Mustafa and his associates' study reported that about 10-25% of mice liver proteins are basally sulfhydrated using the biotin switch assay. 25 % of GAPDH was sulfhydrated, while other proteins, such as MEK1 had less basal sulfhydration level at about 10% (Mustafa et al., 2009). In other words, the baseline sulfhydration of MEK1 is lower than GAPDH. Our results of the biotin switch assay suggested that there was relatively more sulfhydration of the MEK1 than GAPDH after exposure to Na₂S. This result might suggest that the MEK1 protein had a lower basal sulfhydrated level, and it might be more prone to sulfhydration by Na₂S. Therefore, it is possible that we cannot detect sulfhydration of GAPDH after Na₂S treatment because it is already highly sulfhydrated, whereas MEK1 is less sulfhydrated. So there is more MEK1 to be sulfhydrated in the presence of Na₂S. In another study, the biotin switch assay was used to measure sulfhydration of MEK1 by NaHS in human umbilical vein endothelial cells (HUVECs) led to a decrease of DNA damage and an increase of DNA repair and possible cytoprotective and cardiovascular protective effects against

aging-related CVD (Zhao et al., 2014).

Overall, the biotin assay results suggested potential sulfhydration detected in the porcine heart, and it may be a potential signalling pathway for H₂S in the heart. Using the BSA, the results were inconsistent and variable, especially in the liver. Therefore, whether biotin switch assay is a sensitive and reliable method to detect changes in the H₂S in the heart and liver is yet unclear and may need further studies to be clarified.

3.6.2. The red maleimide assay results

As the biotin switch assay (BSA) produced variable data and because the presence of a number of steps means the potential loss of sample and variability of the results, the red maleimide assay (RMA) was tested as an alternative method to measure the sulfhydrylation in pig heart. RMA is an easy, simple, direct assay and needs fewer steps and has less protein loss in comparison with the BSA.

The basis of RMA is that proteins are labelled with fluorescent red maleimide (RM) probe. DTT cleaves the disulfide bond and releases the red maleimide probe from the sulfhydrated cysteines (modified) but not from the non-sulfhydrated cysteines (unmodified/free). Therefore, after exposure to DTT, if there is a decrease in fluorescence signal of band after exposure to DTT, this indicates the presence of sulfhydrylation.

The results of the RMA demonstrated that there was a significant decrease in the RM band signal in the presence of DTT. The RMA detected a number of bands, but whether these correlated with GAPDH or MEK1 is difficult to judge. Therefore, decrease of RM band signal after exposure to Na₂S and in the presence of DTT could indicate potential sulfhydrylation of numerous proteins at different molecular weights (Sen, 2017). The RMA was useful to measure the sulfhydrylation of TRPV channels by Na₂S and this sulfhydrylation plays important role in H₂S-mediated relaxation in rat aorta endothelial cells (Naik et al., 2016). The RMA showed promising properties, sensitivity and selectivity over the biotin switch assay, because in the RMA, there is no interactions with other amino acids, such as methionine, tyrosine and histidine and the RMA particularly targeting thiol groups of cysteine residues of the targeted proteins (Sen et al., 2012).

An interesting observation in the RMA was that there was a clear, consistent shifting of the bands observed with both GAPDH and MEK1 to a lower MW in the presence of Na₂S and absence of DTT when samples were run under non-reducing conditions. This observation may be due to DTT decreasing the intermolecular bonds (reduces disulfide bonds) of protein and interferes with the sulfhydrylation process, as indicated in the RMA. Moreover, sulfhydrylation causes a shift in the MW that the protein runs at and a similar effect is seen with phosphorylation. Therefore, these data suggest that sulfhydrylation may alter the way the proteins run on polyacrylamide gels, which can only be detected under non-reducing conditions. The running of proteins on polyacrylamide gels under non-reducing condition is important because it may keep the protein conformation in the folded structure. Therefore the interaction between

the polypeptides is preserved, which may affect the protein separation on the polyacrylamide gels.

Although the apparent molecular weight of the proteins changed, the total density of the bands remained the same, indicating that there was no change in the amount of protein, or the amount of protein that could be detected by the antibody, just that there are shifts in the MW bands. The shifting to a lower MW band is more obvious with GAPDH than with MEK1, which might be attributed to the prevalence of sulfhydrylation in GAPDH compared to MEK1. Furthermore, the results with MEK1 were more variable than GAPDH.

In some of the RMA results, there was a non-significant difference in the RM signal after exposure to DTT, especially with MEK1 more than GAPDH. This result may be normal and expected since it cannot predict to see the same impact always in the samples with Na₂S and DTT. Also, whether the sulfhydrylation of protein by Na₂S is reversible or permanent is not well established.

In short, RMA seems to work for measuring the sulfhydrylation in the heart because there was a significant decrease of the red maleimide signal in Na₂S treated samples on exposure to DTT, especially with GAPDH. Therefore, the red maleimide assay may be a useful and robust tool for measuring sulfhydrylation. However, the observation that the MW of proteins appears to change in samples exposed to Na₂S and run under non-reducing conditions suggests that this may be a simpler way of detecting changes in sulfhydrylation.

3.6.3. The immunoprecipitation and immunoblotting results

As the results of the red maleimide assay above indicated that running of the proteins in non-reducing conditions might uncover changes in the sulfhydrylation level and observed molecular weight that GAPDH and MEK1 run at, we repeated the experiment in the absence of red maleimide in order to determine whether this shift in MW could be used as a measure of sulfhydrylation. Immunoprecipitation of GAPDH protein was also carried out in order to increase the amount of GAPDH and remove any possible sample contamination by removing other proteins and visualising only the GAPDH protein. In the immunoprecipitation test, there was observable shifting of the GAPDH band to a low MW (increased ratio of the low/high MW signal) in the presence of Na₂S and absence of DTT (non-reducing condition). DTT shifted the apparent MW of the bands back to the usual position. These data lend support to the observation that running samples under non-reducing conditions may allow detection of sulfhydrylation by measuring changes in the apparent molecular weight. Our results demonstrated that the presence of Na₂S is the most essential factor for sulfhydrylation measuring, especially by the ratio of the high/low MW signal, immunoblotting and immunoprecipitation assay. Therefore, we observed that the sulfhydrylation is undetectable in the absence of Na₂S and presence of DTT and buffer.

The level of sulfhydrylation by Na₂S might be the cause of the variability in results, especially with MEK1 rather than GAPDH. These variations could be due to instability of sulfhydrated proteins, or differences in level of sulfhydrylation between proteins.

As the studies in the tissue homogenates indicated that proteins exposed to Na₂S run at a different MW in non-reducing PAGE conditions, we investigated whether this method could be used to pick up differences in proteins in isolated blood vessels, such as PCA (intact blood vessels). As with the tissue homogenates, there were some obvious shifts in the observed MW that GAPDH runs at in the tissues exposed to Na₂S, at least in some PCAs. However, the variability between samples could be attributed to a loss of sulfhydrylation in organ bath and during the homogenisation step during PCA sample preparation.

3.6.4. Summary

Briefly, the different assays for measuring sulfhydrylation could be useful. The biotin switch assay produced variable, and inconsistent results, potentially due to the complicated steps and procedure. The red maleimide assay results were encouraging, more so with GAPDH than MEK1. Also the red maleimide assay is simpler and easier, and it has fewer steps. The shift in the MW using immunoprecipitation and immunoblotting is an even simpler technique for measuring the sulfhydrylation, and it may be comparable to the red maleimide assay and could be explored further.

The development of a reliable assay for measuring the sulfhydrylation is important because it may provide a useful tool to study the signalling pathways for H₂S and as an index for H₂S production in the heart, which may provide an enhanced understanding of the biological role and impact of H₂S in the cardiovascular system, particularly in cardiovascular diseases.

Chapter Four

Comparison of Signalling Pathways of Hydrogen Sulfide Sources (Na_2S , GYY4137 and AP39) in the Relaxation Response of Porcine Coronary Artery

4.1. Introduction:

The results of the present study demonstrated low H₂S generation in the heart, but the addition of the myocardial tissue led to relaxation of PCA. Therefore, H₂S generated in the myocardial tissue could act in a paracrine manner, rather than directly on atrial or ventricular contractility. Thus, comparison the effects of exogenous H₂S using three different H₂S sources on PCA could be useful.

Hydrogen sulfide (H₂S) has been shown to be involved in the cardiovascular system (CVS) of mammals. Thus, H₂S sources (donors, drugs) reduce the damage of particular cardiovascular insults (Geng et al., 2004; Lilyanna et al., 2015; Chen et al., 2016). In 1997, Hosoki's study reported that H₂S has relaxing effects on the vascular tissues, such as rat portal vein and aorta (Hosoki et al., 1997). Moreover, it has been reported that there is an inverse relationship between H₂S level and oxygen level (Olson et al., 2006; Olson, 2013). Therefore, relaxing effects of H₂S could be changed depending on oxygen level (Olson et al., 2006). For example, in rat aorta, low H₂S levels (about 5-100 μM) lead to a contraction response at high oxygen level (about 200 μM), while a relaxation response is at high H₂S levels (about 200-400 μM) and at low oxygen level (about 40 μM) (Koenitzer et al., 2007). Thus, removal of oxygen is suggested to cause relaxation of coronary artery segments and this relaxation response could be mediated via H₂S, at least in part.

Most of the literature of H₂S is based on use of H₂S salts as a source for H₂S. These inorganic salts, such as NaHS and Na₂S release a large amount of H₂S in a short time, a fast release H₂S source and they could lead to supra-physiological concentrations. Therefore, there is a question mark about how useful and reliable these data are. These H₂S salts could be considered as the classical H₂S sources used in many previous studies (Wang, 2012; Powell et al., 2018).

Using three different H₂S donors (sources), which release H₂S by different mechanisms could have an effect on the relaxation response and the mechanism of relaxation. In this study three different H₂S sources were used. First, inorganic H₂S salts, such as NaHS and Na₂S, a fast release H₂S source. Second, GYY4137 [morpholin-4-ium 4 methoxyphenyl (morpholino) phosphinodithioate] is an organic slow-release rate H₂S source, which releases a small amount of H₂S slowly over time, a slow release H₂S source (Li et al., 2008; Karwi et al., 2016). It is unclear whether GYY4137 releases H₂S inside or outside the cells and this may be due to a slow hydrolysis of GYY4137 (Yu et al., 2010).

Third, AP39 [(10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5-yl) phenoxy) decyl) triphenylphosphonium bromide] is a mitochondria-targeted organic H₂S source, which releases H₂S in the mitochondria (Ahmad et al., 2016; Karwi et al., 2017). AP39 has two parts, one part is triphenylphosphonium (TPP⁺), which a mitochondrially targeted part and the other part is dithiolethione, which is the H₂S donor part, and these two parts are connected by an aliphatic linker. GYY4137 and AP39 could be considered as new generation H₂S sources in recent studies.

Signalling mechanisms in response to exogenous H₂S application by H₂S sources in the CVS are relatively established, and multiple signalling mechanisms have been reported in mammals, such as interactions with NO and nitrosothiol formation. Modulation of ion channels, such as the opening of potassium channels, blocking of calcium channels and opening TRPV channels. Moreover, H₂S may mediate the relaxation response at low oxygen level condition (hypoxia) and act as an oxygen transducer (Olson et al., 2006). However, these studies are usually carried out with inorganic H₂S salts, such as NaHS and Na₂S. Therefore, the key aims of this chapter were to determine whether there are any differences in the relaxation response and mechanism of relaxation among different types of H₂S sources. Thus, it would be important to compare the effects of compounds releasing H₂S by different ways on vascular tone.

This chapter aimed to gain a greater insight into the role and molecular mechanism/s of H₂S in the vasculature and identify whether distinct exogenous H₂S salts and sources (donors, drugs) differentially influence tone in the PCA and to compare these effects. Furthermore, to investigate the mechanisms of relaxation responses of these three H₂S sources under different conditions, such as the presence and absence of nitric oxide synthase inhibitor (L-NAME), potassium channels blockers, calcium-free condition, glucose-free condition, hypoxia and reoxygenation.

4.2. Materials and Methods:

4.2.1. Tissue Preparation:

Hearts from large white hybrid pigs of either sex, aged 4-6 months, and weighing about 50 kg were obtained from a local abattoir and transported to the laboratory in ice-cold Krebs'-Henseleit solution ([mM]: NaCl 118, KCl 4.8, CaCl₂ 1.3, NaHCO₃ 25, KH₂PO₄ 1.2, MgSO₄ 1.2, glucose 11.1) (Fisher Scientific, Loughborough, UK) on ice. The Krebs'-Henseleit solution had been pre-gassed with 95% O₂/ 5% CO₂. The left circumflex PCA was then dissected out.

4.2.2. Isolated organ bath experiments:

The PCA was put in Krebs'-Henseleit solution pre-gassed with 95% O₂/ 5% CO₂ for overnight storage at 4 °C. The next day, the fat and connective tissue were removed from the PCA, vessels finely dissected to prepare paired PCA rings from each artery ensuring vessels were not stretched/ or damaged and then cut into rings of ~ 5 mm in length (~2-3 mm internal diameter) and were suspended in a multichannel 20 ml or 5 ml tissue baths. PCA segments were attached to two metal hooks placed through the lumen, ensuring that the hooks were not overlapping. One hook was connected to a glass rod, and the other was connected to an isometric force transducer by a silk thread. Each bath was filled with 20 ml or 5 ml of Krebs-Henseleit solution and maintained at 37 °C and constantly gassed with 95% O₂, 5% CO₂. After about 20 min equilibration period, 8-10 g tension was applied to each segment, and tissues allowed to equilibrate for about 60 min. Previous experiments indicated that 8-10 g was the optimum level of tone for the PCA. This tension was measured by isometric force transducer and recorded using a Power-Lab data acquisition system computer program (AD Instruments, Sydney, Australia). Transducers were calibrated with a 10 g weight on a daily basis.

When a stable baseline was reached, 60 mM KCl was added to allow standardisation of responses and to check tissue viability. After about 10 min, the KCl was washed out with fresh Krebs'-Henseleit solution. Following the return to a stable baseline and a further 20-30 min recovery period, exposure to KCl was repeated. Once again, the tissue was washed out with Krebs'-Henseleit solution, to allow the segment tone to re-stabilise to a baseline.

4.2.3. Effect of different H₂S sources on U46619-induced contractions

These experiments were performed in order to assess the effect of Na₂S, GYY4137 and AP39 on vascular tone, in particular, the relaxation response. U46619, a thromboxane A₂ mimetic contractile agent, was used to induce contraction of PCA rings. PCA rings were exposed to U46619 (1 nM-300 nM) in a cumulative manner to about 40-60% of the final response to KCl contraction. After the contraction had reached a plateau, a single (bolus) concentration of H₂S source was added: Na₂S (300 μM or 1 mM) or vehicle control (distilled water); GYY4137 (100 μM) or vehicle control (0.1 % v/v DMSO); AP39 (1, 5, 10 and 30 μM) or vehicle control (0.1 % v/v DMSO), and then recording tension for about 60 min (Rashid et al., 2013; Donovan et al., 2017; Donovan et al., 2018). Relaxation responses were expressed as the change in contractility expressed as a percentage of the U46619 response as the y-axis.

In some experiments, cumulative concentration-response curves (CRC) of Na₂S or NaHS in U46619 pre-contracted PCA segments were performed. In other experiments, the effects of Na₂S and NaHS as a single addition were compared with cumulative concentrations in U46619 pre-contracted PCA.

4.2.4. The effect of different H₂S sources on calcium-induced contractions

In some experiments, tissues were washed with Krebs-Henseleit solution in which the calcium was replaced with 2 mM ethylene glycol bis (β-aminoethyl ether)-N, N, N', N'- tetra acetic acid (EGTA, calcium chelator). Tissues were then washed with calcium-free Krebs without EGTA. Next, PCA tissue was pre-incubated with each one of the three different H₂S sources, 1 mM Na₂S (or distilled water vehicle control for Na₂S) or 100 μM GYY4137 (or 0.1 % v/v DMSO vehicle control for GYY4137) or 30 μM AP39 (or 0.1 % v/v DMSO vehicle control for AP39) for 15 min in order to determine the effect of H₂S donors on calcium influx induced contractions. Next, 60 mM KCl was added to open voltage-gated calcium channels prior to the addition of a single concentration of calcium chloride to a final concentration 1 mM.

4.2.5. The effect of different H₂S sources on the BayK8644-induced contractions

BayK8644 is a dihydropyridine derivative and L-type calcium channel opener (Thomas et al., 1985). In order to determine the involvement of calcium channels, in particular, L-type voltage-gated calcium channels in the H₂S-mediated relaxation responses, the effect of H₂S sources on BayK8644-induced contractions were determined. The PCA tissues were contracted to 40-60 % of KCl contraction response by BAYK8644 addition. Then, each H₂S sources: 1 mM Na₂S (or distilled water vehicle control for Na₂S) or 100 μM GYY4137 (or 0.1 % v/v DMSO vehicle control for GYY4137) or 30 μM AP39 (or 0.1 % v/v DMSO vehicle control for AP39) was added and then the larger relaxation responses were measured.

4.2.6. Investigating the mechanism/s of relaxation responses to different H₂S sources in the isolated pre-contracted PCA

In this series of experiments, the mechanisms of relaxation evoked by different sources of H₂S were tested.

300 μM L-NAME (N-nitro L-arginine methyl ester) was employed to inhibit nitric oxide synthase activity and the production of nitric oxide from the endothelium. 10 mM TEA (tetraethylammonium) was used to block potassium channels non-selectively. 1 μM glibenclamide or 100 μM tolbutamide was used to inhibit K_{ATP} channels selectively. In order to confirm that glibenclamide was selective for K_{ATP} channels in the PCA, the effect of 1 μM glibenclamide on the response to pinacidil, a K_{ATP} channel opener (Gollasch, 1995; Zhao et al., 2001), was determined. Glucose-free conditions were administered to determine mitochondrial ATP synthesis in comparison to energy supply from glycolysis. Therefore, in order to investigate the cellular energy source, glucose-free Krebs was employed during the whole experiment

FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) acts by uncoupling the mitochondrial respiratory chain and impairing ATP production (Módis et al., 2013; Szczesny et al., 2014). Therefore, in order to determine the role of mitochondria in the relaxation responses to AP39, relaxations to a submaximal concentration of AP39 were determined in the presence of FCCP. The concentration of 1 μM was used based on previous observations in the laboratory, about the role of mitochondria in simvastatin (lipid-lowering drug) mediated relaxation responses in PCA (Almukthar et al., 2016). 10 μM capsazepine was used to block TRPV1 channel, according to study about H₂S-mediated relaxation responses in rat mesenteric arteries (White et al., 2013), which suggests that TRPV1 channels involved in H₂S vasorelaxation responses.

These modulators L-NAME, TEA, glibenclamide, tolbutamide, FCCP, and capsazepine were administered for about 60 min to PCA segments, which were then contracted to 40-60 % of KCl contraction response by U46619 addition, and then exposed to the indicated sources of H₂S.

4.2.7. The effects of oxygen level on the relaxation responses of different sources of H₂S

Since molecular oxygen may oxidise H₂S and decrease H₂S availability (Olson et al., 2006; Olson, 2008), the influence of changing the oxygen level from 95 % O₂/CO₂ mix (control) to 21 % O₂/5 % CO₂ (air-like) throughout the experiment was examined. Tissues were contracted in the presence of U46619 as before, and then the different sources of H₂S were added.

4.2.8. The effects of different sources of H₂S before or after the addition of U46619 on the hypoxia and recovery responses in PCA

In order to determine the effect of the H₂S sources on the hypoxic response in the PCA, tissues were pre-incubated (15 min) with 1 mM Na₂S, 100 μM GYY4137 or 30 μM AP39 before U46619 addition. Then, U46619 was added to get 40-60 % of the KCl contraction. Next, hypoxia (replacing the 95 % O₂/5 % CO₂ gas with 95 % N₂/5 % CO₂) was applied for 15 min. PCA segments were then reoxygenated (oxygen reintroduction). Subsequent hypoxia response, peak (maximum) relaxation response of hypoxia, hypoxia-induced contraction and peak recovery response to reoxygenation were measured (figure 4.2).

Pre-incubation with the different sources of H₂S before U46619 may lead to loss of the H₂S before the induction of hypoxia, because of the time required for U46619 to produce a contraction. Therefore, the experiment was repeated by contracting with U46619 to get 40-60 % of the KCl contraction, prior to addition of the H₂S sources for 15 min and subsequent induction of hypoxia (replacing the 95 % O₂/5 % CO₂ gas with 95 % N₂/5 % CO₂) for 15 min. PCA segments were then reoxygenated (oxygen reintroduction). Subsequent hypoxia response, peak (maximum) relaxation response of hypoxia, hypoxia-induced contraction, and peak recovery response to reoxygenation were measured.

4.2.9. The effects of endogenous H₂S on responses to hypoxia and reoxygenation

There is some evidence in the results of this study, which suggests that H₂S sources, such as GYY4137 could decrease the recovery response to reoxygenation (oxygen reintroduction after hypoxia). As before, U46619 was added to get 40-60 % of the KCl contraction. Next, hypoxia (replacing the 95 % O₂/5 % CO₂ gas with 95 % N₂/ 5 % CO₂) was applied for 15 min before adding 100 μM AOAA and 100 μM PPG for 30 min to inhibit CBS and CSE, respectively. The PCA segments were then reoxygenated (oxygen reintroduction), and subsequent changes in tone monitored.

4.2.10. Defining time-dependent parameters of changes in tone

Two time-dependent parameters for relaxation were quantified: t_{100} was defined as the time latency to reach maximal relaxation (pr, peak relax) after administering the different sources of H₂S, while t_{50} was defined as the time at which 50 % of the peak relaxation was attained (figure 4.1).

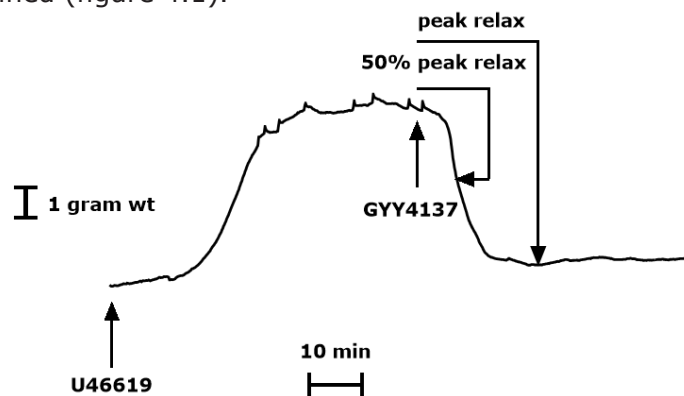


Figure 4.1: a representative trace recording to illustrate the time-dependent relaxation parameters measured following administration of GYY4137.

In the response to reoxygenation, two time-dependent parameters were quantified: tr_{100} was defined as the time latency following switching the gas source to high oxygen levels to reach peak recovery response to reoxygenation (pr_r), while tr_{50} was defined as the time at which 50 % of the peak recovery response to reoxygenation was attained. There was a gap for gas change for about 5 min (figure 4.2).

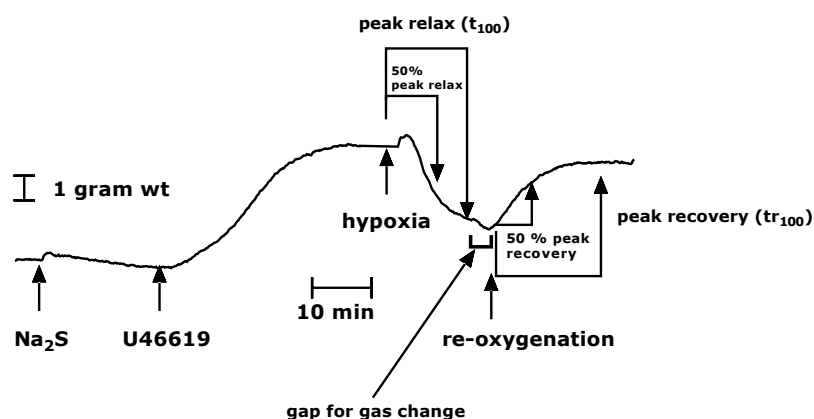


Figure 4.2: a representative trace recording to illustrate the time-dependent relaxation due to hypoxia and the recovery of tone following reoxygenation.

4.2.11. Drugs and Chemicals:

NaHS (296200250, Acros Organics, USA); AP39 (17100, Caymen chemicals, UK); U46619 (1932), BAY K8644 (1544) and pinacidil (1503), were from Tocris Bioscience, Bristol, UK. Na₂S (208043), GYY4137 (SML0100), L-NAME (N5751), TEA (205583), glibenclamide (G0639), tolbutamide (T0891), FCCP (C2920), capsazepine (C191) and EGTA (E4378) were from Sigma-Aldrich, Poole, UK.

A stock solution of Na₂S was freshly dissolved immediately before use in distilled water and kept on ice. A stock solution of GYY4137 or AP39 was prepared in dimethyl sulfoxide (DMSO).

Stock solutions of L-NAME were prepared in distilled water. TEA was dissolved in Krebs'-Henseleit solution. Glibenclamide, tolbutamide and pinacidil were dissolved in DMSO. FCCP and capsazepine were dissolved in ethanol.

Stock solutions of U46619 were made to 10 mM in ethanol, and all further dilutions of the stock solutions were made using distilled water. Except where indicated, stock solutions of reagents were kept frozen at -20 °C.

4.3. Statistical Analysis:

Relaxation responses are presented as the change in contractility expressed as a percentage of the U46619 response. Data analysis and statistics were performed using Graph-Pad PRISM 6 (Software version 6, USA). All data were expressed as means \pm standard error of the mean (SEM). For comparisons between two data sets, a two-tailed unpaired Student's t-test was used, or for paired data, a two-tailed paired Student's t-test. Differences between three or more groups were assessed using one-way ANOVA (analysis of variance) or two-way ANOVA following by Sidak's post-hoc test. The P-value <0.05 was considered statistically significant between the data sets. * represents P-value <0.05 , ** represents P-value <0.01 , *** represents P-value <0.001 , where n= number of experiments from separate animals.

4.4. Results:

4.4.1. The effects of Na₂S and NaHS on vascular tone

The aim of these experiments was to identify the concentration-dependence of responses to Na₂S and NaHS.

Cumulative concentration-response curve experiments for Na₂S and NaHS were carried out. Relaxation responses were only apparent at higher concentrations (0.3 and 1 mM) of both Na₂S and NaHS, although only responses to 1 mM NaHS were statistically significant (figure 4.3).

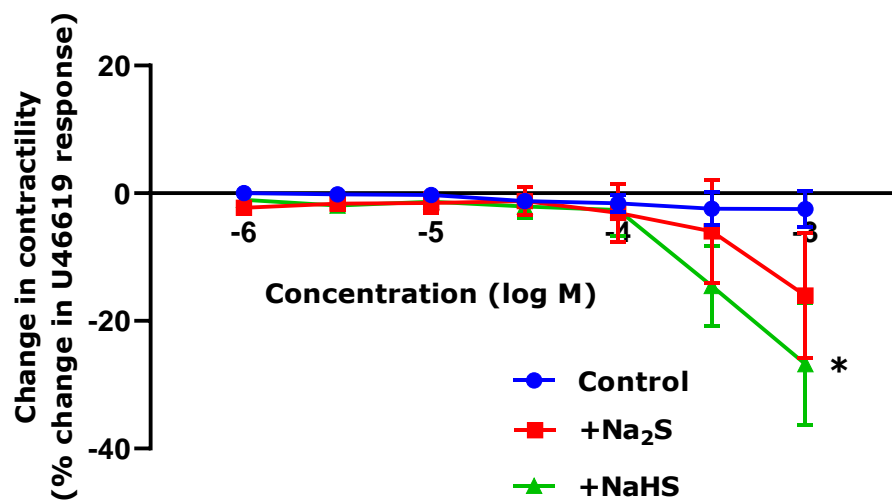


Figure 4.3: Cumulative concentration-response curve (CRC) to Na₂S and NaHS in U46619 pre-contracted PCA. The Y-axis represents the change in contractility expressed as a percentage of the U46619 response. Data are mean +/- SEM of 12 animals. * P<0.05 NaHS vs control (vehicle control, distilled water) using two-way ANOVA followed by Sidak's post hoc test, (n=12).

As H₂S is unstable with a short half-life, the effects of single additions of the two highest concentrations on U46619 pre-contracted PCA were assessed. 300 μM NaHS produced a relaxation numerically larger than 300 μM Na₂S. At 1000 μM, the relaxation response was numerically larger to Na₂S than NaHS (figure 4.4). Both concentrations of the two sources of H₂S were significantly different from the baseline. Subsequently, a single concentration of 1000 μM Na₂S was used in the next experiments.

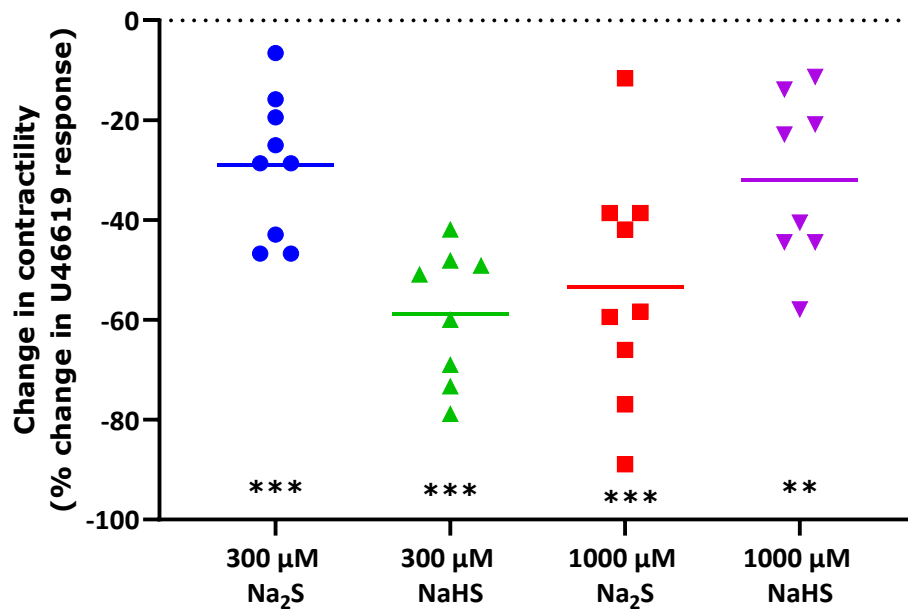


Figure 4.4: The effects of single additions of hydrogen sulfide salts on PCA contractility. Tissues were pre-contracted in the presence of U46619 before single additions of either 300 or 1000 μM NaHS or Na₂S were applied and the maximal change recorded. The Y-axis represents the change in contractility expressed as a percentage of the U46619 response. Data are individual points from 8 (NaHS) or 9 (Na₂S) independent experiments, with the horizontal line indicating the mean value. ** P<0.01, *** P<0.001 one-sample t-test compared to baseline, (n=8-9).

4.4.2. The effects of GYY4137 on vascular tone

The aim of these experiments was to identify the effects of the H₂S donor GYY4137 on responses to U46619 pre-contracted PCA. There were significant relaxations at 25, 50 and 100 μ M GYY4137 vs 0.1 v/v % DMSO (vehicle control) (figure 4.5). Relaxations at 100 μ M GYY4137 were significantly larger than those at 50 μ M indicating a clear concentration dependence of GYY4137-evoked relaxations. In subsequent experiments, 100 μ M GYY4137 was used as a comparator.

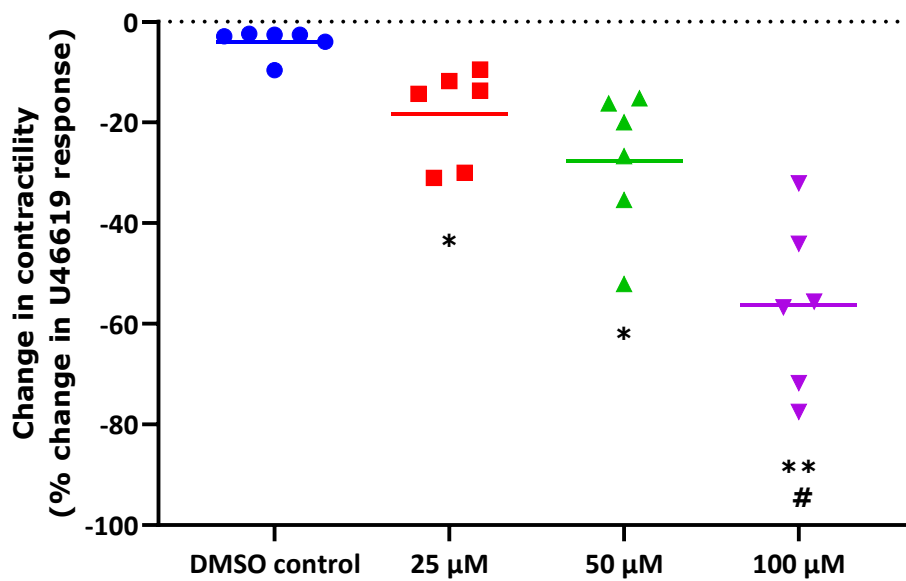
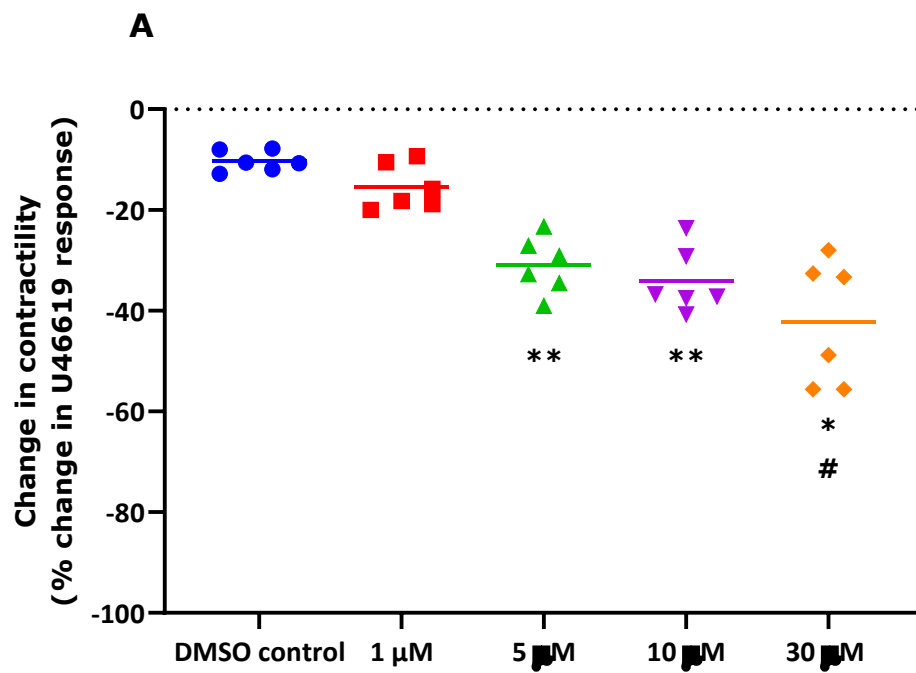


Figure 4.5: The effects of single additions of the hydrogen sulfide donor GYY4137 on PCA contractility. Tissues were pre-contracted in the presence of U46619 before single additions of the indicated concentrations of GYY4137 or the vehicle control DMSO were applied and the maximal change recorded. The Y-axis represents the change in contractility expressed as a % of the U46619 response. Data are individual points from six independent experiments, with the horizontal line indicating the mean value. * $P < 0.05$, ** $P < 0.01$ compared to the DMSO control (vehicle control, 0.1 v/v % dimethyl sulfoxide, DMSO); # $P < 0.05$ compared to 50 μ M, $P < 0.01$ compared to 25 μ M GYY4137, one way repeated measures ANOVA with post-hoc Dunnett's test; $n = 6$.

4.4.3. The effects of AP39 on vascular tone

The aim of these experiments was to identify the effects of the mitochondrial-targeted H₂S donor AP39 on tone of the pre-contracted PCA. There were significant differences compared to the DMSO (0.1 v/v % DMSO, vehicle control) at 5, 10 and 30 μ M AP39 (figure 4.6: A). Relaxations at the highest concentration were significantly larger than the lowest concentration (figure 4.6: B, C, D, E and F). 30 μ M AP39 was used in the following experiments.



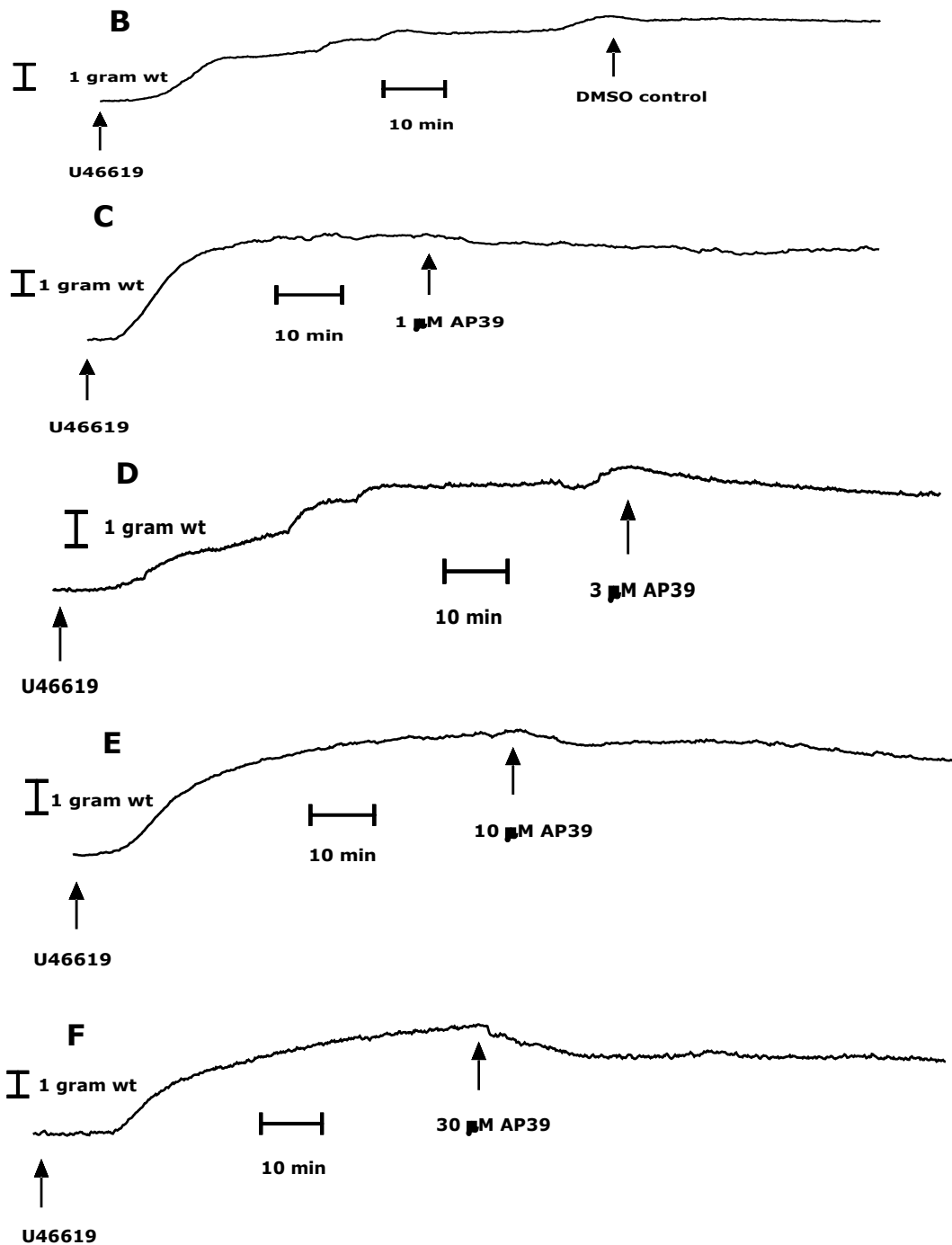


Figure 4.6: The effects of single additions of the mitochondrial targeted hydrogen sulfide donor AP39 on PCA contractility. Tissues were pre-contracted in the presence of U46619 before single additions of the indicated concentrations of AP39, or the vehicle control DMSO (vehicle control, 0.1 v/v % dimethyl sulfoxide) were applied and the maximal change recorded. The Y-axis represents the change in contractility expressed as a % of the U46619 response. Representative traces in the presence of DMSO control (B), 1 μ M AP39 (C), 3 μ M AP39 (D), 10 μ M AP39 (D), 30 μ M AP39 (F). Data are individual points from six independent experiments, with the horizontal line indicating the mean value. * $P < 0.05$, ** $P < 0.01$ compared to the DMSO control, # $P < 0.01$ compared to 1 μ M GYY4137, one-way repeated measures ANOVA with post-hoc Dunnett's test.

4.4.4. The effects of the nitric oxide synthase inhibitor, L-NAME, on responses to three different sources of H₂S

Several studies have reported conflicting results regarding the interaction between H₂S and nitric oxide (NO) in the vasculature (Hosoki et al., 1997; Whiteman et al., 2006; Kubo et al., 2007; Webb et al., 2008; Donovan et al., 2017). Therefore, the aim of these experiments was to investigate the role of NO using 300 μM L-NAME, as a NO synthase inhibitor. L-NAME produced a significant enhancement in the relaxation responses of 1 mM Na₂S, 100 μM GYY4137 and 30 μM AP39 in U46619-precontracted PCA (figure 4.7: A, B and C).

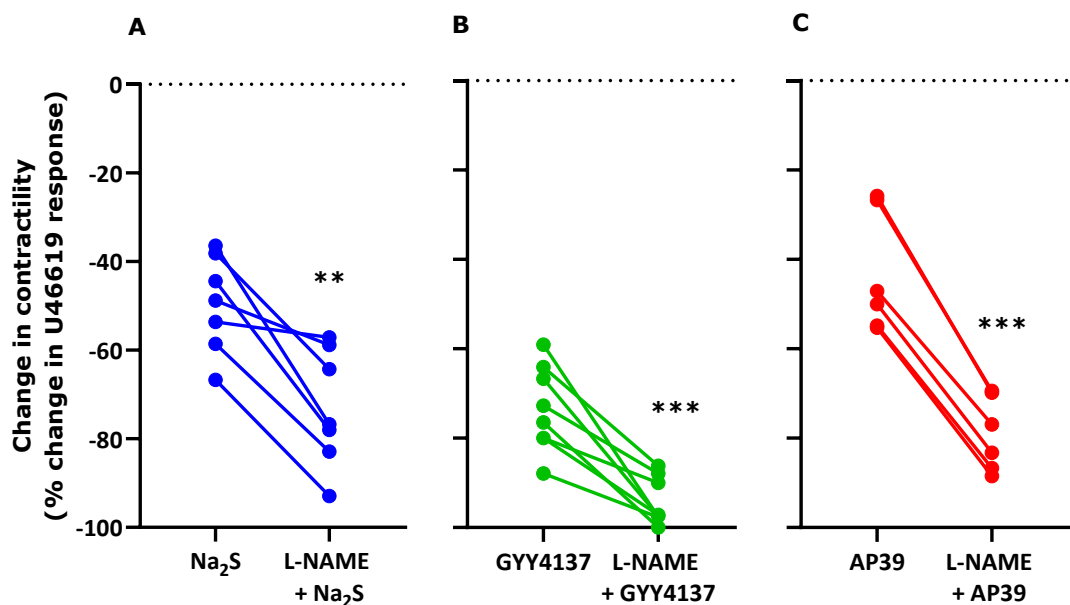


Figure 4.7: The effects of the NOS inhibitor L-NAME (300 μM) on single additions of hydrogen sulfide salt and donors on PCA contractility. Tissues were pre-contracted in the presence of U46619 before single additions of either Na₂S (1 mM) (A), GYY4137 (100 μM) (B) or AP39 (30 μM) (C) or the vehicle control for L-NAME distilled water were applied and the maximal change recorded. The Y-axis represents the change in contractility expressed as a % of the U46619 response. Data are individual points from seven (Na₂S) or eight (GYY4137) or six (AP39), independent experiments. **P<0.001, ***P<0.001 compared to the distilled water vehicle control for L-NAME using a two-tailed paired Student's t-test.

4.4.5. The effects of the spasmogen on responses to different sources of H₂S

U46619 is a thromboxane A₂ mimetic used to induce contraction of PCA rings (Choi et al., 2016). Many studies have reported that the H₂S relaxing effects are mediated by potassium channels, in particular, K_{ATP} channels (ATP-sensitive potassium channels) (Zhao et al., 2001). KCl causes smooth muscle contraction by membrane depolarisation leading to the opening of voltage-gated calcium channels, and the subsequent influx of calcium from the extracellular compartment causing contraction (Ratz et al., 2005). The aim of these experiments was to investigate the role of potassium channels in responses to different sources of H₂S by examining two different contracting agents, U46619 vs KCl. There were no significant differences in the relaxation responses of GYY4137 (-34.1± 3 vs -39.3± 6.1) or AP39 (-32.5± 3.9 vs -45± 5.5) (Figure 4.8: B and C), although there was a significant increase in relaxation to Na₂S (-31.3± 2.8 vs -55.8± 6.5) in KCl-precontracted compared to U46619-precontracted PCA (figure 4.8: A).

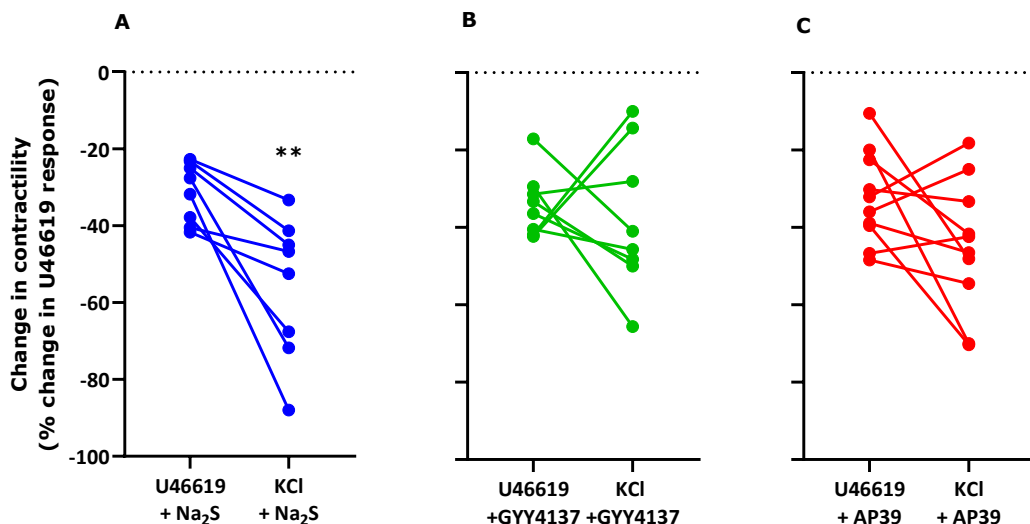


Figure 4.8: The effects of the contractile agent on single additions of hydrogen sulfide salt and donors on PCA contractility. Tissues were pre-contracted in the presence of either U46619 or KCl before single additions of either Na₂S (1 mM) (A), GYY4137 (100 μM) (B) or AP39 (30 μM) (C) were applied and the maximal change recorded. The Y-axis represents the change in contractility expressed as a % of the U46619 response. Data are individual points from eight (Na₂S), eight (GYY4137) or ten (AP39) independent experiments. **P<0.01 compared to the response in the presence of U46619 using a two-tailed paired Student's t-test.

4.4.6. The effects of the potassium channel blocker, tetraethyl ammonium chloride (TEA) on relaxation responses to different sources of H₂S

Tetraethyl ammonium chloride (TEA) is a non-selective potassium channels blocker. (Khodakhah et al., 1997; Lenaeus et al., 2005). The presence of 10 mM TEA unexpectedly enhanced relaxation responses to all three sources of H₂S (figure 4.9: A, B and C). There were no significant effects of 10 mM TEA on the concentration of U46619 needed to achieve 40-60 % of KCl response (data not shown).

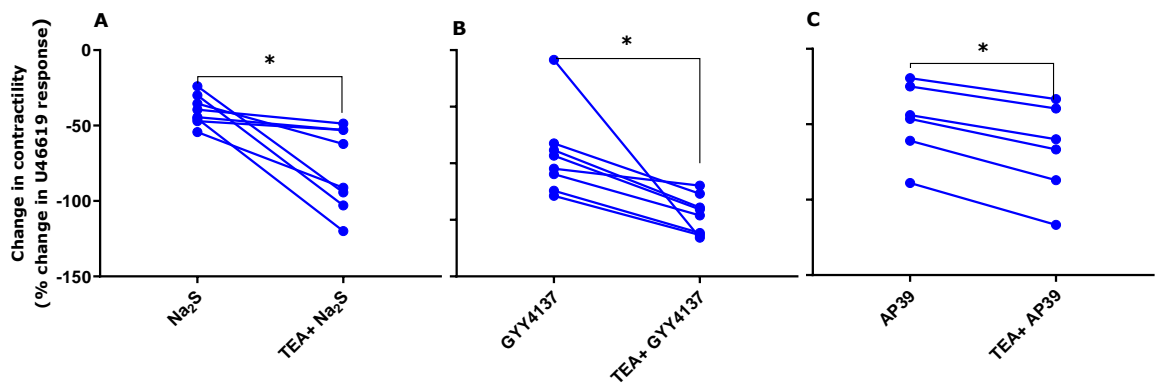
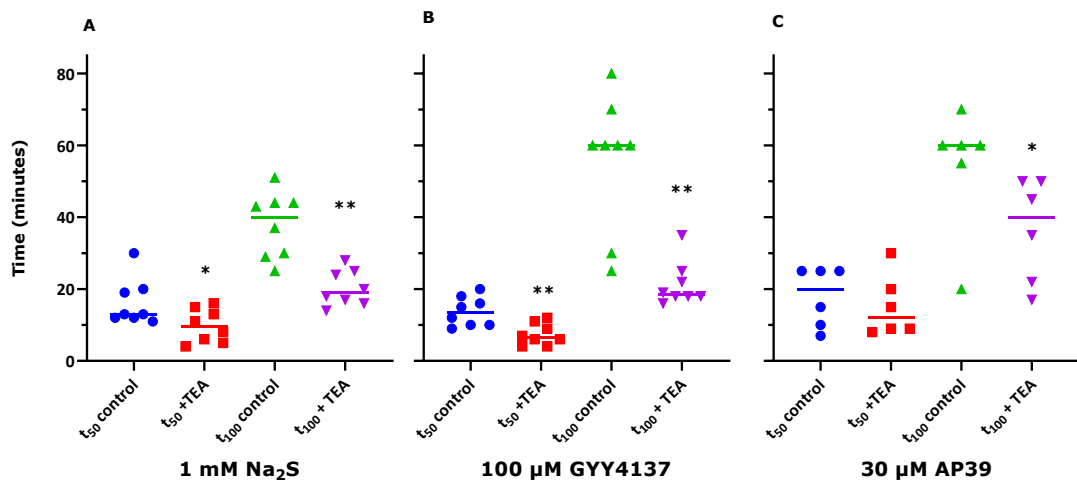


Figure 4.9: The effects of TEA (10 mM, tetraethylammonium, non-selective potassium channel blocker) on single concentration additions of hydrogen sulfide salt and donors on PCA contractility. Tissues were pre-contracted in the presence of U46619 before single additions of either Na₂S (1 mM) (A), GYY4137 (100 μM) (B) or AP39 (30 μM) (C) or the vehicle control for TEA Krebs'-Henseleit solution were applied and the maximal change recorded. The Y-axis represents the change in contractility expressed as a % of the U46619 response. Data are individual points from eight (Na₂S) or eight (GYY4137) or six (AP39), independent experiments. *P < 0.05 compared to the vehicle control for TEA Krebs'-Henseleit solution using a two-tailed paired Student's t-test.

4.4.7. The effects of potassium channel blocker, tetraethylammonium chloride (TEA) on the time dependence of responses to different sources of H₂S

The aim of these experiments was to investigate the effect of 10 mM TEA on the time profile of relaxation responses to the different sources of H₂S in U46619-precontracted PCA segments. Two time-dependent parameters were quantified: t_{100} was defined as the time latency to reach peak relaxation (pr), while t_{50} was defined as the time at which 50 % of the peak relaxation was attained (see 4.2.9) and (figure 4.10: D)

There were significant reductions in t_{50} and t_{100} of responses to Na₂S (figure 4.10: A) and GYY4137 (figure 4.10: B and E) in the presence of TEA. For AP39 (figure 4.10: C), there was only a significant reduction in the t_{100} in the presence of TEA. These results indicating a quicker relaxation response in the presence of TEA (figure 4.10: D and E).



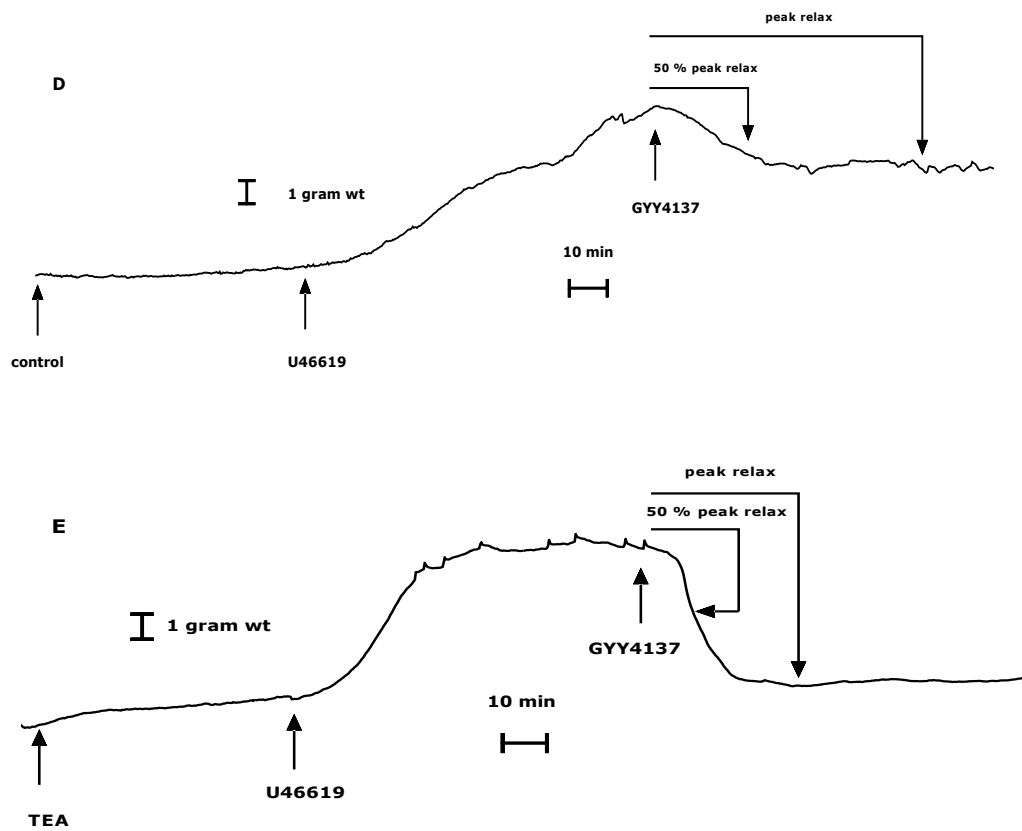


Figure 4.10: The effects of 10 mM TEA (tetraethylammonium chloride, non-selective potassium channel blocker) on the time profile parameters t_{100} (time to reach to the peak relaxation [pr]) or t_{50} (time to reach to 50% of the peak relaxation [pr]) of the relaxation responses of 1 mM Na_2S (A); 100 μM GYY4137 (B); 30 μM AP39 (C); as a single concentration addition in U46619-precontracted PCA. The Y-axis represents the time of the relaxation response in min; control (vehicle control Krebs'-Henseleit solution, in the absence of TEA) group vs +TEA (in the presence of 10 mM TEA). Representative trace recordings for the effects of the absence of 10 mM TEA (control, vehicle control Krebs'-Henseleit solution, in the absence of TEA) (D) or presence of 10 mM TEA (E) on peak relaxation (pr) and 50 % peak relaxation of the relaxation responses of 100 μM GYY4137 as a single concentration addition in U46619-precontracted PCA. * $p < 0.05$ vs control; ** p -value < 0.01 vs control using two-tailed paired Student's t-test; $n=6$.

4.4.8. The effect of K_{ATP} channel blockade on the relaxation evoked by three different sources of H₂S

The effect of TEA was unexpected as previous studies suggested an inhibition of the relaxation, not an enhancement. In vascular tissues, such as rat aorta, it has been reported that H₂S relaxation responses were mediated via K_{ATP} channels opening and glibenclamide was suggested as selective inhibitor of K_{ATP} channels (Zhao et al., 2001; Tang et al., 2005). Therefore, the effect of a selective inhibitor of K_{ATP} channels was investigated. In the presence of 1 μM glibenclamide, there was a significant enhancement of the relaxation responses to Na₂S and GYY4137 (figure 4.11: A and B), but not to AP39 (figure 4.11: C).

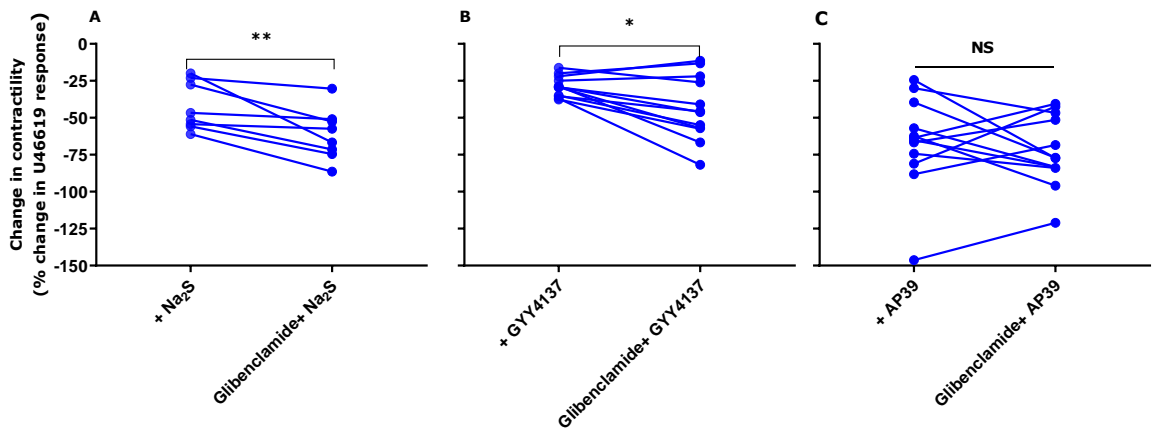


Figure 4.11: The effects of the K_{ATP} channel blocker glibenclamide (1 μM) on single additions of hydrogen sulfide salt and donors on PCA contractility. Tissues were pre-contracted in the presence of U46619 before single concentration additions of either Na₂S (1 mM) (A), GYY4137 (100 μM) (B) or AP39 (30 μM) (C) were applied and the maximal change recorded. The Y-axis represents the change in contractility expressed as a % of the U46619 response. Control (in the absence of glibenclamide, vehicle control, 0.1 % v/v DMSO) group vs +glibenclamide (in the presence of 1 μM glibenclamide); *p-value < 0.05, **p-value < 0.01; NS (non-significant) p-value > 0.05 using two-tailed paired Student's t-test; n=7-12.

In the presence of glibenclamide, the t_{50} values of responses to all three agents were unchanged, while only the t_{100} of GYY4137 and AP39 responses was quicker (figure 4.12: B and C).

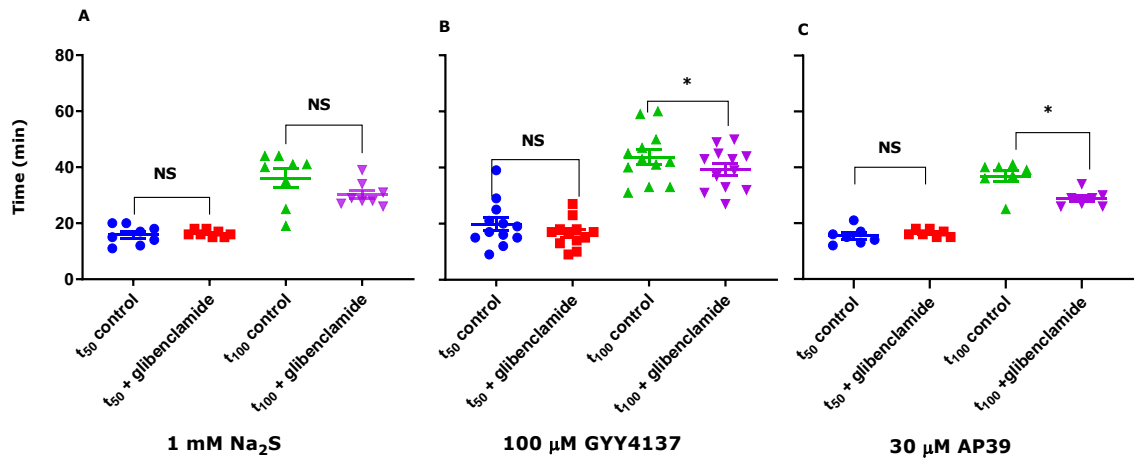


Figure 4.12: The effects of 1 μ M glibenclamide (K_{ATP} channel blocker) on the time profile parameters t_{100} (time to reach the peak relaxation [pr] response) or t_{50} (time to reach 50% of the peak relaxation response) of the relaxation responses of 1 mM Na₂S (A); 100 μ M GYY4137 (B); 30 μ M AP39 (C); as a single concentration addition in U46619-precontracted PCA. The Y-axis represents the time of the relaxation response in min; control (vehicle control 0.1% v/v DMSO, in the absence of glibenclamide) group vs +glibenclamide (in the presence of 1 μ M glibenclamide); data are individual points, and the horizontal line represents mean value. NS represents non-significant $p > 0.05$, $*p < 0.05$ vs control; using a two-tailed paired Student's t-test; $n=7-12$

4.4.9. The effect of tolbutamide on the relaxation responses to different sources of H₂S

Glibenclamide is a sulfonylurea oral antidiabetic agent, acting as a K_{ATP} channel blocker (Schwanstecher et al., 1994; Zünkler et al., 1997). Tolbutamide is also a sulfonylurea oral antidiabetic agent (Zünkler et al., 1997). Therefore, in order to confirm the effects of glibenclamide, the experiment was repeated in the presence of tolbutamide. In the presence of 100 μM tolbutamide there were significant enhancements in relaxation responses of all three different sources of H₂S in U46619-precontracted PCA (figure 4.13: A, B and C).

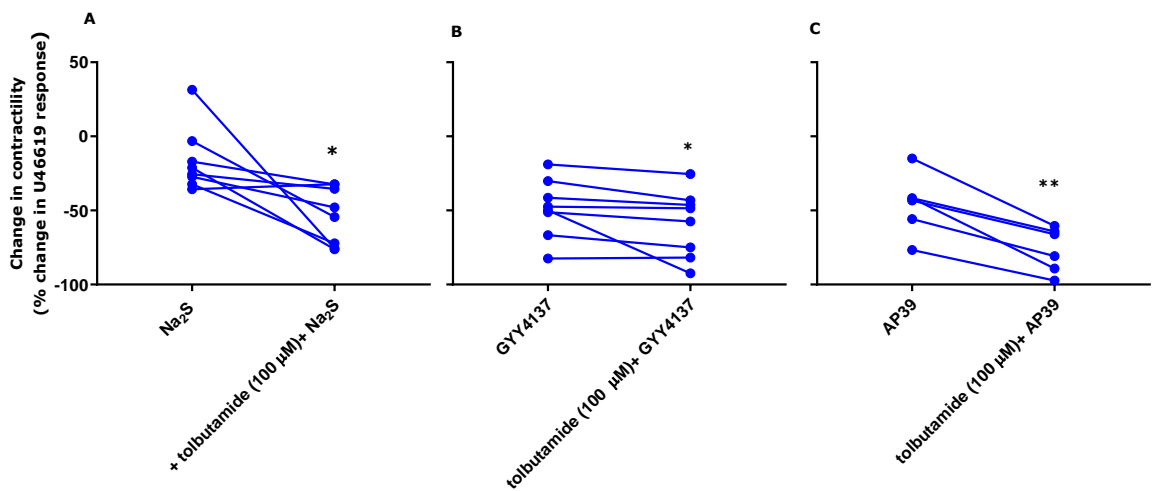


Figure 4.13: The effects of K_{ATP} channel blocker tolbutamide (100 μM) on single concentration additions of hydrogen sulfide salt and donors on PCA contractility. Tissues were pre-contracted in the presence of U46619 before single concentration additions of either Na₂S (1 mM) (A), GYY4137 (100 μM) (B) or AP39 (30 μM) (C) were applied and the maximal change recorded. The Y-axis represents the change in contractility expressed as a % of the U46619 response. Control (in the absence of tolbutamide, vehicle control, 0.1 % v/v DMSO) group vs +tolbutamide (in the presence of 100 μM tolbutamide); *p-value < 0.05, **p-value < 0.01 using two-tailed paired Student's t-test; n=6-8.

There were significant differences in t_{100} values for Na₂S (figure 4.14: A) and GYY4137 (figure 4.14: B), but not to AP39 (Figure 4.14: C). There were significant reductions in t_{50} with GYY4137 (figure 4.14: B) and AP39 (figure 4.14: C), but not Na₂S (figure 4.14: A).

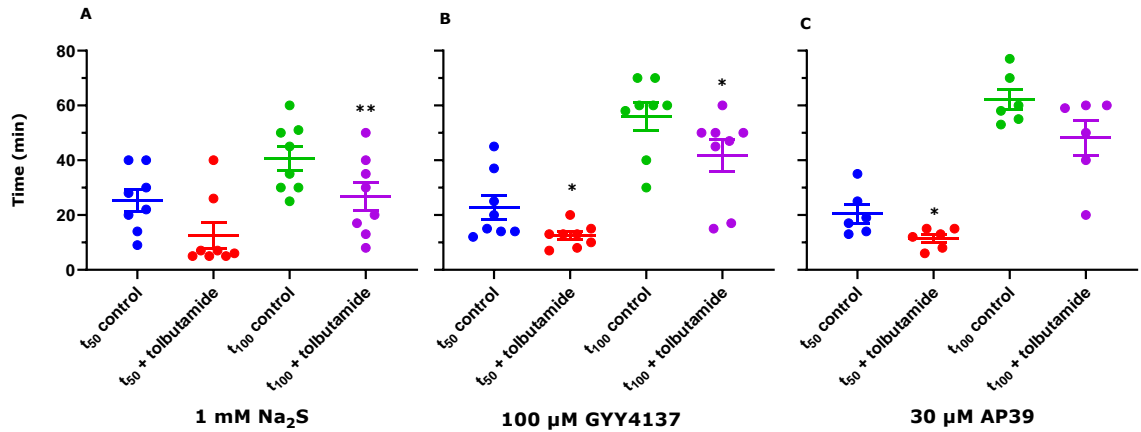


Figure 4.14: The effects of 100 μM tolbutamide (K_{ATP} channel blocker) on the time profile parameters t_{100} (time to reach the peak relaxation [pr] response) or t_{50} (time to reach 50% of the peak relaxation response) of the relaxation responses of 1 mM Na₂S (A); 100 μM GYY4137 (B); 30 μM AP39 (C); as a single concentration addition in U46619-precontracted PCA. The Y-axis represents the time of the relaxation response in min; control (vehicle control 0.1% v/v DMSO, in the absence of tolbutamide) group vs +tolbutamide (in the presence of 100 μM tolbutamide). Data are individual points, and the horizontal line is the mean value. * $p < 0.05$ vs control; ** $p < 0.01$ vs control using two-tailed paired Student's t-test; $n = 6-8$.

4.4.10. The effect of the K_{ATP} channel blocker, glibenclamide on the relaxation responses of the K_{ATP} channel opener, pinacidil

In order to confirm that glibenclamide was acting by blocking K_{ATP} channels, the effect of glibenclamide on the response to pinacidil, a K_{ATP} channel opener (Gollasch, 1995), was determined. Glibenclamide evoked a significant decrease in the relaxation responses to pinacidil at higher concentrations (figure 4.15).

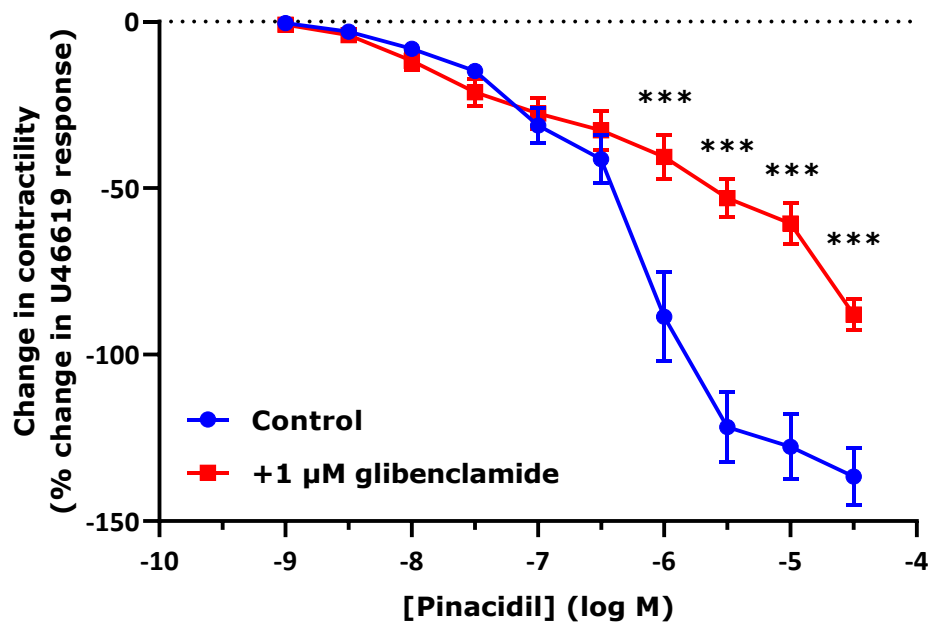


Figure 4.15: The effect of the K_{ATP} channel blocker glibenclamide (1 μ M) on the relaxatory responses to the K_{ATP} channel opener pinacidil. Tissues were pre-contracted in the presence of U46619 in the absence or presence of glibenclamide. The Y-axis represents the change in contractility expressed as a % of the U46619 response, while the X-axis is the logarithm of the pinacidil concentration. Data are mean \pm SEM from ten independent experiments. *** $P < 0.001$ compared to the response in the absence of glibenclamide (0.1 % v/v DMSO vehicle control) using a two-way ANOVA matched for tissue donor, with a post-hoc Sidak's multiple comparison test, $n=10$.

4.4.11. The effect of glucose removal on relaxation responses to three different sources of H₂S

Mitochondria play essential roles in energy production and vascular tone (Polhemus et al., 2014). The absence of glucose from the Krebs'-Henseleit solution should increase the cells dependence on mitochondrial respiration by oxidative phosphorylation more than cytosolic glycolysis for the generation of ATP (Yang et al., 2017). Relaxation responses of GYY4137 and AP39 were unaffected by the removal of glucose (figure 4.16: B and C), while it enhanced the relaxation to Na₂S (figure 4.16: A). The removal of glucose from Krebs's solution necessitated a significant increase in the concentration of U46619 needed to get a contraction to 40-60 % of KCl response (figure 4.16: D).

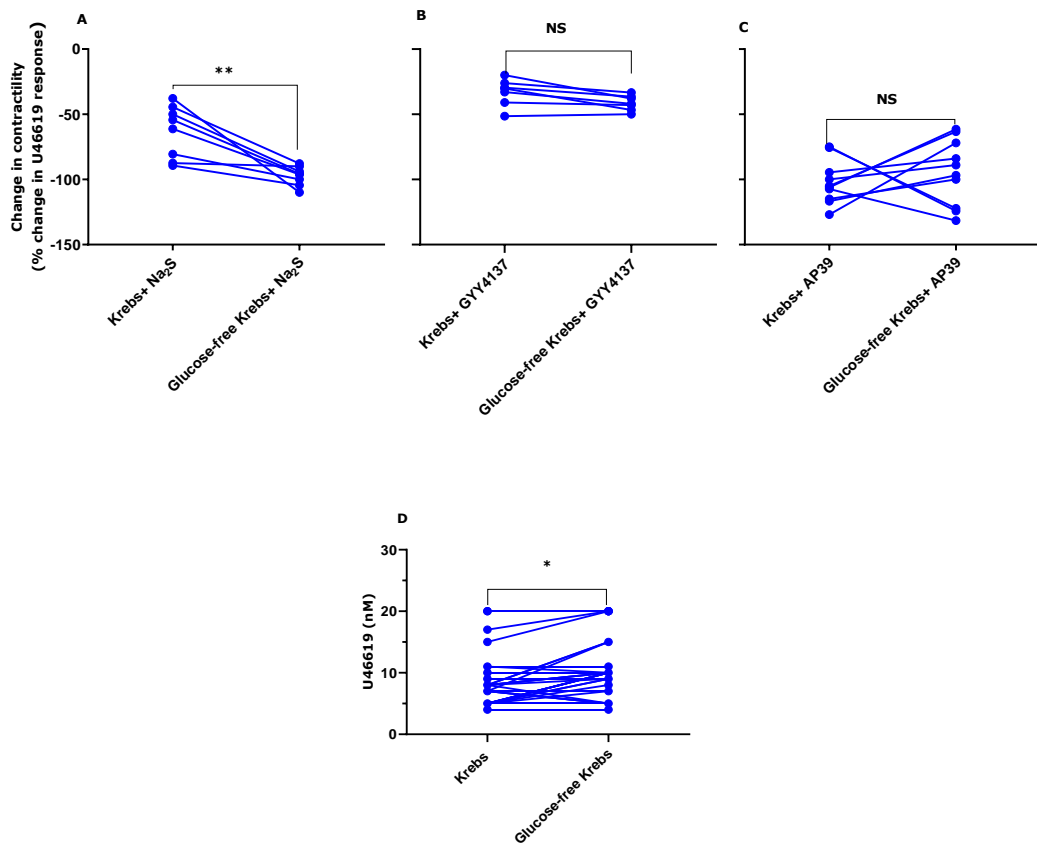


Figure 4.16: The effects of glucose-free Krebs' solution on single additions of hydrogen sulfide salt and donors on PCA contractility. Tissues were pre-contracted in the presence of U46619 before single additions of either Na₂S (1 mM) (A) or GYY4137 (100 μM) (B) or AP39 (30 μM) (C) were applied and the maximal change recorded. The Y-axis represents the change in contractility expressed as a % of the U46619 response. The x-axis represents Krebs' solution (control Krebs, in the presence of glucose) group vs glucose-free Krebs solution (in the absence of glucose). The concentration of U46619 needed to get 40-60 % of KCl response (D); * p-value < 0.05 (n=20-25), ** p-value < 0.01; NS (non-significant) p-value > 0.05 using a two-tailed paired Student's t-test; n=6-8.

4.4.12. The effect of glucose on relaxation responses of submaximal concentrations of the mitochondrial-targeted H₂S donor AP39

There was no effect on the relaxation response to 30 μ M AP39, the mitochondrial-targeted H₂S donor in the absence of glucose (glucose-free Krebs solution) (figure 4.16). However, the relaxation response was near maximal using this concentration in these experiments. Therefore, the effect of removal of glucose on lower concentrations of AP39 was determined. The results of these experiments demonstrated that removal of extracellular glucose had no effect on the relaxation to 10 μ M AP39 (figure 4.17: A), but it resulted in a significant enhancement in the relaxation responses to 5 μ M AP39 in U46619-precontracted PCA (figure 4.17: B).

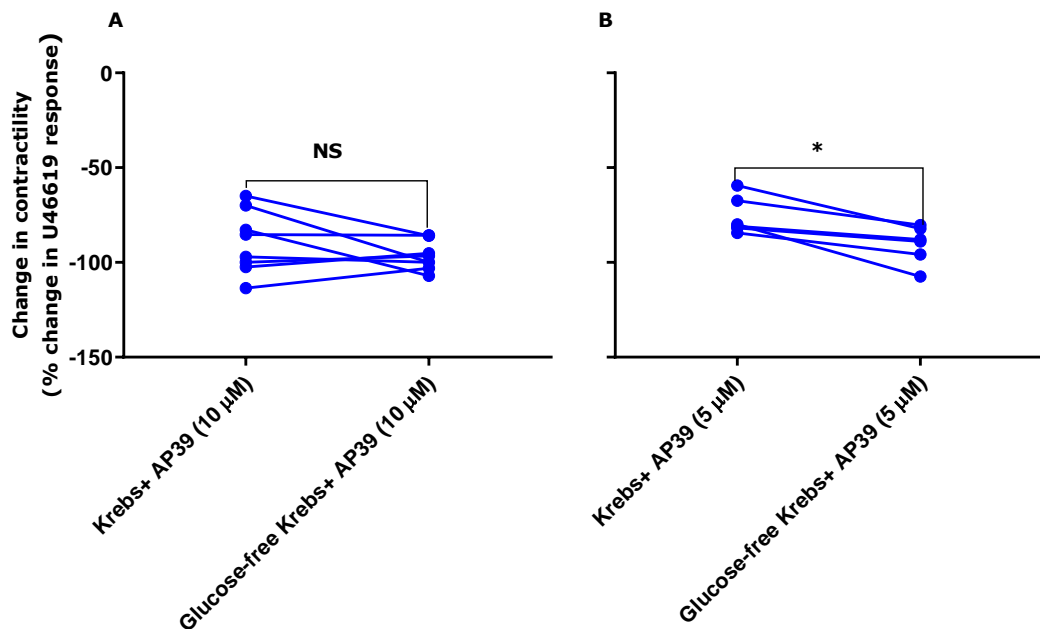


Figure 4.17: The effects of the glucose-free Krebs' solution on single additions of the mitochondrial-targeted H₂S donor AP39 on PCA contractility. Tissues were pre-contracted in the presence of U46619 before single additions of either AP39 (10 μ M) (A) or AP39 (5 μ M) (B) were applied and the maximal change recorded. The Y-axis represents the change in contractility expressed as a % of the U46619 response. The X-axis represents Krebs' solution (control, in the presence of glucose) group vs glucose-free Krebs' solution (in the absence of glucose); NS (non-significant) p-value > 0.05; * p-value < 0.05 using two-tailed paired Student's t-test; n=6-8.

4.4.13. The effect of the mitochondrial uncoupling agent FCCP on relaxation responses of submaximal concentration of the mitochondrial-targeted H₂S donor AP39

FCCP is a mitochondrial uncoupling agent, which impairs ATP production in the mitochondria (Szczesny et al., 2014). Therefore, in order to determine the role of mitochondria in the relaxation responses to AP39, relaxations to a submaximal concentration of AP39 were determined in the presence of FCCP (Szczesny et al., 2014). 1 μ M FCCP had no effect on the relaxation responses of 5 μ M AP39 in U46619-precontracted PCA (figure 4.18: A). 1 μ M FCCP had no effect on the concentration of U46619 needed to get 40-60 % of KCl response (figure 4.18: B).

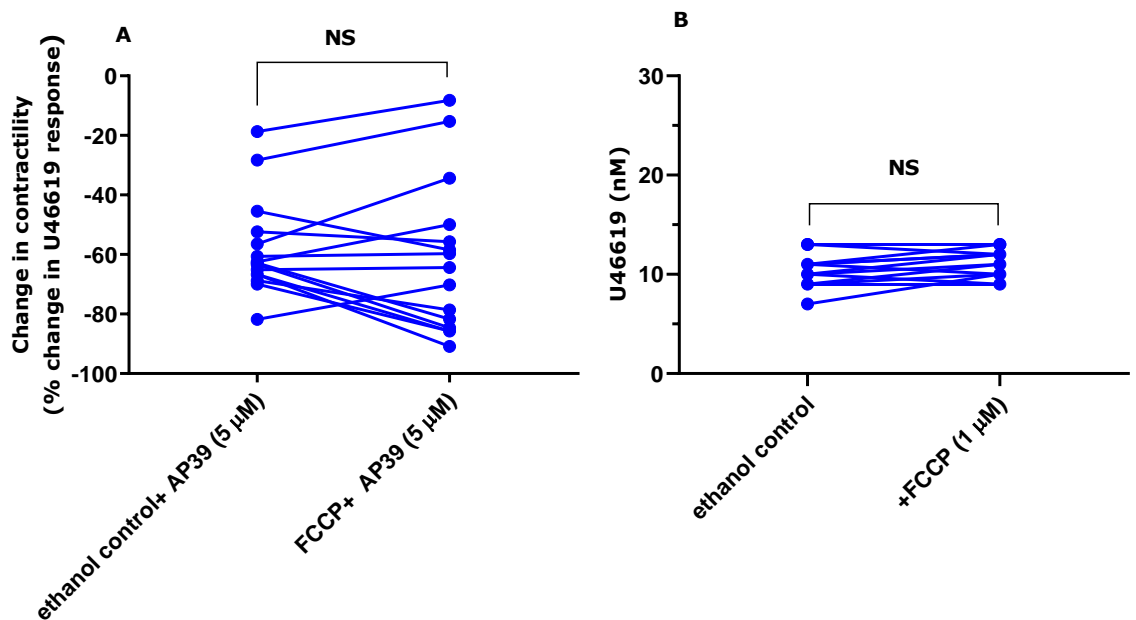


Figure 4.18: The effects of 1 μ M FCCP on single additions of the mitochondrial-targeted H₂S donor AP39 on PCA contractility. Tissues were pre-contracted in the presence of U46619 before single additions of 5 μ M AP39 (A) were applied, and the maximal change recorded. The amount of U46619 needed to get 40-60 % of KCl response (B). The Y-axis represents the change in contractility expressed as a % of the U46619 response. The X-axis represents ethanol control (0.1 % v/v ethanol, vehicle control for FCCP, in the absence of FCCP) group vs FCCP (in the presence of 1 μ M FCCP); NS (non-significant) p-value > 0.05 using two-tailed paired Student's t-test; n=14.

4.4.14. The effect of different sources of H₂S on calcium-induced contractions

It has been reported that H₂S is able to inhibit the influx of calcium by blocking calcium channels (Zhao and Wang, 2002; Al-Magableh and Hart, 2011). Therefore, the aim of these experiments was to determine whether H₂S could inhibit contractions caused by an influx of extracellular calcium. Tissues were pre-incubated for 15 min with each one of the three different sources of H₂S in calcium-free Krebs' solution. Next, 60 mM KCl was added to open voltage-gated calcium channels, and contractions were induced by addition of 1 mM CaCl₂ at the final step of the experiment. Pre-incubation with the three different sources of H₂S led to significant decreases in the contractile responses after addition of 1 mM CaCl₂ in PCA (figure 4.19: A, B and C).

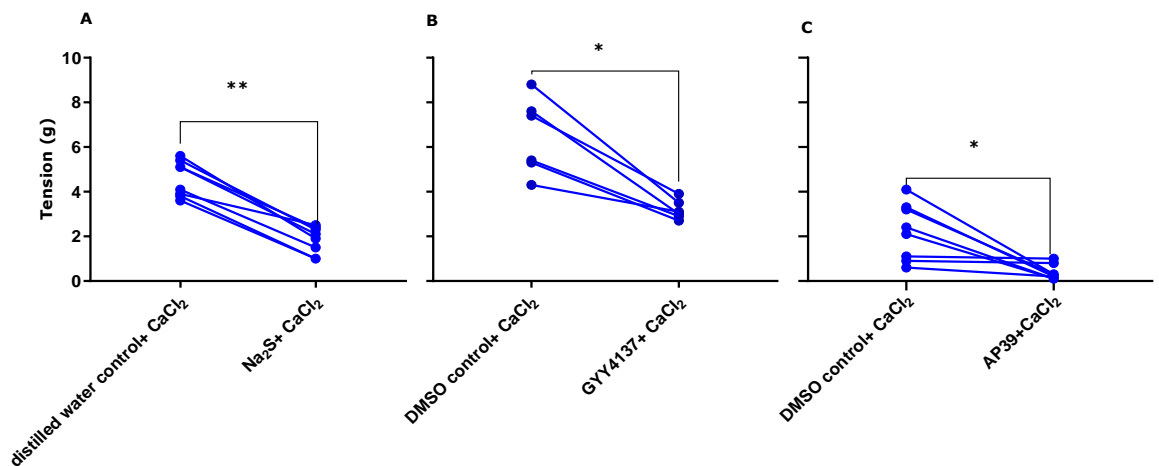


Figure 4.19: The effects of pre-incubation (15 min) with the different sources of H₂S 1 mM Na₂S (A); 100 μM GYY4137 (B); 30 μM AP39 (C) on 1 mM calcium chloride (CaCl₂) induced contraction responses of PCA. The Y-axis represents contractility tension in gram (g) after addition of 1 mM calcium chloride (CaCl₂). The X-axis represents distilled water control+CaCl₂ (vehicle control: distilled water for Na₂S) and Na₂S+CaCl₂ (15 min pre-incubated with 1 mM Na₂S) (A). The X-axis represents DMSO control+ CaCl₂ (vehicle control 0.1 % v/v DMSO for GYY4137) and GYY4137+CaCl₂ (15 min pre-incubated with 100 μM GYY4137) (B). The X-axis represents DMSO control+ CaCl₂ (vehicle control 0.1 % v/v DMSO for AP39) and AP39+CaCl₂ (15 min pre-incubated with 30 μM AP39). *p < 0.05, **p < 0.01 using two-tailed paired Student's t-test; n=6-8.

Investigation of the time-profile of these responses after different periods of pre-incubation (0, 15, 30 and 60 min) with 1 mM Na₂S, 100 μM GYY4137 and 30 μM AP39 may be useful to determine whether the H₂S donors have prolonged effects. There was a significant reduction in the contraction to calcium with Na₂S (figure 4.20: A), GYY4137 (figure 4.20: B) and AP39 (figure 4.20: C) compared to control (vehicle control distilled water for Na₂S or 0.1 % v/v DMSO for GYY4137 and AP39). There was no difference in the size of the reduction when Na₂S or GYY4137 or AP39 was pre-incubated for 15, 30 or 60 min, indicating a maintained response (figure 4.20: A, B and C).

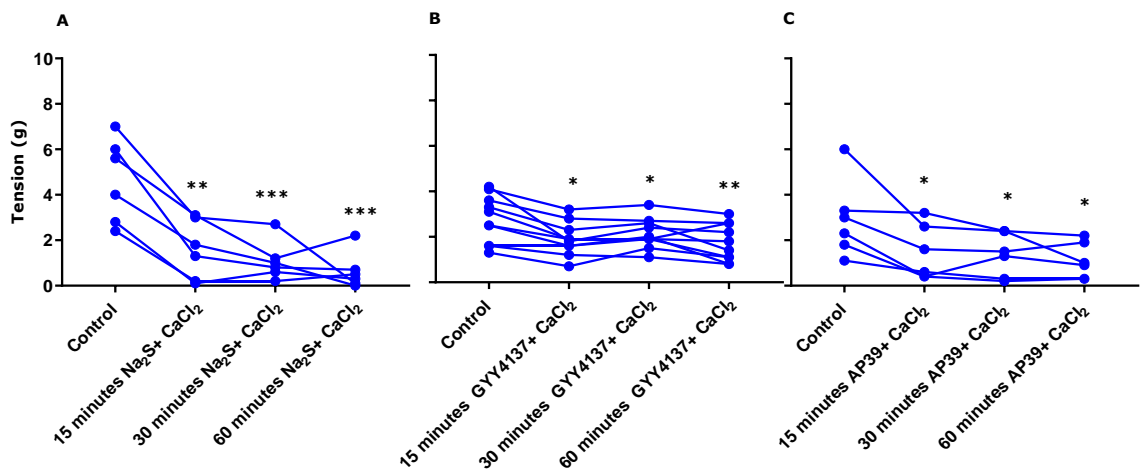


Figure 4.20: The effect of different periods of pre-incubation (15, 30 and 60 min) with different sources of H₂S donors 1 mM Na₂S (A); 100 μM GYY4137 (B); 30 μM AP39 (B) on the addition of 1 mM calcium chloride (CaCl₂) induced contraction responses of PCA. The Y-axis represents contractility tension in gram (g) after addition of 1 mM calcium chloride (CaCl₂). The X-axis represents control (vehicle control: distilled water for Na₂S) and Na₂S+CaCl₂ (15, 30 and 60 min pre-incubated with 1 mM Na₂S) (A). The X-axis represents control (vehicle control: 0.1 v/v % DMSO for GYY4137) and GYY4137+CaCl₂ (15, 30 and 60 min pre-incubated with 100 μM GYY4137) (B). The X-axis represents control (vehicle control: 0.1 v/v % DMSO for AP39) and AP39+CaCl₂ (15, 30 and 60 min pre-incubated with 30 μM AP39) (C). * P < 0.05 vs control, **p < 0.01 vs control, ***p < 0.001 vs control using one-way ANOVA followed by Sidak's post-hoc test; n=6-10.

4.4.15. The effect of three different sources of H₂S on BayK8644-precontracted PCA

The above experiments suggest the involvement of calcium channels, in particular, voltage-gated calcium channels in the H₂S-mediated relaxation responses. BayK8644 is an L-type calcium channel activator (Thomas et al., 1985). Therefore, the effect of the three different sources of H₂S: 1 mM Na₂S, 100 μM GYY4137 and 30 μM AP39 on BayK8644-precontracted PCA was determined. 1 mM Na₂S (figure 4.21: A), 100 μM GYY4137 (figure 4.21 B) and 30 μM AP39 (figure 4.21 C) produced significant relaxations in BayK8644-precontracted PCA.

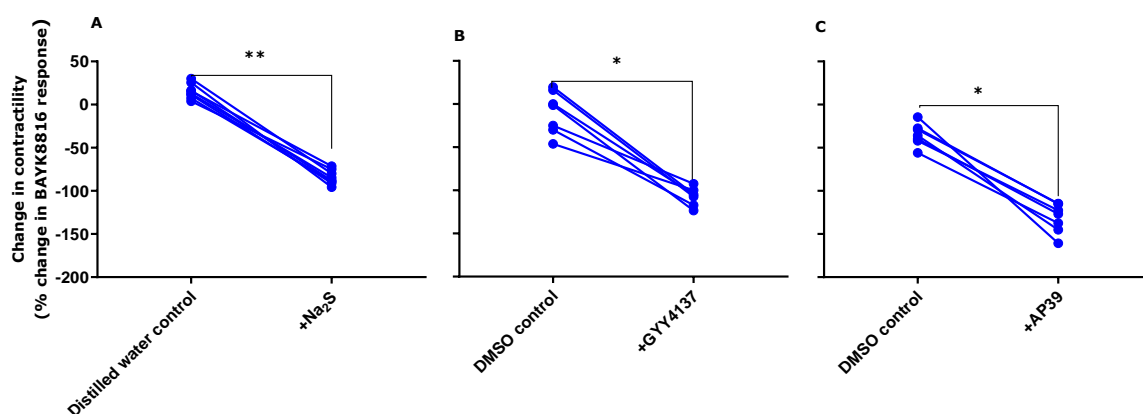


Figure 4.21: The effects of three different sources of hydrogen sulfide on PCA contractility. Tissues were pre-contracted in the presence of BAYK8816 before single concentration additions of either Na₂S (1 mM) or vehicle control (distilled water for Na₂S) (A); GYY4137 (100 μM) or 0.1 v/v % DMSO control (vehicle control for GYY4137) (B), AP39 (30 μM) or 0.1 v/v % DMSO control (vehicle control for AP39) were applied and the maximal change recorded. The Y-axis represents the change in contractility expressed as a % of the BAYK8816 response. Data are individual points; *P<0.05 compared to control, **P<0.01 compared to control using a two-tailed paired Student's t-test; n=6-8.

4.4.16. The effect of capsazepine on relaxation responses to three different sources of H₂S in U46619-precontracted PCA

Recently, there is some evidence, which suggests transient receptor potential (TRP) channel involvement in the relaxation response of blood vessels to H₂S (White et al., 2013; Naik et al., 2016). Therefore, the effect of 10 μM capsazepine, a TRPV1 channel blocker, on relaxation responses to H₂S using 1 mM Na₂S, 100 μM GYY4137 and 30 μM AP39 on U46619-precontracted PCA was determined. 10 μM capsazepine led to a significant decrease in the relaxation responses of all three different sources of H₂S, 1 mM Na₂S (figure 4.22: A), 100 μM GYY4137 (figure 4.22: B), and 30 μM AP39 (figure 4.22: C) in U46619-precontracted PCA.

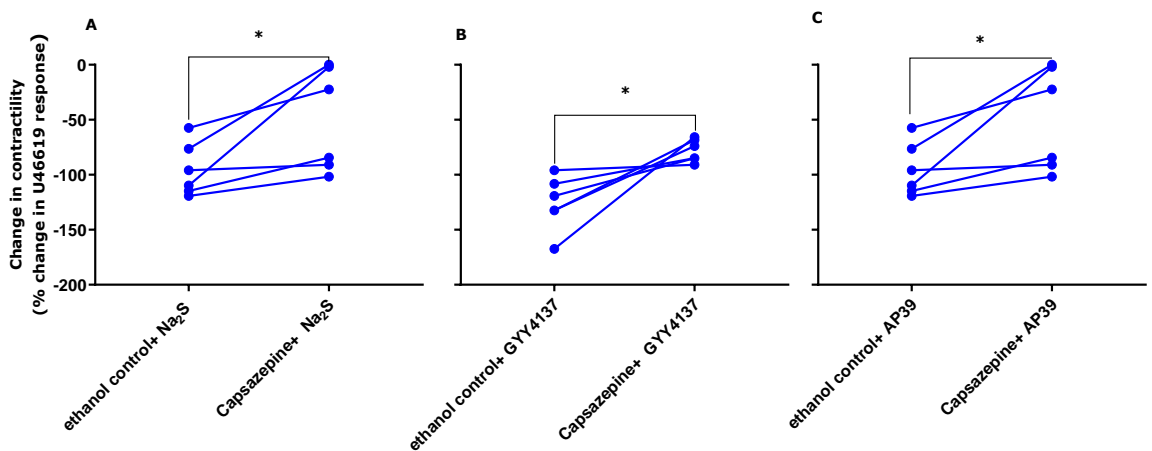
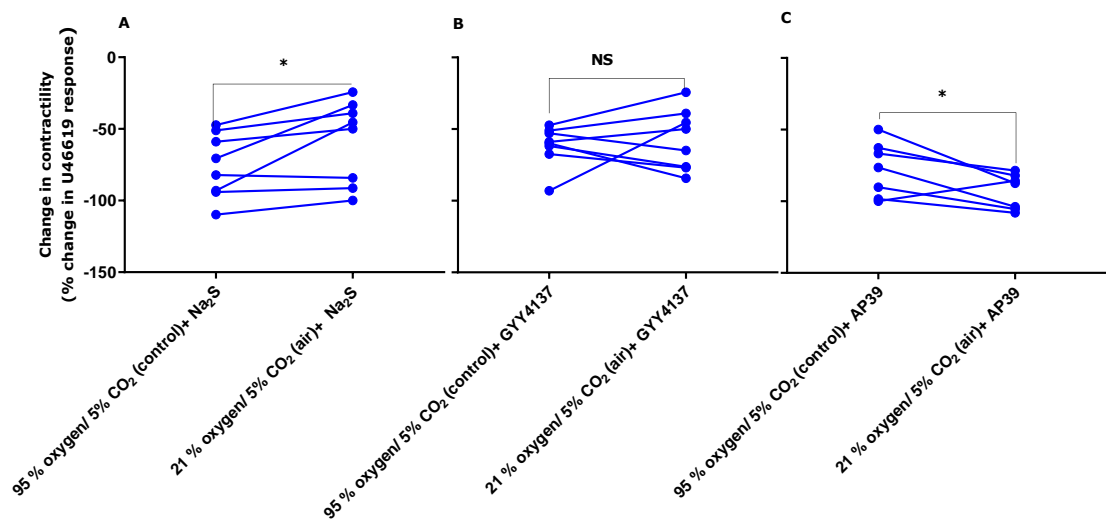


Figure 4.22: The effects of 10 μM capsazepine (TRPV1 blocker) on single concentrations of hydrogen sulfide sources on PCA contractility. Tissues were pre-contracted in the presence of U46619 before single concentration additions of either Na₂S (1 mM) (A), GYY4137 (100 μM) (B) or AP39 (30 μM) (C) were applied and the maximal change recorded. The X-axis represents ethanol control (0.1 % v/v ethanol vehicle control for capsazepine, in the absence of capsazepine) + Na₂S (1 mM) or capsazepine (10 μM) + Na₂S (1 mM) (A). The X-axis represents ethanol control (0.1 % v/v ethanol vehicle control for capsazepine, in the absence of capsazepine) + GYY4137 (100 μM) or capsazepine (10 μM) + GYY4137 (100 μM) (B). The X-axis represents ethanol control (0.1 % v/v ethanol vehicle control for capsazepine, in the absence of capsazepine) + AP39 (30 μM) or capsazepine (10 μM) + AP39 (30 μM) (C). The Y-axis represents the change in contractility expressed as a % of the U46619 response. Data are individual points; *P<0.05 compared to ethanol control using a two-tailed paired Student's t-test; n=6.

4.4.17. The comparison of 95 % oxygen and 21 % oxygen on relaxation responses in U46619-precontracted PCA

It has been suggested that oxygen gas may oxidise H₂S and decrease H₂S availability (Olson et al., 2006). Thus, decrease of oxygen level may lead to increase of H₂S availability. Therefore, the effect of two levels of oxygen, 21% and 95%, on relaxation responses to the three different sources of H₂S, 1 mM Na₂S, 100 μM GYY4137 and 30 μM AP39 in U46619-precontracted PCA was determined. Unexpectedly, there was a significant decrease in the relaxation responses to 1 mM Na₂S in the presence of 21% oxygen compared to 95% oxygen (figure 4.23: A). There was no significant difference in the relaxation responses of 100 μM GYY4137 in the two oxygen conditions (figure 4.23: B). Intriguingly, there was a significant enhancement in the relaxation responses of 30 μM AP39 in U46619-precontracted PCA in the presence of 21% oxygen compared to 95% oxygen (figure 4.23: C). There was no significant difference in the contraction responses of 100 μM GYY4137 and 30 μM AP39 in U46619-precontracted PCA in the presence of 21% oxygen compared to 95% oxygen (figure 4.23: E and F), but there was a significant increase in the contraction responses of 1 mM Na₂S in U46619-precontracted PCA in the presence of 21% oxygen compared to 95% oxygen (figure 4.23: D) and this may be responsible for decrease in the relaxation responses to 1 mM Na₂S in the presence of 21% oxygen compared to 95% oxygen (figure 4.23: A). Furthermore, there was no significant difference in the concentration of U46619 needed to get 40-60 % of KCl response (data not shown).



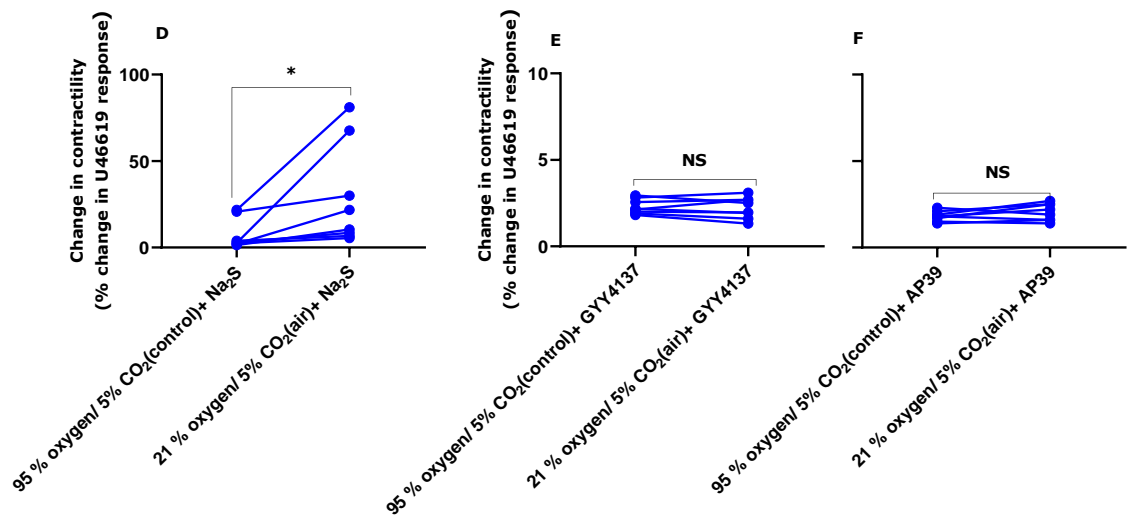


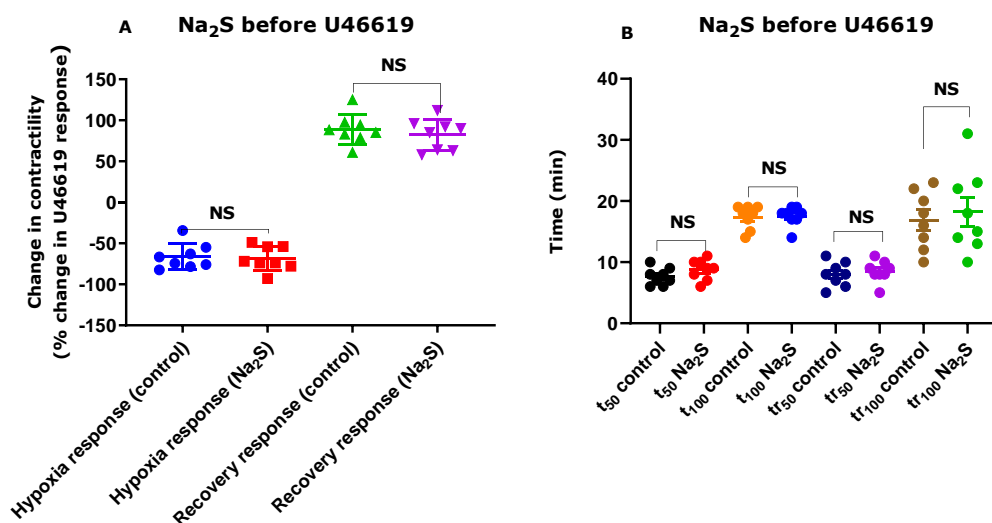
Figure 4.23. Comparison of the effects of 95% oxygen gas/ 5% CO₂ (control) gas mixture and 21% oxygen gas/5% CO₂ (air) gas mixture on single concentration additions of hydrogen sulfide donors on PCA contractility. Tissues were pre-contracted in the presence of U46619 before single concentrations of either Na₂S (1 mM) (A), GYY4137 (100 μM) (B) or AP39 (30 μM) (C) were applied and the maximal change recorded. The X-axis represents 95% oxygen gas/ 5% CO₂ (control) gas mixture+ Na₂S (1 mM) and 21% oxygen gas/5% CO₂ (air) gas mixture+ Na₂S (1 mM)(A). The X-axis represents 95% oxygen gas/ 5% CO₂ (control) gas mixture+ GYY4137 (100 μM) and 21% oxygen gas/5% CO₂ (air) gas mixture+ GYY4137 (100 μM) (B). The X-axis represents 95% oxygen gas/ 5% CO₂ (control) gas mixture+ AP39 (30 μM) and 21% oxygen gas/5% CO₂ (air) gas mixture+ AP39 (30 μM) (C). The contraction responses of the three different H₂S sources 1 mM Na₂S (D), 100 μM GYY4137 (E), and 30 μM AP39 (F). The Y-axis represents the change in contractility expressed as a % of the U46619 response. Data are individual points. * P < 0.05 vs control, NS (non-significant) p > 0.05 using a two-tailed paired Student's t-test; n=7-8.

4.4.18. The effects of different sources of H₂S before or after U46619 on the hypoxia and recovery responses in PCA

In order to determine the effect of the H₂S donors on the hypoxic response in the PCA, tissues were pre-incubated (15 min) with 1 mM Na₂S, 100 μM GYY4137 or 30 μM AP39 before U46619 addition.

4.4.18.1. The effects of 1 mM Na₂S added before U46619 addition

The results of these experiments demonstrated that there were no significant effects of 1 mM Na₂S pre-incubation (15 min) on: the size of the hypoxia response, peak (maximum) relaxation response (figure 4.24: A, F and G), the recovery response to reoxygenation (figure 4.24: A, F and G), the peak relaxation (pr)-time (**t**₁₀₀, the time required to reach the peak relaxation response after hypoxia) (figure 4.24: B), the **t**₅₀-time (the time required to reach 50 % of the peak relaxation response after hypoxia) (figure 4.24: B), the **tr**₁₀₀-time (the time required to reach peak recovery response after 95 % oxygen/ 5 % CO₂ reintroduction/reoxygenation) (figure 4.24: B), the **tr**₅₀-time (the time required to reach 50 % of the peak recovery response after 95 % oxygen/5 % CO₂ reintroduction/reoxygenation) (figure 4.24: B and G). Pre-incubation with 1 mM Na₂S before U46619 led to no significant difference in the hypoxia-induced contraction response (figure 4.24: C). Pre-incubation with 1 mM Na₂S before U46619 led to no significant difference in U46619 contraction (figure 4.24: D); however 1 mM Na₂S led to a significant increase (about double) in the concentration of U46619 required to reach 40-60 % contractility response of KCl (figure 4.24: E).



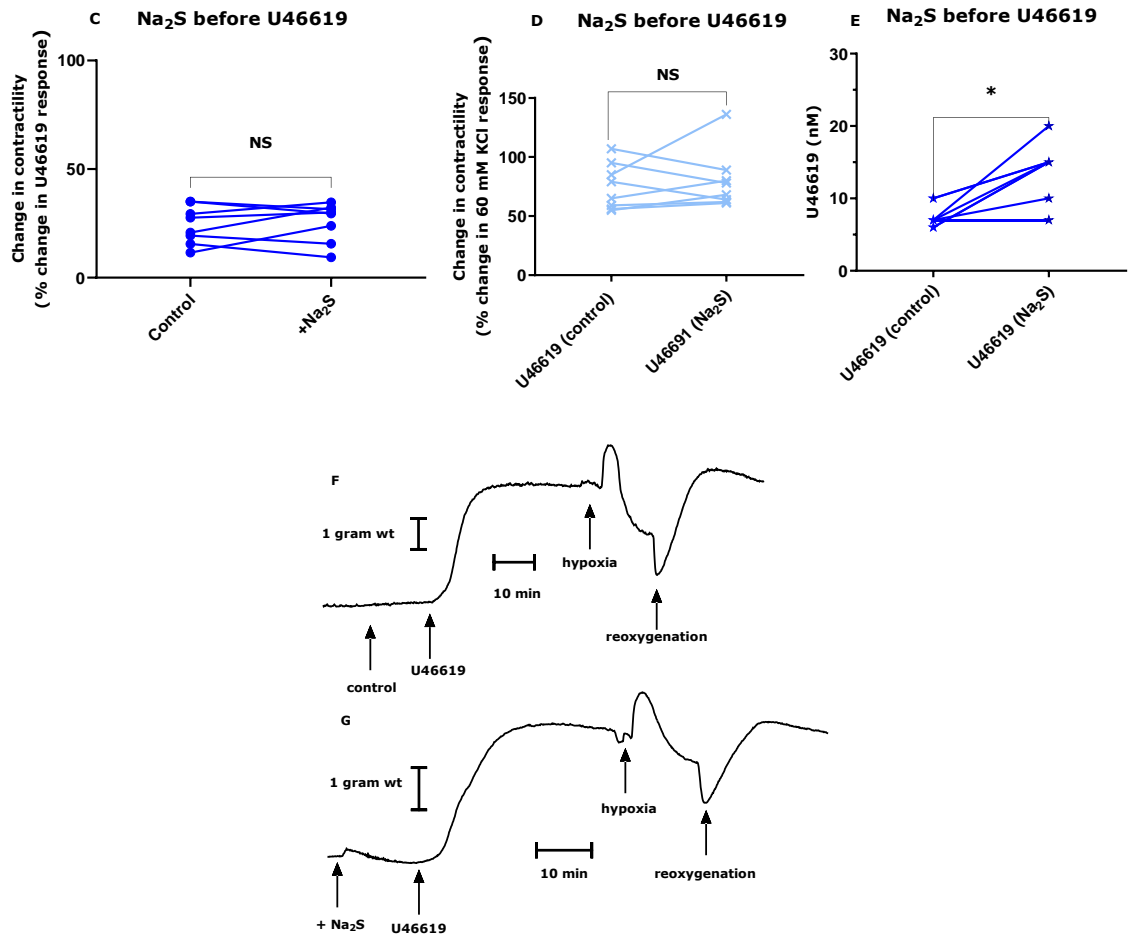
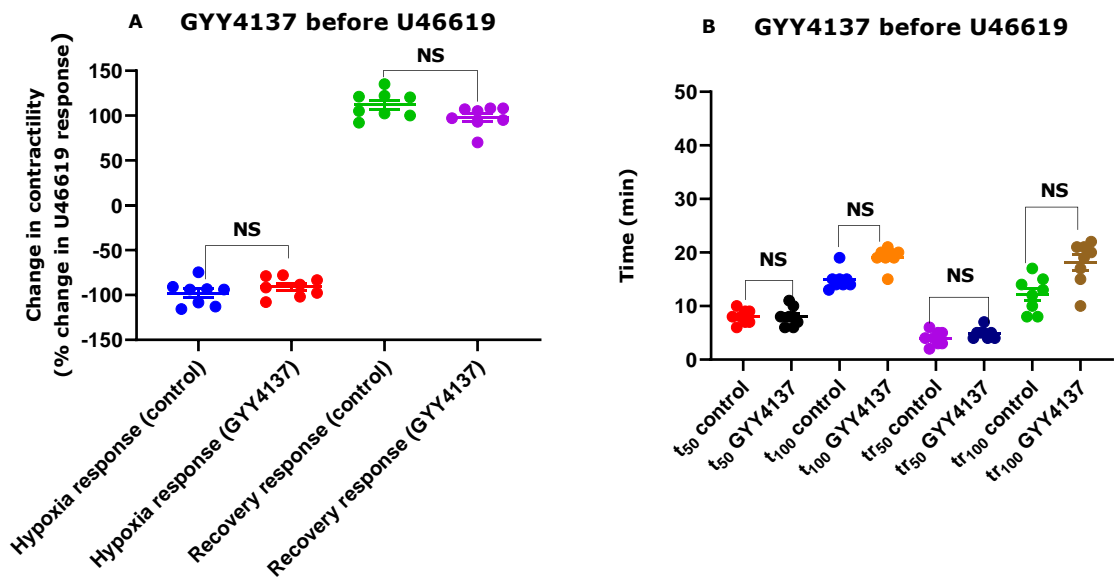


Figure 4.24. The effects of 1 mM Na_2S pre-incubation (15 min) on the hypoxia response and recovery response (A); the t_{100} -time (the time required to reach the peak relaxation response after hypoxia), the t_{50} -time (the time required to reach 50 % of the peak relaxation response after hypoxia), tr_{100} -time (the time required to reach the peak recovery response after 95 % oxygen/5 % CO_2 reintroduction/reoxygenation), the tr_{50} -time (the time required to reach 50 % of the peak recovery response after 95 % oxygen/5 % CO_2 reintroduction/reoxygenation) (B). The contraction response of hypoxia with control (vehicle control for Na_2S distilled water) or Na_2S (1 mM) (C). U46619 contraction response with control (vehicle control for Na_2S distilled water) or Na_2S (1 mM) (D). The concentration of U46619 required to reach 40-60 % contractility response of 60 mM KCl response with control (vehicle control for Na_2S distilled water) or Na_2S (1 mM) (E). The representative trace of the hypoxia relaxation response and recovery response in the presence of control (vehicle control for Na_2S distilled water) (F) or Na_2S (1 mM) (G). The control represents in the absence of 1 mM Na_2S (vehicle control distilled water); Na_2S represents in the presence of 1 mM Na_2S (Na_2S pre-incubated for 15 min before U46619 addition). NS (non-significant) $p > 0.05$, * $p < 0.05$ using two-tailed paired Student's t-test; $n = 7-8$.

4.4.18.2. The effects of 100 μM GYY4137 added before U46619 addition

The results of these experiments demonstrated that there were no significant effects of pre-incubation with GYY4137 on: the size of hypoxia response, peak (maximum) relaxation response (figure 4.25: A and F), recovery response after reintroduction of oxygen (figure 4.25: A and F), peak relaxation (pr)-time (t_{100} -time, the time required to reach the peak relaxation response after hypoxia)(figure 4.25: B), t_{50} -time (the time required to reach 50 % of the peak relaxation response after hypoxia) (figure 4.25: B), tr_{100} -time (the time required to reach the peak recovery response after 95 % oxygen/5 % CO_2 reintroduction/reoxygenation) (figure 4.25: B), tr_{50} -time (the time required to reach 50 % of the peak recovery response after 95 % oxygen/5 % CO_2 reintroduction/reoxygenation) (figure 4.25: B), the concentration of U46619 required to reach 40-60 % contractility response of KCl response (figure 4.25: D). Pre-incubation with 100 μM GYY4137 before U46619 led to significant decrease in the size of the hypoxia-induced contraction response (figure 4.25: C). Representative traces in the presence of control (figure 4.25: E) and 100 μM GYY4137 (figure 4.25: F).



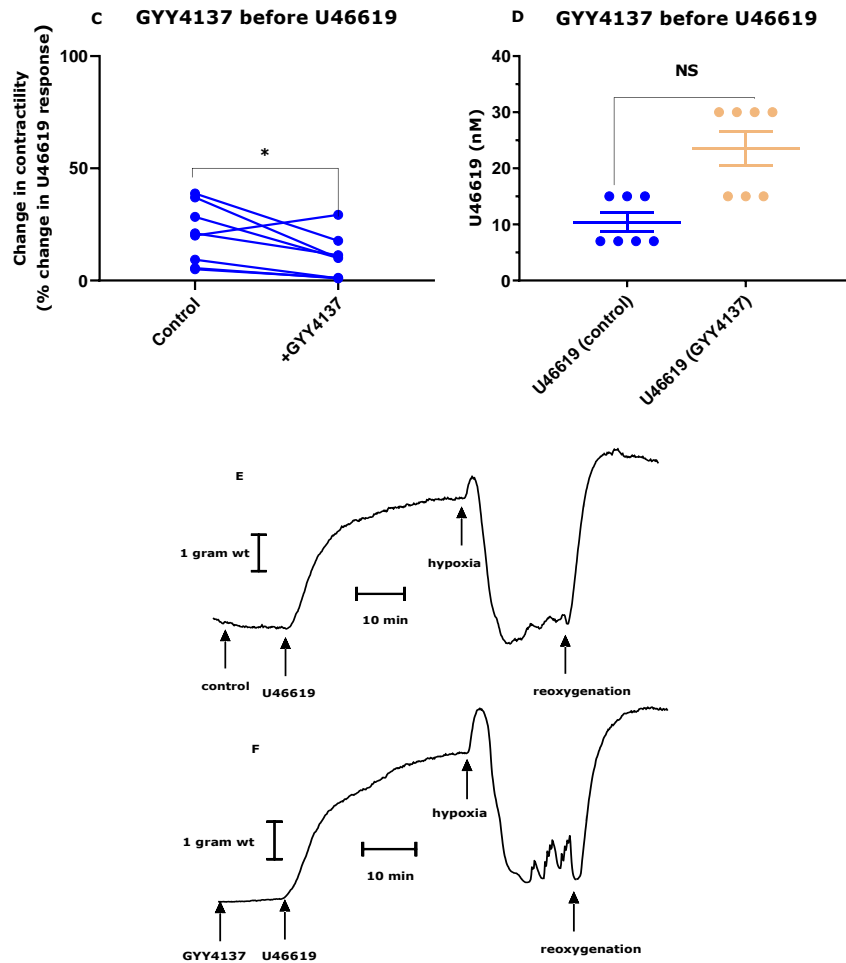
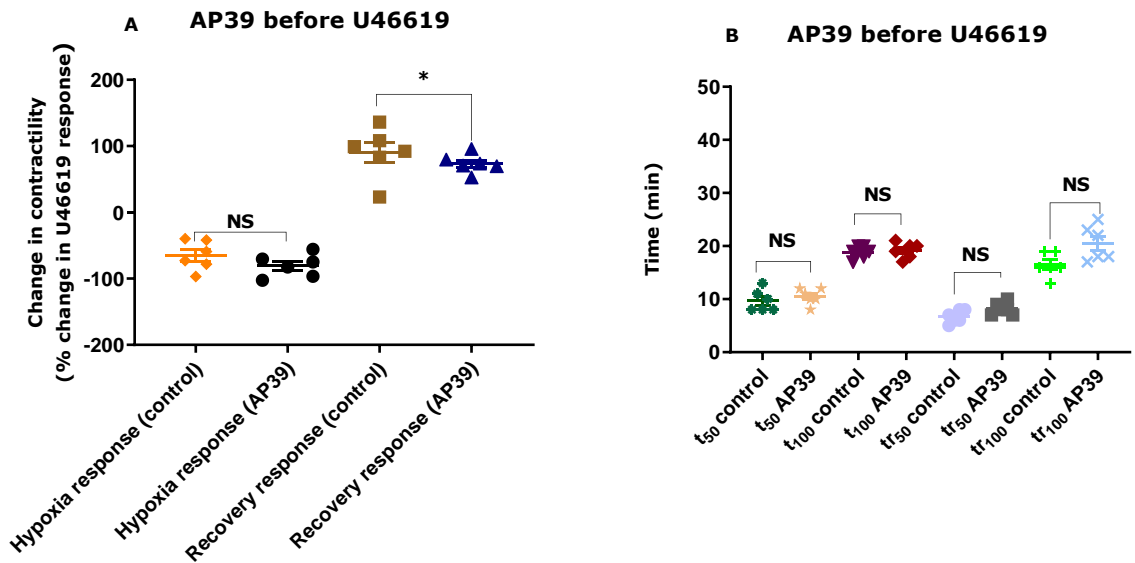


Figure 4.25: The effects of 100 μM GYY4137 pre-incubation (15 min) before U46619 on the hypoxia response and recovery response (A); t_{100} -time (the time required to reach the peak relaxation response after hypoxia), t_{50} -time (the time required to reach 50 % of the peak relaxation response after hypoxia), tr_{100} -time (the time required to reach the peak recovery response after 95 % oxygen/5 % CO_2 re-introduction/re-oxygenation), tr_{50} -time (the time required to reach 50 % of the peak recovery response after 95 % oxygen/5 % CO_2 re-introduction /re-oxygenation) (B); the contraction response of hypoxia with control (vehicle control for GYY4137 0.1 % v/v DMSO) or GYY4137 (100 μM) (C). The amount of U46619 required to reach 40-60 % contractility response of 60 mM KCl response with control (vehicle control for GYY4137 0.1 % v/v DMSO) or GYY4137 (100 μM) (D). The representative trace of the hypoxia relaxation response and recovery response in the presence of control (vehicle control for GYY4137 0.1 % v/v DMSO) (E) or GYY4137 (100 μM) (F). The control represents in the absence of 100 μM GYY4137 (vehicle control, 0.1 % v/v DMSO); GYY4137 represents in the presence of 100 μM GYY4137 (GYY4137 pre-incubated for 15 min before U46619 addition); NS (non-significant) $p > 0.05$, * $p < 0.05$ using a two-tailed paired Student's t-test; $n=7$.

4.4.18.3. The effects of 30 μ M AP39 addition before U46619 addition

There were no significant effects of pre-incubation with 30 μ M AP39 on: the size of hypoxia response, peak (maximum) relaxation response (figure 4.26: A), the peak relaxation (pr)-time (t_{100} -time, the time required to reach the peak relaxation response after hypoxia) (figure 4.26: B), the t_{50} -time (the time required to reach 50 % of the peak relaxation response after hypoxia)(figure 4.26: B), the tr_{100} -time (the time required to reach the peak recovery response after 95 % oxygen/ 5 % CO₂ reintroduction/reoxygenation)(figure 4.26: B), the tr_{50} -time (the time required to reach 50 % of the peak recovery response after 95 % oxygen/ 5 % CO₂ reintroduction/reoxygenation)(figure 4.26: B), the hypoxia-induced contraction response (figure 4.26: C), U46619 response (figure 4.26: D), the concentration of U46619 required to reach 40-60 % contractility response of 60 mM KCl response (figure 4.26: E). Interestingly, 30 μ M AP39 addition before U46619 led to a significant decrease in the size of the recovery response after reintroduction of oxygen (reoxygenation) (figure 4.26: A, F and G).



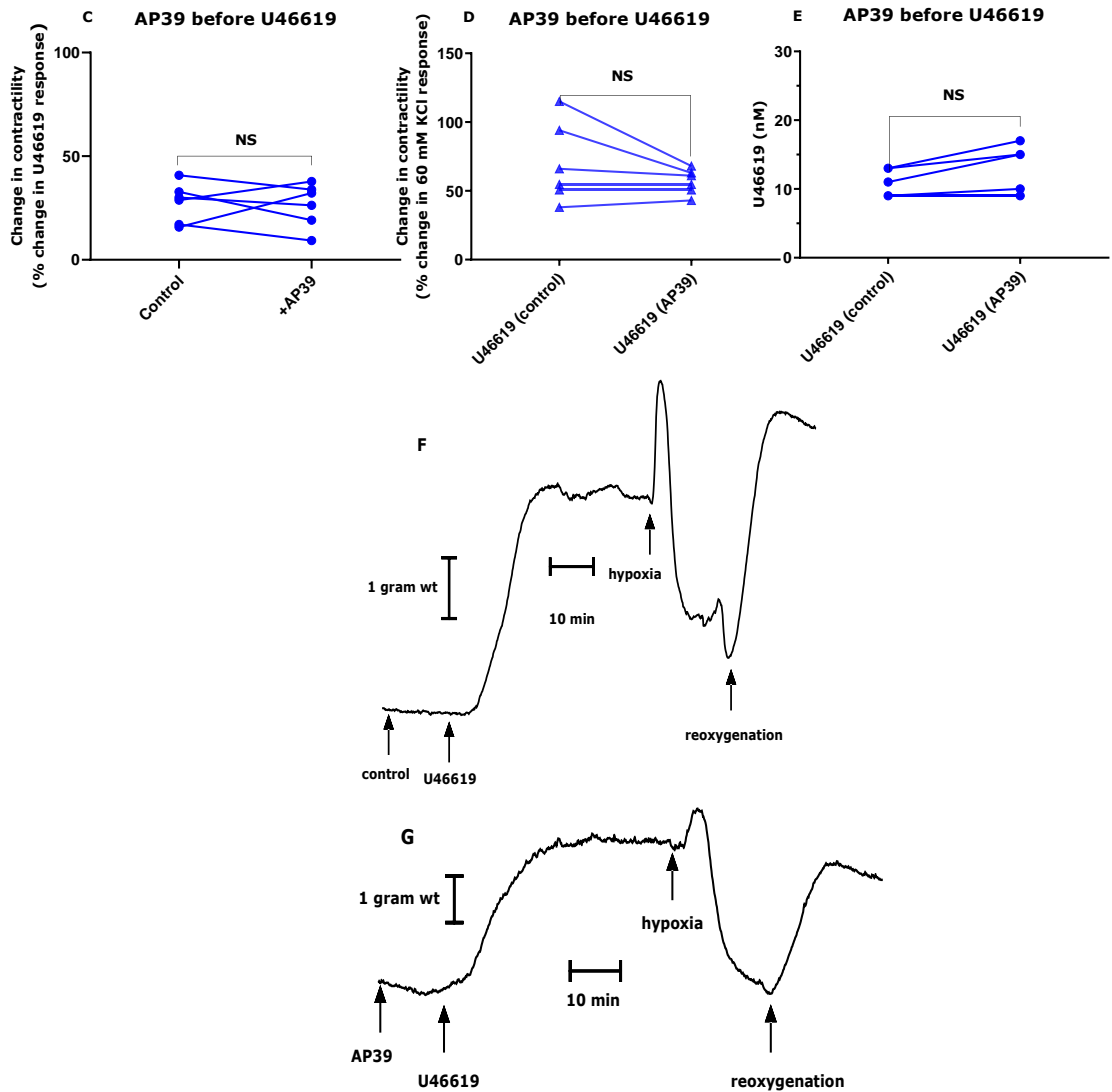
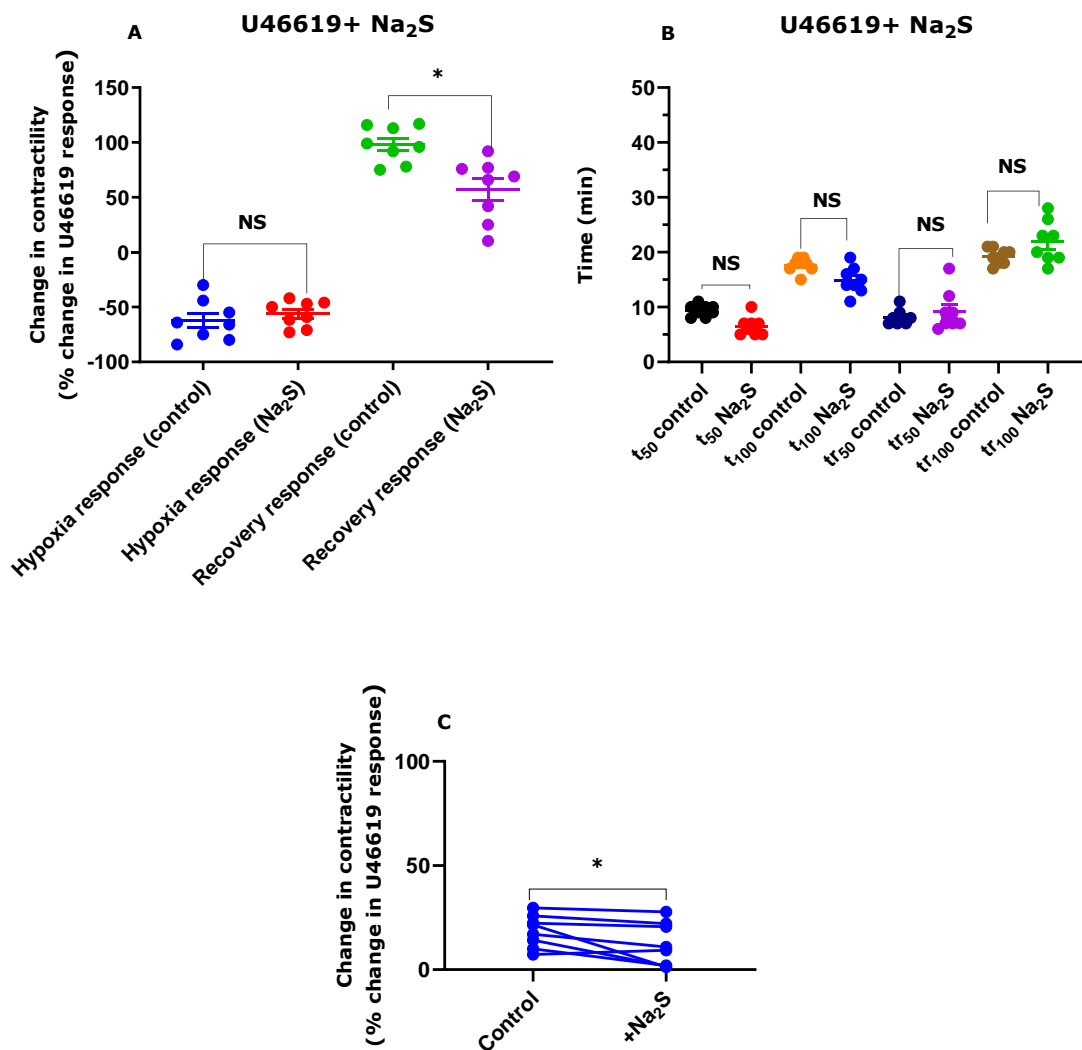


Figure 4.26: The effects of 30 μM AP39 added (15 min) before U46619 on the hypoxia response and recovery response (A); the t_{100} -time (the time required to reach the peak relaxation response after hypoxia), the t_{50} -time (the time required to reach 50 % of the peak relaxation response after hypoxia), the tr_{100} -time (the time required to reach the peak recovery response after 95 % oxygen/5 % CO_2 reintroduction/reoxygenation), the tr_{50} -time (the time required to reach 50 % of the peak recovery response after 95 % oxygen/5 % CO_2 reintroduction/ reoxygenation) (B). The contraction response of hypoxia with control (vehicle control for AP39 0.1 % v/v DMSO) or AP39 (30 μM) (C). U46619 response with control (vehicle control for AP39 0.1 % v/v DMSO) or AP39 (30 μM) (D). The concentration of U46619 required to reach 40-60 % contractility response of 60 mM KCl response with control (vehicle control for AP39 0.1 % v/v DMSO) or AP39 (30 μM) (E). The representative trace recording of the hypoxia relaxation response and recovery response with control (vehicle control for AP39 0.1 % v/v DMSO) (F) or AP39 (30 μM) (G). The control represents in the absence of 30 μM AP39 (vehicle control, 0.1% v/v DMSO); AP39 represents in the presence of 30 μM AP39 (AP39 incubated for 15 min before U46619 addition); NS (non-significant) $p > 0.05$, $*p < 0.05$ vs control using a two-tailed paired Student's t-test; $n=6$.

Pre-incubation with the different sources of H₂S before U46619 may lead to loss of the H₂S before the induction of hypoxia, because of the time required for U46619 to produce a contraction. Therefore, the experiment was repeated by contracting with U46619, prior to addition of the H₂S sources and subsequent induction of hypoxia.

4.4.18.4. The effects of 1 mM Na₂S addition after U46619 addition

1 mM Na₂S incubation after U46619 had no significant effect the time-profile parameters of the hypoxia response (t_{100} and t_{50}) or the recovery response (tr_{100} and tr_{50}) after reintroduction of oxygen (reoxygenation) (figure 4.27: B), and had non-significant effect on the size of the relaxation response to hypoxia (figure 4.27: A, D and E). However, 1 mM Na₂S incubation after U46619 led to a significant decrease in the size of the recovery response after reintroduction of oxygen (reoxygenation) (figure 4.27: A, D and E) and a significant decrease in the size of the hypoxia-induced contraction response (4.27: C).



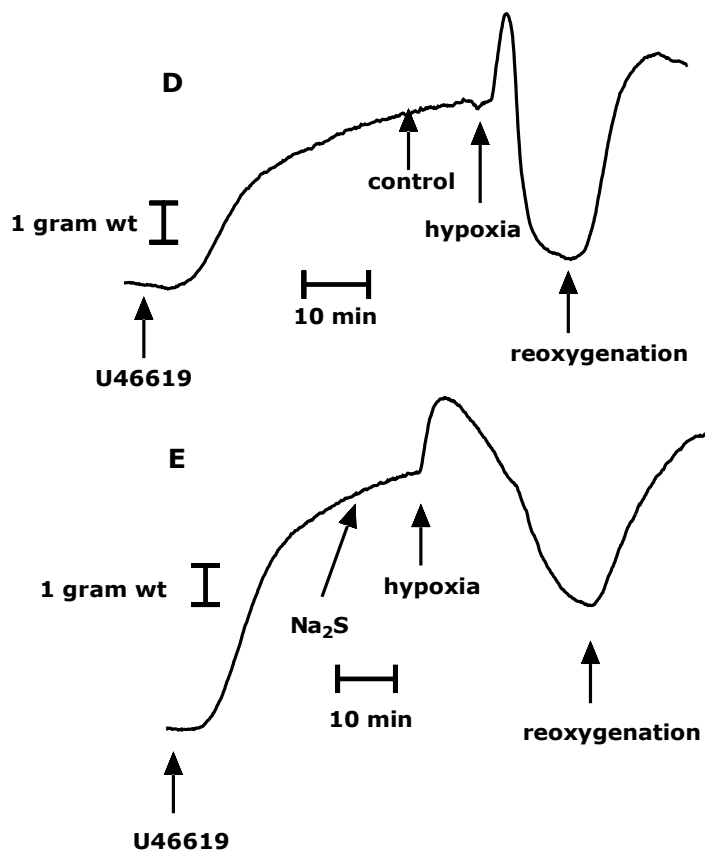
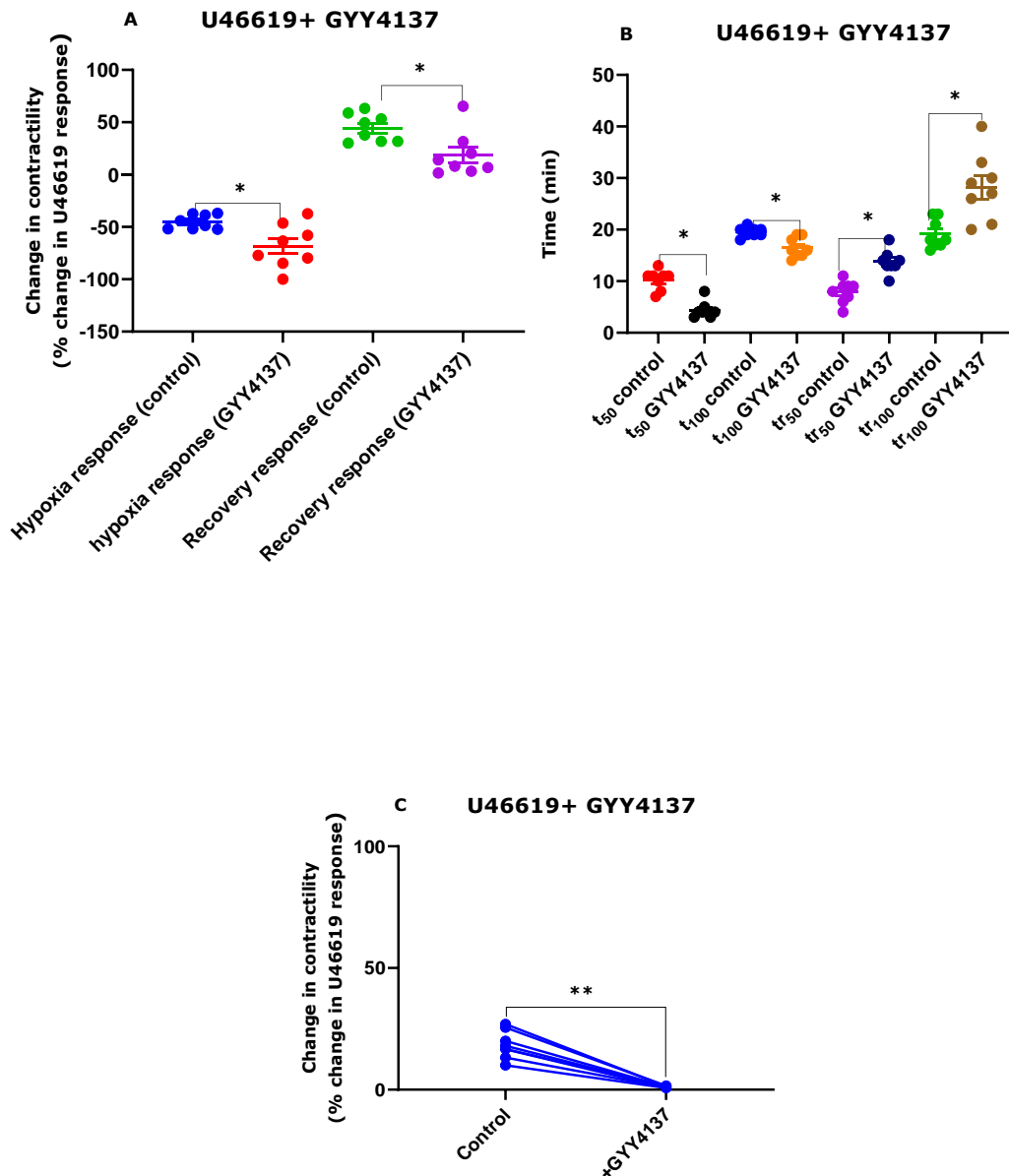


Figure 4.27: The effects of 1 mM Na₂S incubation (15 min) after U46619 addition on the hypoxia response and recovery response (A); the t_{100} -time (the time required to reach the peak relaxation response after hypoxia), the t_{50} -time (the time required to reach 50 % of the peak relaxation response after hypoxia), the tr_{100} -time (the time required to reach the peak recovery response after 95 % oxygen/5 % CO₂ reintroduction/reoxygenation), the tr_{50} -time (the time required to reach 50 % of the peak recovery response after 95 % oxygen/5 % CO₂ reintroduction/reoxygenation) (B). The contraction response of the hypoxia in the presence of control (vehicle control, distilled water) or 1 mM Na₂S (C). The representative trace of the hypoxia relaxation response and recovery response in the presence of control (vehicle control, distilled water) (D) or 1 mM Na₂S (E). Control represents in the absence of 1 mM Na₂S (vehicle control, distilled water); Na₂S represents in the presence of 1 mM Na₂S (Na₂S incubated for 15 min after U46619 addition); NS (non-significant) $p > 0.05$, * $p < 0.05$ vs control using two-tailed paired Student's t-test; $n=8$.

4.4.18.5. The effects of 100 μ M GYY4137 addition after U46619 addition

Incubation with 100 μ M GYY4137 after U46619 addition led to significant enhancing of the peak (maximum) relaxation response of hypoxia (figure 4.28: A). Interestingly 100 μ M GYY4137 incubation after U46619 addition led to a significant decrease in the recovery response after reintroduction of oxygen (reoxygenation) (figure 4.28: A and C) and moreover, there was a significant decrease in the time-profile parameters of the hypoxia response (t_{100} and t_{50}) (figure 4.28: B) and also there was a significant delay in the recovery response (tr_{100} and tr_{50}) (figure 4.28: B). Thus, it seemed that 100 μ M GYY4137 made the hypoxia relaxation response quicker (figure 4.28: B and D). Furthermore, 100 μ M GYY4137 incubation after U46619 led to a significant decrease in the hypoxia-induced contraction response (figure 4.28: C and E).



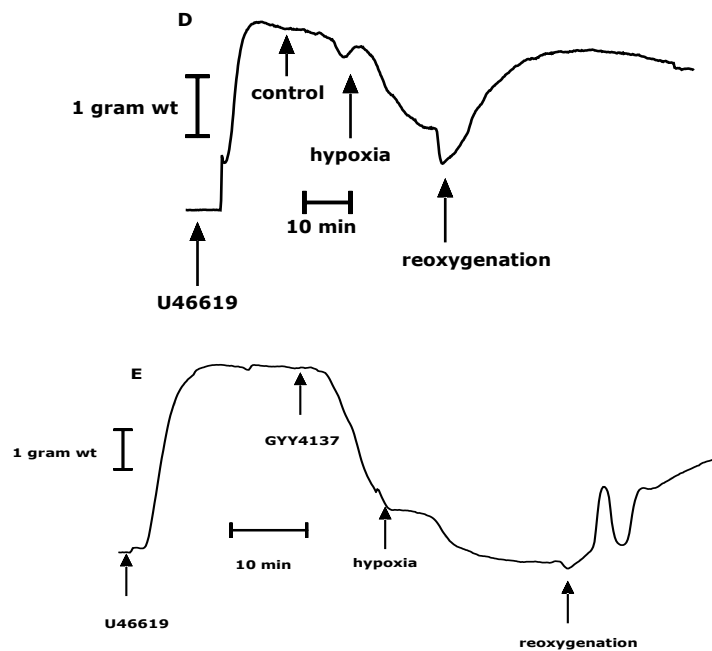
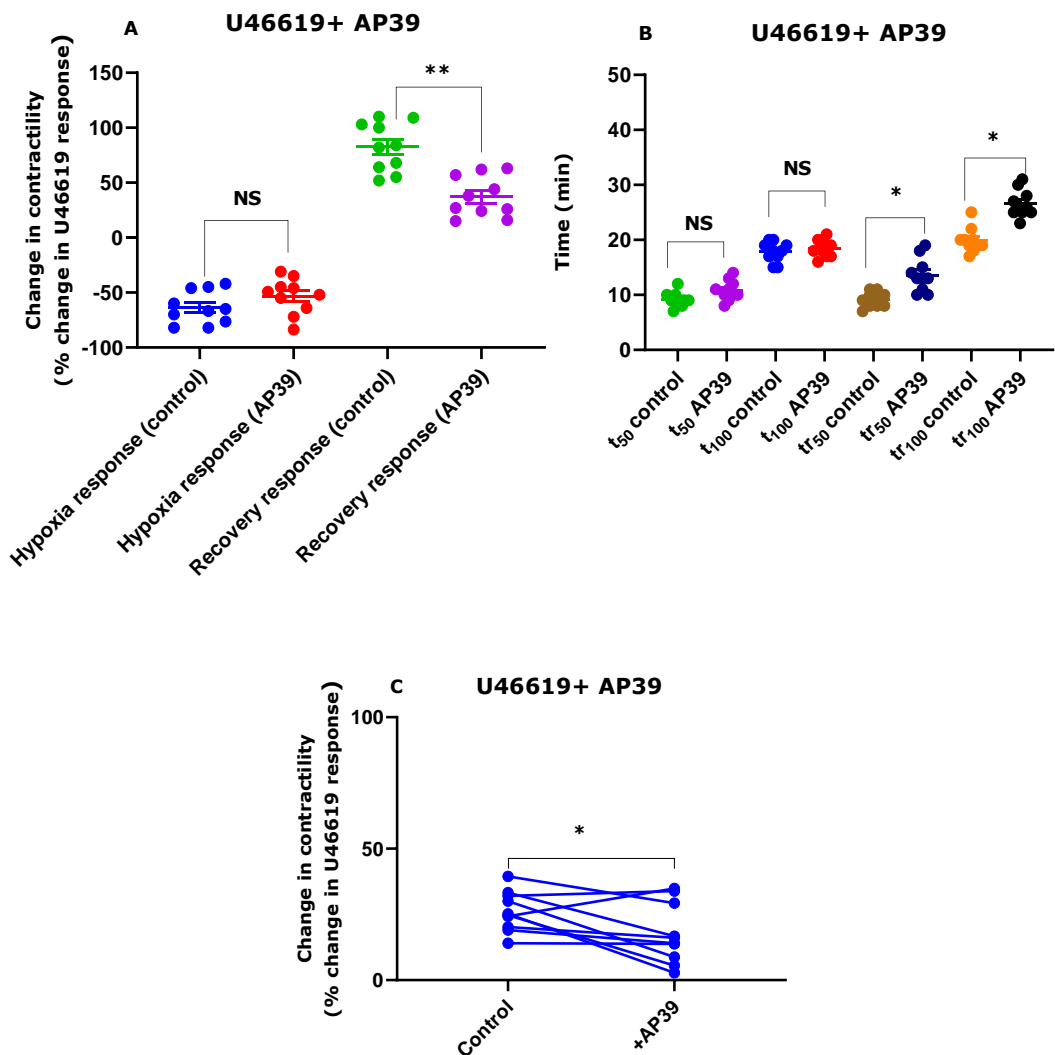


Figure 4.28: The effects of 100 μM GYY4137 incubation (15 min) after U46619 addition on the hypoxia response and recovery response (A); the t_{100} -time (the time required to reach the peak relaxation response after hypoxia), the t_{50} -time (the time required to reach 50 % of the peak relaxation response after hypoxia), the tr_{100} -time (the time required to reach the peak recovery response after 95 % oxygen/5 % CO_2 reintroduction/reoxygenation), the tr_{50} -time (the time required to reach 50 % of the peak recovery response after 95 % oxygen/ 5 % CO_2 reintroduction/reoxygenation) (B). The contraction response of hypoxia in the presence of control (vehicle control for GYY4137 0.1% v/v DMSO) or GYY4137 (100 μM) (C). The representative trace of the hypoxia relaxation response and the recovery response after oxygen reintroduction (reoxygenation) in the absence of GYY4137 control (vehicle control, 0.1% v/v DMSO) (D) or in the presence of GYY4137 (100 μM) (E). The control represents in the absence of 100 μM GYY4137 (vehicle control, 0.1% v/v DMSO); GYY4137 represents in the presence of 100 μM GYY4137 (GYY4137 incubated for 15 min after U46619 addition); NS (non-significant) $p > 0.05$, * $p < 0.05$, ** $p < 0.01$ using a two-tailed paired Student's t-test; $n=8$.

4.4.18.6. The effects of 30 μ M AP39 addition after U46619 addition

The results of these experiments demonstrated that 30 μ M AP39 incubation led to no significant difference in the time-profile parameters of the hypoxia response (t_{100} and t_{50}) (figure 4.29: B). Moreover, there were no significant effects of 30 μ M AP39 incubation after U46619 addition on the size of the hypoxia response, peak (maximum) relaxation response (figure 4.29: A and E). However, there was a significant decrease in the size of the recovery response after reintroduction of oxygen (reoxygenation) (figure 4.29: A). Also, there was a significant delay in the time-parameters of the recovery response after reintroduction of oxygen (reoxygenation) (tr_{100} and tr_{50}) (figure 4.29: B). The above results indicate that there were significant effects of 30 μ M AP39 incubation after U46619 addition on the time profile of the recovery response (figure 4.29: B and D). Moreover, 30 μ M AP39 incubation after U46619 led to a significant decrease in the size of the hypoxia-induced contraction response (figure 4.29: C and E).



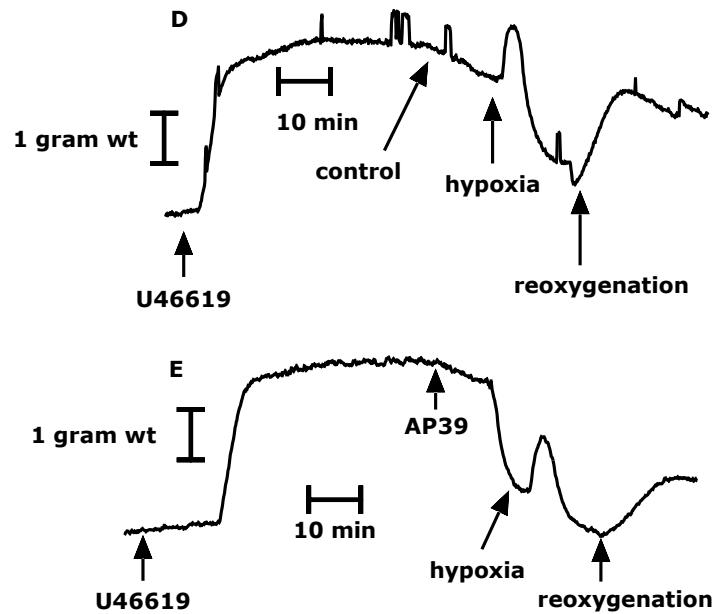
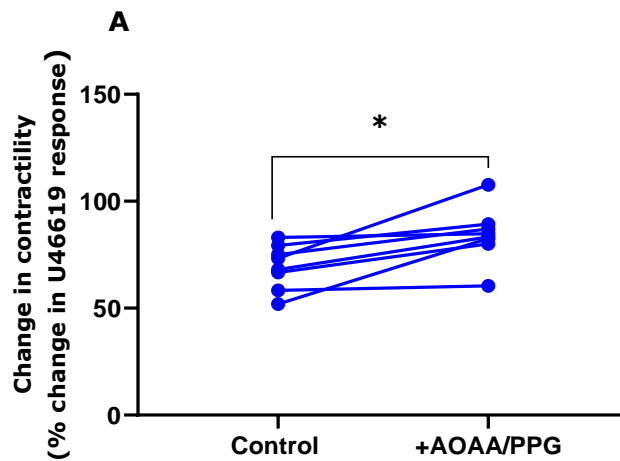


Figure 4.29: The effects of 30 μM AP39 incubation (15 min) after U46619 addition on the hypoxia response and the recovery response (A); the t_{100} -time (the time required to reach the peak relaxation response after hypoxia), the t_{50} -time (the time required to reach 50 % of the peak relaxation response after hypoxia), the tr_{100} -time (the time required to reach the peak recovery response after 95 % oxygen/5 % CO_2 reintroduction/reoxygenation), the tr_{50} -time (the time required to reach 50 % of the peak recovery response after 95 % oxygen/5 % CO_2 reintroduction/reoxygenation) (B). The contraction response of hypoxia in the presence of control (vehicle control for AP39 0.1% v/v DMSO) or AP39 (30 μM). The representative trace of the hypoxia relaxation response and recovery response in the absence of AP39 control (vehicle control for AP39, 0.1 v/v % DMSO) (D) and in the presence of AP39 (30 μM) (E). The control represents in the absence of 30 μM AP39 (vehicle control for AP39 0.1% v/v DMSO); AP39 represents in the presence of 30 μM AP39 (AP39 incubated for 15 min after U46619 addition); NS (non-significant) $p > 0.05$, $*p < 0.05$ vs control, $**p < 0.01$ vs control using a two-tailed paired Student's t-test; $n = 10$.

4.4.19. The effects of inhibition of endogenous H₂S production on the recovery response after 15 min hypoxia in the U46619-precontracted PCA

Depending on the above results, there is some evidence which suggests that the H₂S sources may decrease the size of the recovery response after reoxygenation (oxygen reintroduction) after hypoxia. Therefore, the aim of this experiment was to determine the effect of inhibiting endogenous H₂S synthesis using enzyme inhibitors AOAA (CBS inhibitor) and PPG (CSE inhibitor) on the recovery response to reoxygenation after 15 min of hypoxia. In the presence of AOAA/PPG, there was a significant increase in the size of the recovery response to reoxygenation by 95 % oxygen/5 % CO₂ (95 % oxygen/ 5 % CO₂ reintroduction/reoxygenation) (figure 4.30: A, B and C).



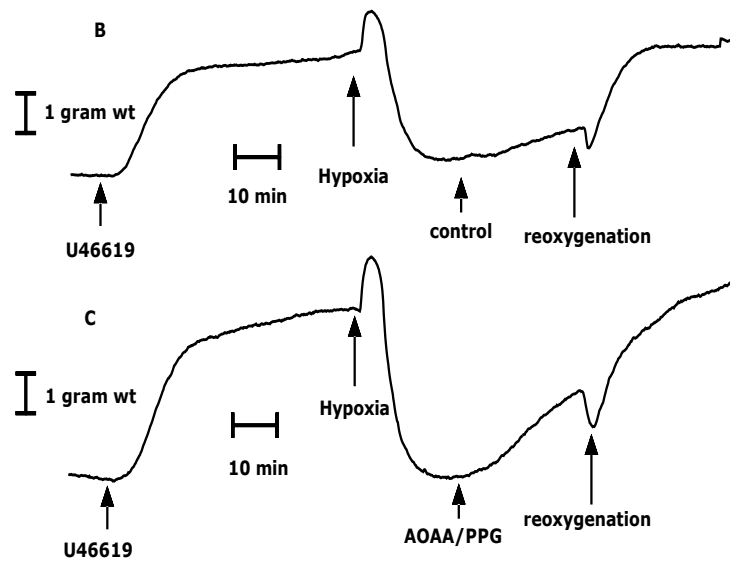


Figure 4.30: The effects of H₂S synthesising enzymes inhibitors in combination, 100 μ M AOAA (aminooxyacetic acid, CBS inhibitor) and 100 μ M PPG (propargylglycine, CSE inhibitor) on the recovery response to reoxygenation by 95 % oxygen/ 5 % CO₂ (95 % oxygen/ 5 % CO₂ reintroduction/reoxygenation) after 15 min hypoxia in U46619 precontracted PCA (B) compared to control (vehicle control distilled water for AOAA/PPG) (A). The X-axis represents control (in the absence of AOAA/PPG, vehicle control distilled water for AOAA/PPG) vs + AOAA/PPG (PCA pre-incubated with 100 μ M AOAA and 100 μ M PPG for 30 min, presence of AOAA/PPG). The Y-axis represents the change in contractility expressed as a percentage of the U46619 response. Data are individual points. * $p < 0.05$ using a two-tailed paired Student's t-test; $n=6$. The representative trace for the effects of H₂S synthesising enzymes inhibitors in control (vehicle control distilled water for AOAA/PPG) (B) and presence of combination, 100 μ M AOAA and 100 μ M PPG on the recovery response to reoxygenation (C).

4.5. Discussion

Most of the literature of H₂S is based on the use of salts as a source of H₂S. These salts release a large amount of H₂S in a short time and this could lead to supra-physiological concentrations. Therefore, there is a question mark about how useful and reliable these data are.

Therefore, the key aims of this chapter were to determine whether there are any differences in the relaxation responses and mechanism of relaxation among three different types of H₂S sources. Thus, in this chapter, we compared the effects of three different H₂S sources on the relaxation response in the PCA. Next, we compared mechanisms of relaxation of these three different H₂S sources in the PCA. First, Na₂S salt, which releases H₂S in a large amount in a short time, a fast release H₂S salt, which may be regarded as the classical H₂S salt/source used in many previous studies. Second, GYY4137, which releases H₂S slowly over time, a slow-release H₂S source. Third, AP39, which releases H₂S in the mitochondria, mitochondrial-targeted H₂S source. GYY4137 and AP39 could be regarded as new generation H₂S sources in recent studies.

Thus, this chapter aimed to gain greater insight into the role and molecular mechanism/s of H₂S in the vasculature and to identify whether distinct exogenous H₂S sources differentially influence the tone in the PCA by comparison of signalling pathways of these three different H₂S sources: Na₂S (fast release), GYY4137 (slow-release) and AP39 (mitochondrial-targeted) in the relaxation response of PCA under different conditions.

The results of this study demonstrated there was no difference in the relaxation responses between the three different H₂S sources, Na₂S, GYY4137 and AP39. In other words, there was no difference in the relaxation response in PCA between the non-specific targeted H₂S sources (Na₂S and GYY4137) and the mitochondrial-targeted H₂S source AP39. Thus, the previous studies looking at the vascular responses to H₂S using the salts might be useful in determining the relaxation response to H₂S in PCA.

Next, there was no difference in the mechanism of relaxation response among the three different H₂S sources. Moreover, results in this chapter demonstrated that the potassium channels, in particular, K_{ATP} channels and NO do not play a major role in H₂S-mediated vasorelaxation in PCA. Our results suggested that H₂S vasorelaxation is potentially mediated by blocking voltage-gated calcium channels and opening TRPV1 channels.

First, we did the cumulative response curve (CRC) for H₂S sources, and we observed that a single (bolus) concentration (dose) of H₂S is more effective than cumulative additions of H₂S because H₂S has a rapid loss and unstable properties. Therefore, we used a single (bolus) concentrations of 1 mM Na₂S, 100 μM GYY4137 and 30 μM AP39 in our next experiments. Although these concentrations of 1 mM Na₂S, 100 μM GYY4137 and 30 μM AP39 may seem higher than the concentrations used previously in other studies in cells (Wang, 2012, Powell et al., 2018), the possible explanation for this difference may be due to multiple layer structure of PCA tissues which may impede and slow the access of H₂S in tissues in comparison to monolayer structure of cells though H₂S is lipid soluble, or it may be due to different effect of H₂S on PCA tissues in comparison to cells, or that PCA tissues may interact with H₂S in a different way compared to cells. For example, AP39 in about 30-300 nanomolar concentrations was effective to protect against oxidative stress and maintain mitochondria function in murine brain endothelial cells (Szczyzny et al., 2014), rat renal epithelial cells (Ahmad et al., 2016) and murine microvascular endothelial cells (Geró et al., 2016).

Zhao and co-workers reported that NaHS produced vasorelaxation with the measured H₂S level of about 50 μM in rat aorta (Zhao et al., 2001). These concentrations are reported by Hosoki's study that 50 μM-160 μM are physiological levels (Hosoki et al., 1997) and also the measured H₂S concentrations under physiological conditions are about 1 μM-160 μM in the blood and tissue of mammals (Abe and Kimura, 1996; Zhao et al., 2001). However, in the present study these concentrations failed to produce significant relaxation responses in PCA. This could be due to the H₂S escaping the tissue bath very quickly, so the concentration that actually reaches the tissue is very low.

1 mM concentration of inorganic salts, such as NaHS was used in many studies and produced hypotension and relaxation in a dose-dependent manner in vascular tissues, such as human internal mammary artery (Webb et al., 2008), aorta and portal vein (Hosoki et al., 1997; Zhao and Wang, 2002; Ali et al., 2006; Kubo et al., 2007; Al-Magableh and Hart, 2011; Bucci et al., 2012), coronary artery (Kuo et al., 2015; Donovan et al., 2017), and mesenteric artery (Cheng et al., 2004; di villa Bianca et al., 2011; Materazzi et al., 2017).

100 μM to 1 mM GYY4137 concentrations led to anti-inflammatory effect in LPS-induced endotoxic shock in rats and mouse macrophages via a significant decrease of TNF-α level and NF-κB activation (Li et al., 2009; Whiteman et al., 2010). In a concentration-dependent manner, 50 μM, 100 μM, 200 μM and 500 μM GYY4137 concentrations produced an increase of cell viability, antioxidant and mitochondrial protective effects against high-glucose levels in H9c2 cells (Wei et al., 2014). 100 μM

GY4137 led to a relaxation of mouse aorta (Bucci et al., 2012) and bovine ciliary artery in a dose-dependent manner (Chitnis et al., 2013).

Generally, H₂S concentrations of more than 100 μM led to vasorelaxation in rat aorta and mesenteric arteries (Whiteman et al., 2011). These blood vessels, rat aorta and mesenteric arteries are very much smaller than the PCA and this may make a difference. These differences in concentration of H₂S donors used may be attributed to variations in the methodology, such as tissue or cells types, species rat or pig and experimental approaches: measurement assay, organ-bath or patch-clamp, conditions hyperoxic or hypoxic. These factors may lead to crucial effects on the concentrations of H₂S sources used to induce an effect on vasculature. Similarly, low concentrations of L-cysteine (H₂S precursor) led to contractions, while high concentrations of L-cysteine led to relaxations of rat mesenteric artery (Cheng et al., 2004) and mouse aorta (Al-Magableh and Hart, 2011).

The effects of H₂S on the vasculature are complex and sometimes contradictory (Wang, 2012). For example, H₂S may lead to a relaxation in some vessels and contraction in other vessels, or relaxation or contraction in the same tissue depending on the oxygen level. The size of the relaxation or contraction responses are also variable between studies. H₂S can lead to both contraction and relaxation, depending on the H₂S level. For example, Kubo and his colleagues reported that a low NaHS concentrations (100 μM-300 μM) produced contraction in rat aorta by inhibition of NO synthase. These contraction responses at low H₂S concentrations are commonly associated with H₂S salts rather than other H₂S sources, such as slow-release H₂S sources GY4137. In contrast, high NaHS concentrations (1000 μM-3000 μM) resulted in relaxation (Kubo et al., 2007). Moreover, NaHS in low concentration (dose) (10 μM -100 μM) led to a contraction in rat aorta while relaxation in rat aorta was produced by higher concentration (400 μM- 1600 μM) (Ali et al., 2006). Donovan's study reported that NaHS and Na₂S produced contraction at low concentrations and relaxations at concentrations in PCA (Donovan et al., 2017). Similarly, NaHS and GY4137 led to a vasorelaxation relaxation response in a concentration-dependent pattern in bovine ciliary artery (Chitnis et al., 2013). Materazzi's study reported that NaHS led to relaxation in human mesenteric arteries in a concentration-dependent pattern (Materazzi et al., 2017).

Ali and co-workers reported that low H₂S concentrations led to an increase in blood pressure. In contrast, high H₂S concentrations of H₂S led to a decrease in blood pressure which may be via vasoactive effects of H₂S on blood vessels of anaesthetised rats (Ali et al., 2006). The results of the present study demonstrated that contraction responses at low H₂S concentrations are observed with H₂S salts rather than other H₂S sources, such as slow-release H₂S sources GYY4137 and mitochondrial-targeted H₂S sources AP39. Furthermore, low concentrations of GYY4137 and AP39 led only to small relaxation responses.

4.5.1. L-NAME effects: NO role

The results of the interaction between H₂S with NO are contradictory. Some studies reported the interaction as enhancement of the relaxation, such as Zhao and Wang's study, which reported that endothelial-denudation and L-NAME led to decrease of NaHS relaxation response in rat aorta (Zhao and Wang, 2002). On the other hand, other studies demonstrated the interaction of H₂S with NO is antagonistic via formation of inactive nitrosothiol, which did not relax or contract blood vessels. They depended on biochemical and physiological evidence (Whiteman et al., 2006). This observation could be due to physiological antagonism between H₂S and NO, and that nitrosothiol formation might be a mechanism to control the prolonged NO vasorelaxation effect. Therefore, H₂S may modulate NO effects on vascular tone by controlling the availability of NO via chemical interaction and the formation of inactive nitrosothiol. For example, mixing of NaHS with NO donor, sodium nitroprusside led to a decrease in the relaxation effect of NaHS in rat aorta (Ali et al., 2006).

The results of the current study showed that inhibition of NO synthesis by L-NAME led to a significant enhancement in the relaxation response of all our three different H₂S sources in PCA. Moreover, transient contraction was observed with H₂S salt Na₂S rather than GYY4137 and AP39. In line with these results, Donovan's study found that inhibition of NO synthesis by L-NAME led to a decrease in the transient contraction phase and increase in relaxation response of Na₂S in PCA. They suggested that this observation may be due to antagonism in physiological functions between NO and H₂S (Donovan et al., 2017). These observations could be due to NO with H₂S could lead to the formation of inactive nitrosothiol, which presumably prevents the relaxation of H₂S as well as NO (Whiteman et al., 2006). Thus, formation of nitrosothiol blocked by L-NAME. Therefore, removal of NO by L-NAME led to a higher detectable relaxation caused by H₂S in PCA. Similarly, co-administration of AP39 with L-NAME *in vivo* led to augmentation of the hypotensive effect of AP39 and decrease of heart rate in rat hearts (Tomasova et al., 2014). In another study, the interaction of NO and NaHS (H₂S salt) leads to contraction responses in rat and mouse aorta by direct inhibition of NO synthase activity in the endothelium (Geng et al., 2007; Kubo et al., 2007). They also reported that denudation lead to presence of only vasorelaxation in response to NaHS. Similar observation was reported in and human internal mammary artery (Webb et al., 2008). Therefore, these results could suggest a physiological antagonism between H₂S and NO. Thus, in this case NO is reducing the H₂S relaxation response, which could be happened in the current study.

L-NAME produced no difference in IK-1001 (H₂S donor) relaxation response in rat aorta (Kiss et al., 2008). Similarly, Chitnis's study reported that L-NAME led to no difference in NaHS and GYY4137 relaxation responses in bovine ciliary artery muscle and they suggested an alternative mechanism for H₂S mediated relaxation possibly via inhibition production of excitatory-prostanoids, such as PGF₂α by flurbiprofen (NSAIDs) (Chitnis et al., 2013).

4.5.2. KCl, TEA and glibenclamide effects: K⁺ channels role and K_{ATP} channels roles

Some studies in the literature reported that H₂S vasorelaxation is mediated by opening potassium channels, in particular, K_{ATP} channels and the effect of NaHS (H₂S salt) was similar to pinacidil (K_{ATP} channel opener) and reduced by glibenclamide (K_{ATP} channels blocker) (Zhao et al., 2001; Cheng et al., 2004; Tang et al., 2005). Moreover, Kubo and his colleagues reported that 10 μM glibenclamide led to a partial decrease of NaHS-mediated relaxation in rat aorta (Kubo et al., 2007). Moreover, blocking of potassium channels, BK_{Ca} and K_v channels resulted in a partial decrease in NaHS-mediated relaxation in mouse aorta (Al-Magableh and Hart, 2011).

In contrast to these previous studies about the role of K_{ATP} channels in H₂S mediated relaxation, the results of the present study demonstrated that vasorelaxation in PCA is not principally mediated by K_{ATP} channels opening. Furthermore, blocking of potassium channels by TEA (non-selective potassium channels blocker) and by glibenclamide (K_{ATP} channels blocker)/tolbutamide (K_{ATP} channels blocker) interestingly produced a significant enhancement in the relaxation response to Na₂S and GYY4137 in PCA. Regarding AP39 results, there was no apparent effect of glibenclamide, but there was a significant enhancement with tolbutamide. K_{ATP} channels are reported to be involved in relaxation responses and they are likely to be the plasma membrane channels, not the mitochondrial ones. Therefore the question is, does releasing H₂S in the mitochondria affect K_{ATP} channels on the plasma membrane. Donovan et al's study reported that 10 mM TEA led to an inhibition of the contraction phase of the hypoxia response to both NaHS and Na₂S, and also 10 mM TEA led to an increase in relaxation response of Na₂S in PCA. They indicated that 10 μM glibenclamide did not change the relaxation response of Na₂S in PCA (Donovan et al., 2017) and these results might be due to non-specific effects of glibenclamide at this concentration.

It has been reported that glibenclamide needs to be in high concentrations of 10-50 μM to be effective in blocking K_{ATP} channels (Zhao et al., 2001; Cheng et al., 2004; Kubo et al., 2007; Webb et al., 2008; Chitnis et al., 2013). Some studies that have used these high concentrations of glibenclamide have indicated that H₂S-mediated relaxation mechanisms are independent of K_{ATP} channels (Zhao et al., 2001; Cheng et al., 2004; Kubo et al., 2007). However, in the present study, 10 μM glibenclamide inhibited the contraction to U46619 and this suggesting non-selective effects of glibenclamide at these concentrations. Therefore, we used a submaximal concentration of 1 μM glibenclamide. Kiss's study reported that 100 μM glibenclamide led to no significant change in IK-1001 mediated relaxation in rat aorta (Kiss et al.,

2008). They suggested alternative mechanisms of H₂S relaxation via inhibition of ATP generation and intracellular acidification.

The results of the present study indicated that 1 μM glibenclamide inhibited the pinacidil induced relaxations (K_{ATP} channel opener), indicating that this concentration is effective in inhibiting K_{ATP}-mediated relaxations. This supports our conclusion that H₂S relaxations are not mediated via K_{ATP} channel opening. It is possible that the inhibitory effects seen with glibenclamide in other studies could be due to other mechanisms of actions of glibenclamide other than K_{ATP} channels blocking, such as calcium channels blocking effect (Lee and Lee, 2005) and this could be the reason that glibenclamide inhibits the U46619 induced contraction at the higher concentration. In agreement with our results, glibenclamide led to enhancement of NaHS-mediated relaxations in human mesenteric arteries (Materazzi et al., 2017). They suggested alternative mechanisms of H₂S relaxation via decrease of prostanoid biogenesis.

The other possible explanation for TEA and glibenclamide results may be due to variation in animal species or tissue between rat and pig and also because of most of the studies of potassium/K_{ATP} channels opening role were in rat aorta, mesenteric and vascular smooth muscle cells (Zhao et al., 2001; Cheng et al., 2004). Therefore, in our results in the presence of potassium/K_{ATP} channels blockers, there was a significant enhancement of H₂S source-mediated relaxation responses. This observation might be attributed to that blocking of potassium/K_{ATP} channels might sensitise the PCA to H₂S-mediated relaxation via calcium channels, such as voltage-gated calcium channels blocking, or it might alter the effects of H₂S on other contracting systems of blood vessels, such as inhibition of the angiotensin-converting enzyme (ACE) (Laggner et al., 2007; Al-Magableh et al., 2015). Another possible interpretations that the delay in tissue transport and tissue deterioration might lead to excessive potassium/K_{ATP} channels opening and that TEA might have other effects, such as anti-inflammatory and cardiovascular protective effects in sepsis in rats (Sordi et al., 2011).

4.5.3. The role of calcium channels

The results of the present study demonstrated that the three different H₂S sources, Na₂S, GYY4137 and AP39 blocked calcium-induced contractions, which could be through the blocking of voltage-gated calcium channels (VGCC). Interestingly, we observed that the inhibition of the calcium-induced contraction was maintained after 15, 30 and 60 min periods of incubation of PCA with all the three different H₂S sources, even though levels of H₂S in the buffer are likely to have dropped. This observation may be attributed to the sulfhydrylation of calcium channels, which resulted in prolongation of H₂S-mediated relaxation in PCA. The sulfhydrylation of ion channels is a suggested mechanism for H₂S-mediated relaxation in blood vessels as a post-translational modification (Mustafa et al., 2009). In agreement with our results, NaHS caused a significant inhibition of voltage-gated calcium channels in mouse aorta via direct blockade of calcium channels (Al-Magableh and Hart, 2011). Similarly, H₂S resulted in blockade of calcium channels potentially via sulfhydrylation in vascular smooth muscle cells (Dai et al., 2019) and in cardiomyocytes (Zhang et al., 2012).

In the line of these results, our results showed that all three different H₂S sources Na₂S, GYY4137 and AP39 led to a significant decrease in the Bay K8644-induced contractions in PCA and these results indicate that H₂S-mediated relaxation is dependent on calcium channel blockade. Thus, these data indicate the calcium channels blockade is principally implicated in H₂S-mediated relaxation response in PCA.

Other studies have also indicated that H₂S could cause inhibition of calcium channels (L and T type dihydropyridine voltage-gated calcium channel), and therefore H₂S leads to inhibition of extracellular calcium influx (figure 1.6) (Sun et al., 2008; Li et al., 2011; Al-Magableh and Hart, 2011; Zhang et al., 2012). In 2002, Zhao and Wang study was the first study which revealed that H₂S produced direct blockade of calcium channels in rat aorta (Zhao and Wang, 2002).

Moreover, it was reported that the relaxation responses of NaHS in mouse and rat aorta was decreased in calcium-free conditions or in the presence of calcium channel blockers, such as nifedipine (Zhao and Wang, 2002; Al-Magableh and Hart, 2011; Zhang et al., 2012; Avanzato et al., 2014). In a similar study, NaHS directly relaxes vascular smooth muscle cells via calcium channel blockade (Dai et al., 2019). Therefore, H₂S caused calcium channel blockade, which was mimicked by nifedipine, a calcium channels blocker, and antagonised by Bay K8644 (L-type calcium channels opener).

Similarly, NaHS caused inhibition of contraction and negative inotropic effect measured by an electrophysiological method in rat cardiomyocytes by inhibition of L-type calcium channels (Sun et al., 2008; Zhang et al., 2012). Additionally, Xu's work revealed that NaHS reduced calcium influx, which was reduced by Bay K8644 in human atrial fibres (Xu et al., 2011). In line with these results, Zhang's study found a similar result; they found that NaHS resulted in a suppressive effect on L-type voltage-gated calcium channels currents in rat cardiomyocytes (Zhang et al., 2012). Moreover, in rat cerebral artery, NaHS caused relaxation via calcium channel blockade but not K_{ATP} channel (Streeter et al., 2012; Tian et al., 2012). Furthermore, AP39 (mitochondria-targeted, H_2S donor) produced sustained decrease of blood pressure via blocking calcium channels in rat hearts (Tomasova et al., 2015). Additionally, application of NaHS to oxidative stress condition induced by H_2O_2 leads to decrease of cell death and increase of cell survival via blockade of calcium channels (Avanzato et al., 2014). Therefore, the blockade of calcium channels could participate in the H_2S antioxidant effect. In the lung Na_2S and GYY4137 decreased the intracellular calcium level by attenuation of release of calcium from endoplasmic reticulum through $InsP_3$ (inositol-1, 4, 5 triphosphate) and therefore, it led to smooth muscle relaxation (Castro-Piedras and Perez-Zoghbi, 2013). Therefore, H_2S may lead to the closure of the calcium channels and then decrease in the intracellular calcium concentration and decrease in muscle tone in the blood vessels and heart and decrease of the blood pressure (Yang et al., 2008; Zhang et al., 2012; Dai et al., 2019).

Increase of intracellular calcium level has been reported to play a pivotal role in IRI (ischaemia- reperfusion injury) because an increase of calcium level leads to leakage of cytochrome c oxidase (complex IV) from the mitochondria via increase of mitochondrial permeability, swelling of mitochondria, triggering of apoptosis and necrosis (Ott et al., 2002; Boehning et al., 2003). Thus, calcium accumulation may lead to dysfunction of the mitochondria in heart and blood vessels. H_2S decreases intracellular calcium (Zhao and Wang, 2002; Pan et al., 2008; Sun et al., 2008; Al-Magableh and Hart, 2011, Xu et al., 2011, Avanzato et al., 2014). Therefore, a decrease of intracellular calcium level by H_2S may be beneficial to IRI (Morris et al., 1996; Pan et al., 2009) via possibly maintaining calcium homeostasis, preserving mitochondria function and structure, increasing of blood flow during ischaemia by relaxation, and decreasing of over-contraction injury during reperfusion. Therefore, these results suggest that H_2S acts by blocking calcium channels and also inhibiting a calcium-induced contraction pathway.

4.5.4. The role of TRPV1 channels

Transient receptor potential (TRP) channels are non-selective ion channels, which play a role in the influx of cations, such as calcium, sodium and potassium. TRPV1, the capsaicin receptor participates in the signalling of inflammatory pain (Phan et al., 2020). Recently, there is some evidence, which suggests TRP ankyrin (TRPA1) and TRP vanilloid (TRPV1) channel involvement in the relaxation response of blood vessels to H₂S (White et al., 2013; Naik et al., 2016).

The results of the present study demonstrated that blocking of TRPV1 channel by capsazepine (TRPV1 channel blocker) led to a significant decrease in the H₂S-mediated relaxation in PCA with all three different H₂S sources Na₂S, GYY4137 and AP39 and these results suggest the involvement, at least in part, of TRPV1 channel opening in H₂S-mediated relaxation in PCA. These results of TRPV1 channels together with calcium channels results might suggest that there might be a possible link between calcium channels and TRPV1 channels which led to H₂S-mediated relaxation in PCA. This might be due to that blocking of calcium influx by blocking calcium channels by H₂S might sensitise PCA to relaxation by TRPV1 channel opening. The other suggested explanation is that H₂S blocks TRPV1, which then leads to inhibition of calcium influx, or H₂S isn't acting on calcium channels directly. White et al's study reported that activation of TRPA1 channels led to the release of neurotransmitters/relaxing neuropeptide from sensory nerves and this could lead to indirect blockade of calcium channels and result in relaxation in rat mesenteric arteries (White et al., 2013) and the case might be similar in TRPV1 channels in PCA.

NaHS led to the activation of TRPV1 channels, contraction and inflammation in guinea pig bronchi via the release of neuropeptide from sensory nerves (Trevisani et al., 2005) and in 2008, Lu et al's group reported that NaHS produced transient activation and prolonged blockade of TRPV1 channels in the smooth muscle contraction in rat duodenum and they suggested that might involve of the release of substance P (tachykinin) as a relaxing factor (Lu et al., 2014). It has been reported that substance P produces a relaxation through the endothelium. In the results of the present study, blocking of NO synthesis led to enhancement of relaxation in PCA with all the three different H₂S sources. Therefore, further studies with endothelial denudation are required to test whether the case is similar in PCA.

4.5.5. The role of mitochondria

It has been reported that H₂S acts to maintain the mitochondrial electron transport chain at low concentrations and inhibit the mitochondrial electron transport chain at high concentrations by inhibition of cytochrome c oxidase, complex IV of the electron transport chain in the mitochondria (Gubern et al. 2007; Módis et al., 2013). The absence of glucose should make the cell depend on the mitochondria for energy production via oxidative phosphorylation (Yang et al, 2018). Therefore, glucose-free conditions were administered to emphasise mitochondria-evoked cellular ATP production in comparison to energy supply from glycolysis.

The results of the present study demonstrated that there were no significant differences in the relaxation responses of the three different H₂S sources in the absence of glucose. This result might suggest that oxidative phosphorylation does not play a major role in H₂S mediated relaxation and that alteration of mitochondrial function may not be involved in the effects of H₂S in blood vessels. However, at low concentration of AP39 (5 µM) there was a significant enhancement of the AP39-mediated relaxation in the PCA, which may be via maintaining the mitochondria roles, such as control of the cellular bioenergetics, and reduction of ROS and calcium store/uptake, or that in low glucose condition there may be more dependence on the mitochondria and so AP39 is acting in the mitochondria to inhibit function, leading to relaxation.

In order to investigate the role of the mitochondria further, we used FCCP a mitochondrial uncoupling agent. Our results with 1 µM FCCP and 5 µM AP39 indicated that FCCP had no effect on the relaxation seen with 5 µM AP39. In contrast to the effects of removal of glucose, these observations suggest that the AP39 relaxation is not dependent on mitochondrial function. Alternatively, it could be that FCCP was less effective to inhibit mitochondrial function at 1 µM FCCP concentration because this concentration is used in isolated mitochondria studies to depolarise the mitochondrial membrane, or that AP39 protected the mitochondria and reduced the FCCP impact.

4.5.6. The effects of hypoxia

Ischaemic heart diseases (IHD) are one of the common causes of death in the developed world (Murray and Lopez, 1997; Polhemus et al., 2014). Ischaemia is the decrease in the blood flow to a tissue leading to hypoxia. Hypoxia is a harmful situation, and it leads to a negative impact on heart function. The hypoxic relaxation response in the porcine coronary artery during hypoxia is vital to maintain the blood flow and heart function (Donovan et al., 2017).

There are several factors which have been suggested in the hypoxia-induced relaxations in the coronary arteries, such as pathways mediated by NO, K_{ATP} channel opening, prostaglandins (PGs), calcium channel inhibition (Smani et al., 2002), adenosine release (Berne, 1985), decrease of ATP synthesis, increase of lactic acid and decrease in the intracellular pH (Gupte and Wolin, 2006). Gupte and Wolin revealed that hypoxia produced relaxation in the bovine coronary artery is mediated by decreased levels of calcium and NADPH (nicotinamide adenine dinucleotide phosphate), and that the hypoxia-induced relaxation was less dependent on NO, prostaglandins or ROS (Gupte and Wolin, 2006). Moreover, hypoxia may inhibit the oxidative phosphorylation in the mitochondria by inhibition of cytochrome c oxidase, which leads to decrease in ROS levels to a low level (physiological level) and decrease in ATP synthesis. These effects are suggested to be mediated by H_2S (Sun et al., 2012; Olson, 2013). Similarly, Kiss's study suggested that inhibition of oxidative phosphorylation and decrease of ATP levels by H_2S lead to vasodilation in rat aorta (Kiss et al., 2008) and also in human mesenteric arteries (Materrazi et al., 2017).

This reversible and competitive inhibition of the cytochrome c oxidase in the mitochondria could lead to hypo-metabolic condition, hypothermia and decrease in the oxygen consumption as a suspended animation-like phenomenon, hibernation-like in a non-hibernating animal, and this phenomenon may help the mice to resist and survive after lethal hypoxia (Blackstone et al., 2005; Roth and Nystul, 2005; Blackstone and Roth, 2007; Elrod et al., 2007). Moreover, the hypoxia response is mediated via many other factors, such as chloride channels via activation of Cl^-/HCO_3^- exchange, which results in cellular acidification (Lee et al., 2007; Donovan et al., 2017). Additionally, hypoxia relaxation may be mediated via cyclic GMP and soluble guanylate cyclase which may be activated by H_2S . H_2S synthesising enzyme activity increased in hypoxia, especially CBS in PCA and therefore, CBS has a crucial contribution in hypoxia relaxation in PCA (Donovan et al., 2017).

H₂S has been implicated as an important mediator in the hypoxia relaxation response as an oxygen transducer for several reasons, such as the similarity in the relaxation responses of H₂S and hypoxia (Olson 2008; Olson, 2013; Olson, 2015; Donovan et al., 2017). In lamprey aorta, bovine pulmonary artery, rat aorta and pulmonary artery, H₂S was reported to increase the hypoxic response, while H₂S-synthesising enzyme inhibitors decreased the hypoxia response (Olson, 2006). Moreover, CBS was reported to play a pivotal part in hypoxia response and AOAA inhibited hypoxia response in PCA (Donovan et al., 2017). Decreasing of the oxygen level may cause a decrease in H₂S oxidation and increase in H₂S availability because H₂S level is the net of H₂S generation minus H₂S oxidation. Moreover, low concentration of Na₂S led to relaxation at low oxygen level in rat aorta; in other words, the vasoactive effects of H₂S are reported to be oxygen-dependent (Koenitzer et al., 2007). Additionally, there was an enhancement of the relaxation response of IK-1001 (H₂S source) in rat aorta under low oxygen circumstances (Kiss et al, 2008). Moreover, Wang's study demonstrated that hypoxia could lead to an increase in enzymatic biosynthesis of H₂S and relaxation response in PCA by increase of CBS/CSE within about 4 h (Wang et al., 2014). In other words, there is an inverse relationship between the H₂S level and oxygen level. Low oxygen levels may also influence stores of H₂S. Thus, hypoxia lead to increase of H₂S synthesis/accumulation and enhancement of H₂S-mediated effects. Therefore, H₂S may be considered as an oxygen sensor (Olson, 2008; Olson, 2013). Thus, H₂S could protect the heart and blood vessels from ischaemic injury during hypoxia.

Hypoxia could lead to transient contraction responses, which may be due to alteration in the local levels of endogenous metabolites and their effects due to alteration in their accumulation and production over period of hypoxia (Donovan et al., 2017). The results of the present study demonstrated that the decrease in oxygen level to 21% led to a decrease in Na₂S mediated relaxation in PCA. This result might be due to a transient contraction, which is observed in the results with Na₂S, and this transient contraction of Na₂S became detectable with 21% oxygen level. This transient contraction may affect the maximal detected relaxation level of Na₂S in PCA, and also the decrease in the oxygen level may lead to a decrease in the H₂S oxidation and an increase in the H₂S level. All of these events may lead to accumulation of high H₂S levels in a short time and lead to contraction. Moreover, the contraction might reduce the relaxation response and this contraction may be due to fast H₂S release rate properties of Na₂S, which releases a high concentration of H₂S in a short time period and this may be less physiologically relevant. This transient contraction was not observed with GYY4137 and AP39, which release H₂S over a longer period of time. Therefore, the time frame of H₂S release is important.

In contrast, the results of the present study demonstrated that the decrease of oxygen gas to 21% led to an increase in AP39 mediated relaxation in PCA. This result could be attributed to an increase in H₂S level availability in the mitochondria and maintaining of mitochondria function, and this may lead to a decrease of reactive oxygen species to physiological levels and increase of glutathione levels (antioxidant effects) (Szczesny et al., 2014). Mitochondria-targeted H₂S sources, such as AP39 has been reported to be safe and produce antioxidant effect about 1000 times more than H₂S salts, Na₂S (Geró et al., 2016).

In the present study we investigated the effects of the three different H₂S sources, Na₂S, GYY4137 and AP39 on the hypoxia response, peak (maximum) relaxation response and peak (maximum) recovery response (contraction) after reoxygenation in two different ways. First way, we preincubated PCA with the three different sources for 15 min before the addition of U46619 and then hypoxia application for 15 min and next reoxygenation. Second way, after the addition of U46619, PCA was incubated with the three different sources for 15 min and then the hypoxia application for 15 min and next reoxygenation.

The results of the present study demonstrated that pre-incubation of PCA with Na₂S, and GYY4137 before the addition of U46619 caused no significant effect on the size and time of both the hypoxia and recovery responses. Pre-incubation of PCA with AP39 before the addition of U46619 caused no significant effect on the size and time of the hypoxia response and time of the recovery response, but pre-incubation with AP39 caused significant decrease in the size recovery response, which suggests prolonged effect of H₂S via AP39 possibly via sulfhydration of calcium channels.

The results of the present study demonstrated that the incubation of PCA with Na₂S after the addition of U46619 caused no significant effect on the size and time of the hypoxia response and time of the recovery response, but incubation with Na₂S caused a significant decrease in the size of the recovery response. The incubation of PCA with GYY4137 after the addition of U46619 caused significant increase in the size of the hypoxia response, significant decrease in time of the hypoxia response, significant decrease in the size of the recovery response, and significant increase in the time of the recovery response. The incubation of PCA with AP39 after the addition of U46619 caused no significant effect on the size and time of the hypoxia response, but incubation with AP39 caused significant decrease in the size of the recovery response, and significant increase in the time of the recovery response.

These different results of Na₂S, GYY4137 and AP39 could be due the differences in the release of H₂S. For example, GYY4137 is reported to be a slow-release H₂S source and therefore, GYY4137 may have prolonged actions, or that GYY4137 might have additional properties, such as antioxidant, anti-inflammatory effects and blood pressure-lowering effects more than Na₂S and AP39. Whiteman's study reported that NaHS Led to contradictory and inconsistent effects on inflammation in comparison to consistent anti-inflammatory effects of GYY4137 (Whiteman et al., 2010). Thus, the time frame of H₂S release is important.

The reperfusion, reoxygenation could lead to complex and detrimental impacts. For example, reoxygenation may lead to the generation of high concentrations of ROS, such as superoxide and hydrogen peroxide (Kashiba et al., 2002), and a decrease in the NO level and endothelial dysfunction (Olson, 2013). Oxidative stress is an important cause of tissue injury (Avanzato et al., 2014). Moreover, reoxygenation and high level of ROS lead to an increase in calcium influx and smooth muscle contraction (Olson, 2013). Therefore, excessive production of ROS led to a cellular insult in rat cardiomyocytes (Sun et al., 2012). H₂S has been reported to decrease ROS levels via antioxidants effects, in particular through mitochondrial protection from ROS. For example, treatment of rat cardiomyocytes with NaHS produced a decrease in ROS level and an increase in the activity of ROS scavenging mechanisms of the mitochondria, such as superoxide dismutase, catalase and glutathione peroxidase (Sun et al., 2012). Additionally, Geng's study reported that NaHS application in rat myocytes produced a decrease in the oxidative injury induced by isoprenaline via ROS scavenging and decrease in the malondialdehyde (MDA) levels, one of end-metabolites of lipid peroxidation, which is commonly used as a biomarker of oxidative stress (Geng et al., 2004).

Many studies have reported that cardioprotective effects of H₂S in IRI conditions are mediated by the antioxidants effects of H₂S (Karwi et al., 2017) and similar effects could be in the vascular tissues. Susuki's study reported that NaHS protected rat aorta from hyperglycemic-oxidative stress by mitochondria protection via antioxidant effects of NaHS (Susuki et al., 2011). In line with this idea, AP39 reduced oxidative stress in murine brain endothelial cells via mitochondria DNA protection and maintenance of cellular energy. Therefore, it could be useful for mitochondria-related pathology (Szczesny et al., 2014). Also, AP39 reduced oxidative stress after cardiac-arrest by antioxidant and mitochondria protective effects; therefore, it led to cardioprotective and cytoprotective effects on mice heart and brain (Ikeda et al., 2015). Moreover, AP123 and AP39 decreased the hyperglycemia-induced oxidative stress in murine vascular endothelial cells.

Moreover, Predmore's study revealed that the use of DATS, diallyl trisulfide long-acting natural H₂S source in mouse hearts with IRI led to increase in the transcription level of the Nrf2, which enhances antioxidant expression at the genetic level and this effect likely needs more than 30 min to occur, by increasing its localisation in the nucleus in myocardial ischaemia model in mouse hearts. Therefore, Nrf2 is reported to play essential roles in the increase of the antioxidant defences and reduction of oxidative stress (Predmore et al., 2012). Similarly, pre-treatment of mouse hearts with IRI, with Na₂S as a preconditioning before IRI lead to cytoprotective effects via augmentation of Nrf2 signalling and they reported that Na₂S resulted in a beneficial decrease in caspase-3 activity (Calvert et al., 2009). These results could be attributed to H₂S antioxidant effects, which protect the mitochondria from oxidative stress and preserve mitochondria function and structure.

H₂S may directly augment Nrf2 signalling, which might indirectly control H₂S production by enhancing CSE expression as a positive feedback loop and this process likely takes hours to occur. Similarly, GYY4137 decreased oxidative stress and inflammation in the aorta of mice with diabetes and atherosclerosis via augmentation of Nrf2 signalling (Xie et al., 2016). Additionally, Elrod's study demonstrated that application of Na₂S to mouse hearts with IRI led to decrease of inflammation, apoptosis and maintenance of mitochondrial function, architecture, and mitochondrial recovery after reperfusion (reoxygenation) (Elrod et al., 2007). Moreover, they reported that H₂S leads to a decrease of the mitochondrial enlargement and enhancement of mitochondrial structure using electron microscopy. Therefore, mitochondrial protection could play an important role in H₂S-mediated cytoprotection.

AP39 produced antioxidant effects by decreasing the oxidative stress in rat kidney epithelial cells and renal IRI (Ahmad et al., 2016). Application of NaHS in rat H9c2 cardiomyocytes with H₂O₂-induced oxidative stress leads to increase of cell viability, survival and decrease of apoptosis (Wu et al., 2015). Moreover, in oxidative stress condition induced by H₂O₂, the application of NaHS leads to a decrease of the cell death and increase of cell survival via blockade of calcium channels (Avanzato et al., 2014). Therefore, H₂S antioxidant effect may be mediated by blockade of calcium channels, and this effect may lead to a decrease of mitochondrial suffering and dysregulation under oxidative stress and high calcium level circumstances. Therefore, H₂S decreases oxidative stress and increases antioxidant defences and therefore, H₂S decreases the heart or blood vessel injury via its antioxidant, antiapoptosis, and anti-inflammatory, cardiovascular protective and cytoprotective actions.

The reoxygenation could lead to injury via multiple mechanisms, such as increase of ROS generation, calcium accumulation, mitochondrial dysregulation and inflammation. Thus, H₂S can decrease the injury of reoxygenation by several possible mechanisms, such as an increase in the level of cyclic GMP and protein kinase G and RISK pathway (reperfusion injury salvage kinase) and SAFE pathway (survival activating factor enhancement). H₂S might also cause a decrease of the apoptosis and maintenance of the mitochondrial function, permeability integrity, and adjustment of the sarco endoplasmic reticulum calcium-ATPase (SERCA) activity to prevent calcium overload (Polhemus et al., 2014).

The results of the present study demonstrated that the presence of three different H₂S sources Na₂S, GYY4137 and AP39 led to significant decrease in the recovery response to reoxygenation in PCA and this result may suggest extended effect of H₂S and this effect may be by sulfhydration of calcium channels and/or TRPV1 channels as post-translational modifications. Decrease of the recovery response might be beneficial to decrease the possible excessive and uncontrolled contractions induced by reoxygenation, which might lead to IRI, which is likely due to increase of ROS and calcium overload. Moreover, the three different H₂S sources produced a decrease in the size of the recovery response and this may be due to the decrease of the ROS generation and excessive calcium accumulation, and maintenance of the mitochondrial function and structure integrity.

In isolated rat hearts, Hu et al's study suggested that the mechanism of NaHS cardioprotection in IRI is by decreasing the infarction area via an increase in the activity of PI3K/Akt (phosphoinositide 3-kinase/protein kinase B) and phosphokinase G pathway, which led to inhibition of Na⁺/H⁺ exchanger-1 (NHE-1) and inhibition of excessive calcium accumulation (Hu et al., 2011). In another study in rat isolated hearts, the cardioprotection mechanism of NaHS are mediated through stimulation of PKG/ERK1/2 (protein kinase G/extracellular signal regulated kinase) and Akt pathway (risk pathway) to produce an antiapoptotic effect (Hu et al., 2008). Na₂S reduced the infarction size in pig hearts by a decrease of lactate and enhanced the response to noradrenaline (Simon et al., 2008). Na₂S produced vascular protection during IRI by anti-inflammatory effects in PCA (Sodha et al., 2009). Furthermore, H₂S may decrease the autophagy, apoptosis and mTOR (mammalian target of rapamycin) in pig hearts during IRI (Osipov et al., 2009). The mitochondrial protection by H₂S leads to a decrease in the level of apoptosis and cell death (Polhemus et al., 2014). The effects of H₂S sources on the hypoxia and recovery responses during IRI in organ bath (*ex vivo*) may be similar to the cardioprotective effects of H₂S against ischaemia reperfusion injury (Shen et al., 2015). The results

of the present study showed that the incubation of PCA with AOAA/PPG after hypoxia enhanced the contraction in the recovery phase, and this effect is opposite of the H₂S sources effect. These effects suggest the effects of endogenous H₂S on maintaining the hypoxia relaxation response and decreasing of the contraction response in the recovery phase.

4.5.7. Summary

To summarise, first, there were no differences in the relaxation responses among the three different H₂S sources Na₂S, GYY4137 and AP39 in PCA. Thus, the studies looking at the vascular responses to H₂S using the salts might be useful in determining the response to H₂S in PCA. Second, there were no differences in the signalling mechanisms, such as NO, potassium channels, K_{ATP} channels, calcium channels, TRPV1 channels, among the three different H₂S sources Na₂S, GYY4137 and AP39 in PCA. NO, and potassium channel/K_{ATP} opening do not play a major role in H₂S-mediated relaxation by the three different H₂S sources in PCA. H₂S sources have several potential signalling pathways, notably via calcium channel blocking and might be by TRPV1 opening. Third, GYY4137 could be relatively more effective in PCA with ischaemia reperfusion injury than Na₂S and AP39.

Chapter Five

General Discussion

H₂S appears to play a role in the regulation of vascular tone. However, little is known about H₂S role in the heart. Moreover, although many signalling pathways for H₂S have been proposed, they are still unclear. Therefore, there were three main aims of this study:

First, the measurement of endogenous H₂S production in the heart by investigating the expression level of H₂S synthesising enzymes (CBS, CSE and MST) in different parts of the heart and the parallel activity of these enzymes under different conditions in order to develop assays for measuring H₂S generation.

Second, the development of an assay to measure the sulfhydration level of target proteins, such as GAPDH and MEK1 as an index of H₂S production and potential signalling mechanism for H₂S in the heart.

Third, investigating the effects of different types of H₂S sources (Na₂S, GYY4137 and AP39) on vascular tone in PCA and also under different conditions in order to determine and investigate the potential signalling pathways of the three different H₂S sources.

All these aims could help to develop an understanding of H₂S generation, regulation, signalling, vascular reactivity and mechanism of action of different sources of H₂S in the heart and blood vessels. These results may hopefully help in the development and translation of H₂S-relating sources (H₂S-based medicines) as potential drugs for treatment of some CVD. Therefore, this study could define the potential for novel signalling pathways which may regulate cardiac function and allow future studies to measure any changes in cardiac dysfunction.

CBS and MST were detected in the heart, but low enzyme activity was detected in the heart compared to the liver. The possible explanation might be that low enzyme activity is related to the physiological state, or that the H₂S produced may be stored as bound forms, such as sulfane sulfur (persulfides) and therefore not measured. Therefore, the sulfhydration could be as a potential signalling pathway for H₂S in the heart. Our data show that H₂S is endogenously produced in the myocardium and addition of the myocardial tissue led to relaxation of the coronary artery and so could be important in the maintenance of blood flow. This is because the coronary artery is vital for the blood supply to the myocardium and coronary artery pathologies lead to many CVD. Thus, H₂S generated in the myocardial tissue could act in a paracrine pattern, rather than directly on atrial or ventricular contractility. Further investigations about the myocardial tissue contribution will be discussed next.

In coronary artery diseases H₂S production is reduced. Therefore, the application of H₂S sources as an exogenous sources of H₂S can lead to cardiovascular protection against ischaemic injury. Our data in PCA suggest that the presence of the three different H₂S sources Na₂S, GYY4137 and AP39 led to a significant enhancement in the hypoxia relaxation response and decrease in the contraction response in the recovery phase after reoxygenation.

It has been reported that sulfhydration of proteins results in conformational alterations in the targeted proteins, which lead to the alteration of protein activity. For example, sulfhydration of some proteins, such as GAPDH by NaHS may lead to an increase in the enzyme activity of GAPDH by about seven-fold (Mustafa et al., 2009). Conversely, sulfhydration of other proteins may lead to decreasing of protein activity and closing of ion channels. The results of the present study demonstrated that the three different H₂S sources, Na₂S, GYY4137 and AP39 blocked calcium-induced contractions, which could be through the blocking of voltage-gated calcium channels. Interestingly, we observed that the inhibition of the calcium-induced contraction was maintained after 15, 30 and 60 min periods of incubation of PCA with all the three different H₂S sources, even though levels of H₂S in the buffer are likely to have dropped. This observation may be attributed to the sulfhydration of calcium channels, which resulted in prolongation of H₂S-mediated relaxation in PCA as long-term effects. Further investigations about the measurement of sulfhydration of calcium channels will be discussed next. Zhang's study suggested that sulfhydration is the responsible mechanism for the blockade of calcium channels and negative inotropic effects induced by H₂S. This is because in rat cardiomyocytes, diamide is an oxidant sulfhydryl modifier decreases the availability of free sulfhydryl and therefore, diamide decreases the calcium channel blockade induced by NaHS in rat cardiomyocytes (Zhang et al., 2012). In another study, sulfhydration of the heart protein phospholamban (PLN) led to relaxation of the myocardial tissue of rats and frogs likely via decrease of intracellular calcium level (Mazza et al., 2013). Similarly, sulfhydration may be responsible for NaHS mediated calcium channel blockade in rat vascular smooth muscle cells (Dai et al., 2019).

5.1. Measurement of Hydrogen Sulfide Generation in the Heart

The development of a reliable assay for the measurement of H₂S level is crucial to understanding the physiological importance of H₂S. Therefore, in this chapter, we tried to develop a sensitive and reliable assay to measure H₂S production in the heart. We found that CBS and MST as H₂S synthesising enzymes were expressed in the heart, but CSE was not detected. Interestingly, the CBS detected in the heart was consistently present at a high MW band, suggesting the less active propeptide, in comparison with the liver that had higher expression of the low MW bands active form. This may reflect the difference in biological effect of H₂S and post-translational modifications in the heart compared to the liver.

We found that there was a low amount H₂S produced in the heart compared to the liver using both the methylene blue and SF7-AM assays. There are several possible explanations for the low level of H₂S synthesis.

The results of the present study demonstrated that there was low H₂S production in the heart compared to the liver. The simple explanation may be that the heart just did not generate much H₂S as liver. However, the addition of the myocardial tissue led to relaxation of PCA and this effect blocked by H₂S-synthesising enzyme inhibitors. Therefore, further investigations are needed in order to confirm the link. For example, stimulation of the endogenous H₂S generation in the myocardial tissue via incubation with L-cysteine and after that addition of the myocardial tissue to the U46619-precontracted PCA. Stimulation of the endogenous H₂S generation in PCA tissue via incubation with L-cysteine, or inhibition of the endogenous H₂S generation in PCA tissue via incubation with H₂S-synthesising enzyme inhibitors and then, PCA contracted to 40-60 % of KCl contraction response by U46619 addition, which could determine the effect of endogenous H₂S generation on U46619 induced-contractions. Determination of the effect of myocardial tissue addition on U46619-precontracted myocardial strips (atria/strips) of pigs and humans could demonstrate the functional consequences of H₂S on the myocardial tissue tone in an autocrine manner. Measuring H₂S concentration/enzyme activity in tissue and in Krebs's solution after hypoxia as the following: making PCA only hypoxic and then, measuring H₂S concentrations/enzyme activity in the Krebs's solution and PCA tissue; making the myocardial tissue hypoxia and then, measuring H₂S concentrations/enzyme activity in the Krebs's solution and the myocardial tissue. It has been reported that CBS, CSE and MST were expressed in porcine PVAT and also H₂S could be released from PVAT and increase PCA relaxation during hypoxia. Therefore, investigation the effect of PVAT on the myocardial tissue-mediated PCA relaxation by incubation of the

myocardial tissue without/with PVAT could help to determine the role of PVAT in H₂S-mediated PCA relaxation.

Therefore, the produced H₂S by myocardial tissue could be in bound forms. Thus, most of the biological H₂S may be stored as a bound form in the heart, which may be releasable under specific conditions. Therefore, it could be important to detect and consider these stored forms of H₂S, such as reductant-labile stores (sulfane sulfur), which releases stored H₂S under reducing or alkaline conditions, and acid-labile, which releases stored H₂S under acidic condition (Ubuka et al., 2002; Whitfield et al., 2008). Sulfhydrated proteins (persulfides, reductant-labile) can be considered as stores of H₂S, which can release H₂S under specific conditions, such as reducing conditions (Ubuka, 2002; Modis et al., 2013). Therefore, sulfhydrated drugs, could be regarded as H₂S prodrugs, which promising medical use for the treatment and prophylaxis of cardiac and vascular diseases. These storage forms could affect the level of H₂S detection and tell us how much H₂S is stored, but not the activity of the enzymes. Thus, measurement of these forms of H₂S stores would be useful to measure and understand H₂S generation in the heart.

It is possible that low enzyme activity was due to the delay in the time taken for tissues to come from the abattoir. Indeed, enzyme activity in rat tissues which were frozen immediately after death, was higher than porcine tissues. Although we found that rapidly frozen sample also had low H₂S synthesis level, it may be useful to measure CBS and MST activity by the robust SF7-AM assay in rapidly collected and frozen tissue on dry ice if collected soon after slaughter of the pig, to decrease any possible tissue deterioration and optimise tissue quality.

We found that H₂S production and in the pig and rat heart mitochondrial fraction was higher than cytosol, because there was a decent MST activity in the mitochondrial fraction. The possible explanation that MST could be abundant in the mitochondria compared to cytosol, or that the delay in tissue storage and transport may lead to tissue hypoxia/stress and migration of CBS and CSE to the mitochondria, although CBS was only detected in the pig heart cytosol. Therefore, more investigation about CBS and CSE expression in the mitochondrial fraction of pig, rat and human hearts could explain the low H₂S production in pig heart cytosol compared to rat liver and biological role of H₂S in the heart. Moreover, it has been reported that H₂S is an oxygen sensor and that hypoxia could lead to an increase of enzyme expression and activity of H₂S-synthesising enzyme/s to protect the tissue from hypoxia. Therefore, the investigation of whether the expression and activity levels of MST and CBS/CSE are altered under stress circumstances, such as hypoxia may be helpful to elucidate the biological role of H₂S-synthesising enzymes and H₂S production under various

circumstances, such as hypoxia and oxidative stress in the PCA, human coronary artery, and heart (Fu et al., 2012; Teng et al., 2013). Moreover, extension of this investigation CBS, CSE and MST translocation from the cytosol to mitochondria at the cellular level in cells, such as H9c2 rat cardiomyocytes and rat, porcine, human vascular smooth muscle cells, which are important part of blood vessels and play important role in control of vascular tone, blood pressure and blood flow, may help to understand this phenomenon at the cellular level in the heart and vasculature (Wu et al., 2015; Yang et al., 2017).

To my knowledge, the detection of which H₂S synthesising enzymes (CBS, CSE, and MST) are present in human heart has not looked yet. The presence of CBS and CSE was detected in human tissues, such as liver, brain, aorta, internal mammary artery, mesenteric artery, umbilical vein, lung, eye, joint, colon, ovary and uterus has been demonstrated (Webb et al., 2008; Whiteman et al., 2011; Yang et al., 2011; Wang, 2012; di Villa Bianca et al., 2015; Leucker et al., 2017; Materazzi et al., 2017). Less extent of MST was reported in human brain and red blood cells (Whiteman et al., 2011). Therefore, future studies about the responsible enzymes for H₂S generation in human heart are needed in order to confirm the roles of H₂S pathways in the physiology and treatment of cardiovascular diseases in humans.

In the results of present study CSE was not detected in the pig heart. It has been proposed that the use of L-cysteine compared to homocysteine in combination with L-cysteine might underestimate CBS activity and bias H₂S production towards CSE (Singh et al., 2009; Kabil et al., 2011). They proposed that CBS is more efficient to produce H₂S from a combination of L-cysteine with homocysteine than L-cysteine alone, but this would not be a problem in the present study because CSE was not detected in pig heart. CBS is also responsible for homocysteine detoxification. Therefore, it would be worth looking at different substrates, such as homocysteine using the robust SF7-AM assay to determine whether homocysteine could lead to an increase of CBS enzyme activity.

Moreover, it could be useful to assess positive controls, such as the recombinant enzymes (Asimakopoulou et al., 2013) or CBS-transfected cell-lines, such as H9c2 rat cardiomyocytes (Szabo and Hellmich, 2013; Szabo et al., 2013) which have high CBS expression, which may be a useful tool to investigate and evaluate CBS activity and effect of different CBS inhibitors. This could help to evaluate the effect of some factors, such as SAM on CBS activity. The results of the present study found that there was no significant effect of SAM on CBS activity. Also, in our results there was no selective inhibition by AOAA and PPG. Thus, the development of selective CBS inhibitors is important to determine and confirm the role of CBS.

MST needs alkaline pH and a reducing agent, such as DTT *in vitro* and thioredoxin and dihydrolipoic acid (DHLA) *in vivo* to produce H₂S in the presence of α-ketoglutarate (Kimura, 2014). Therefore, it could be useful to compare the results of DTT versus the proposed endogenous reducing agents thioredoxin and DHLA. These could increase the activity of MST. Aspartic acid (AA, aspartate) was used a potential inhibitor of CAT activity, which could help to determine the contribution of CAT/MST pathway in H₂S production (Shibuya et al., 2009; Donovan et al., 2017; Donovan et al., 2018). Our data show that AA produced no significant effect on L-cysteine-derived H₂S production. Therefore, it may be helpful to investigate the effects of our new candidate MST inhibitor 1-(3,4-Dihydroxyphenyl)-2-[(4-hydroxy-6-methyl-2-pyrimidinyl)sulfanyl] ethanone on MST activity.

Intriguingly, at pH 9 there was an increase in H₂S detection which may be due to an increase in H₂S stability and detectability. Moreover, H₂S loss/volatilisation is higher at physiological or acidic pH. Therefore, the preparation of heart samples at alkaline pH, such as pH 9 is recommended to enhance H₂S detection.

Next step, was to use a recent and sensitive assay to measure enzyme activity by SF7-AM assay. By using this assay there was an increase in H₂S detection level in the heart, but it was still less than the liver. An increase of pH to pH 9 led to an increase in H₂S detection via CBS enzyme activity only. At the same time, there was no significant difference with the MST enzyme activity. This might be due to instability or decomposition of SF7-AM with mercaptopyruvate at high pH, or that SF7-AM with L-cysteine might be more stable at high pH.

Dialysis of samples of the heart led to an increase in the activity of the CBS and MST enzymes in both males and females, and this observation might be due to the removal of endogenous inhibitor, which might interfere with SF7 probe assay. Therefore, further experiments about dialysis of samples with the methylene blue assay could help to determine whether endogenous inhibitor interferes with H₂S or SF7-AM, which could affect H₂S detection.

PPG and AOAA have non-selective actions. Thus, there is no selective and potent inhibitor for CBS, or CSE. This dilemma of the absence of selective and potent inhibitors for CBS, CSE, impedes the detailed understanding of specific role and participation of each H₂S-synthesising enzyme in H₂S production. Therefore, the discovery and development of new candidate of CBS, CSE and MST inhibitors are important to confirm or exclude the role of each one of these enzymes in H₂S production. Phenylpyruvic acid was not useful as MST inhibitor due to non-specific and off-target effects. Therefore, it would be useful to investigate the effect of our new candidate MST inhibitor on MST activity in the heart.

It may be useful to compare H₂S measurement using SF7-AM assay with the recent methods, such as monobromobimane (MBB)-fluorimetric assay combined with HPLC, which has some advantages, such as it is sensitive to low-oxygen level and also it has a detection limit about 5 nM higher than PHSS (polarographic H₂S sensor) (Olson et al., 2014; Shen et al., 2015).

Regarding the use of rat tissues, many studies have validated their use in studying H₂S effect on vascular tone, blood pressure and flow. Moreover, rat genetic, behavioural and biological properties are similar to human. Investigation of the presence of this phenomenon in human tissue could be important to understand H₂S role in control of vascular tone during hypoxia in human tissues, which may lead to possible translation of H₂S potential use in the treatment of cardiovascular diseases, such as ischaemia in humans.

Genetic manipulation of H₂S synthesising enzymes by knock-out of CBS, CSE and MST, although it is a costly experimental approach, would be an alternative approach to confirm the contribution of each of H₂S-synthesising enzyme in H₂S biosynthesis. Moreover, genetic manipulation could be useful for studying of H₂S-based drugs, such as H₂S sources application, because H₂S sources may lead to improvement of symptoms depending on the knocked out enzyme. For example, in animal with CSE knocked out there is hypertension. Therefore, use of H₂S sources, such as Na₂S leads to improvement of hypertension and restoring normal blood pressure. CBS knock out leads to death due to cardiac issues. Therefore, it could be useful to investigate the effect of MST knockout on vascular tone and H₂S production in the heart.

Although our results demonstrated that there was no significant effect for the gender on H₂S production using both the methylene blue and SF7-AM assays in pigs, it may be useful to reappraise gender difference effect in humans because H₂S levels are reported to be affected by gender in rats and mice and the case could be similar in humans. In CSE-deficient mice, hyperhomocysteinemia was about 9 fold higher in females than males (Yang et al., 2008). H₂S levels in human plasma was higher in males compared to females (Brancaleone et al., 2015). Therefore, further studies devoted to H₂S-related gender difference, in human hearts and the effect of testosterone and oestrogen on H₂S production may need investigation and evaluation to illustrate the regulation of H₂S synthesis in the cardiovascular system.

In the absence of PLP, there would be no conversion of L-cysteine to H₂S via CBS/CSE and mercaptopyruvate via CAT. Therefore, in situations associated with low or restriction vitamin B6 and L-cysteine nutrition. This would lead to decrease of endogenous H₂S production via these enzymes in tissues and these situations were reported to be associated with increase of cardiovascular pathologies (DeRatt et al., 2014; Mys et al., 2017). Thus, supplementation of PLP (vitamin B6 derivative) and L-cysteine in the wider context leads to stimulation of the endogenous H₂S production via CBS and CSE as well as CAT, and cardiovascular protective effects. Therefore, the development and refining of H₂S detection methods would be useful for the diagnosis of diseases associated with disturbance of H₂S levels and monitoring of H₂S levels during treatment of CVD.

5.2. Development of an Assay to Measure Sulfhydration in the Pig Heart

Sulfhydration of proteins and ion channels has been reported as a potential signalling pathway for H₂S. Therefore, in this chapter, we tried to develop a reliable assay to measure the sulfhydration level, which could be implicated as an index for H₂S production and potential signalling pathway for H₂S in the pig heart. In the present study, sulfhydration of proteins, such as GAPDH and MEK1 was measured in the heart, PCA and liver. Therefore, sulfhydration could be an important signalling pathway for H₂S in the heart. Thus, sulfhydrated proteins (persulfides, reductant-labile) can be considered as endogenous stores of H₂S, which can release H₂S under specific conditions, such as reducing conditions (Ubuka, 2002; Modis et al., 2013). Therefore, sulfhydrated drugs, could be regarded as H₂S prodrugs, which have an promising medical use in the treatment of cardiovascular diseases (CVD).

In the biotin switch assay, there was an increase of sulfhydration level of GAPDH and MEK1 bands, but the results were variable. These results may be due to the prolonged procedure and multiple steps of precipitations which may lead to H₂S loss and then decrease in the detected sulfhydration.

Next, we moved to test a recent technique called the red maleimide assay to measure sulfhydration. In the results of red maleimide assay, there was a decrease of the fluorescence in the presence of Na₂S and presence of DTT of GAPDH and MEK1 protein. However, whether these detected sulfhydrated bands corresponded to GAPDH and MEK1 is difficult to judge, and this observation may indicate the sulfhydration of several proteins by H₂S in the heart. The red maleimide assay is a simple technique, and the results were less variable and promising in comparison to the biotin switch assay.

Interestingly, we observed the shifting of GAPDH and MEK1 bands to a lower MW with the presence of double bands in the presence of Na₂S and absence of DTT. The same observation was also detected by the immunoprecipitation and Western blotting. The possible explanation of this observation might be due to changes in the protein structure in the presence of Na₂S and absence of DTT (non-reducing condition) with sulfhydration causing a shift in the MW that the protein runs at. A similar effect is seen with phosphorylation.

Therefore, all these results indicate the presence of sulfhydration in the heart, in particular, the presence of low MW bands by the immunoprecipitation and Western blotting method, which is a simple technique and its results were even comparable to the red maleimide assay results. The presence of the shift in the apparent MW

may play an important role in the sulfhydration of protein, which may change the protein structure and biological function. Application of mass spectrometry to look at the shift in MW might be helpful to determine whether this shift in MW is due to sulfhydration.

Using the shift in MW as a simpler assay could be explored further in the PCA, human coronary artery, and human coronary artery smooth muscle cells, and rat cardiomyocytes H9c2 cells because in our study H₂S production was detected in rat heart and sulfhydration might be an index of H₂S production. These experiments may illustrate the sulfhydration biological effects, such as sulfhydration of calcium channels as a potential signalling of H₂S in the heart and vasculature, and at the cellular level. Therefore, the development of a reliable assay for the measurement of the sulfhydration is crucial to understanding H₂S signalling and biological effects of H₂S in humans. Using the red maleimide assay/shift in MW to investigate the sulfhydration level of mitochondrial proteins, such as Bcl-XI and Bad of human cardiomyocytes, heart tissue and blood vessels with H₂S donors, in particular AP39 could be useful to demonstrate the signalling pathway/s of H₂S in the heart and blood vessels.

Mass spectrometry is reported to be a useful tool for studying PTMs of proteins by determination of which protein is modified and which cysteine residues are modified, such as presence of sulfhydrated GAPDH, which is sulfhydrated at cysteine residue 150. Therefore, mass spectrometry could characterise sulfhydration of proteins and their sulfhydration locations (Aracena-Parks et al., 2006; Mustafa et al., 2009; Park et al., 2015; Longen et al., 2016). Therefore, application of mass spectrometry analysis to look at the shift in MW might be useful to determine that this shift in MW is due to sulfhydration. Thus, it may be helpful to subject the red maleimide gel, or biotin labelled protein gel to mass spectrometry analysis, which may reveal more details about the sulfhydrated proteins at the molecular level (Meng et al., 2017). Then, depending on the proteomic database, the sulfhydrated proteins can be characterised, and particular targeted sulfhydrated cysteine locations can be identified (Park et al. 2015; Meng et al., 2017). Therefore, this study could provide more molecular insight into the sulfhydration in biological systems (Longen et al., 2016).

The change in the observed MW in proteins run under non-reducing conditions in the red maleimide assay is potentially due to sulfhydration, which could be proved by the mass spectrometry. First, it is important to confirm that the protein is sulfhydrated using presence of low MW bands, immunoprecipitation with immunoblotting, and the red maleimide assay under the presence of H₂S source and non-reducing conditions.

In the proteomic analysis, the identification of protein is the principal goal. We can use the tryptic digestion in order to digest the protein into peptides and then the peptides with persulfides are eluted with tris(2-carboxyethyl)phosphine. Next the persulfides containing peptides are analysable using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and this method is simple and sensitive and it is especially useful for low visibility in-gel proteins with Coomassie brilliant stain (Aracena-Parks et al., 2006; Huynh et al., 2009; Mustafa et al., 2009; Longen et al., 2016). Alternatively, we could do immunoprecipitation for the protein of interest and then immunoblotting and next cut the band and subject it to mass spectrometry.

There were several sulfhydrated proteins detected by the red maleimide assay. In our results, there were some bands observed with the molecular weight correlated to about 90 kDa other than GAPDH and MEK1. Therefore, it may be useful to detect other presumably sulfhydrated proteins, which were reported by many studies such as heat-shock protein90 (HSP90) at ~90 kDa (Mustafa et al., 2009; Meng et al., 2017). Thus, it might be helpful to detect this presumably sulfhydrated protein in PCA, PHH and human heart and blood vessels.

A tag-switch is suggested to have relatively higher selectivity and sensitivity than the biotin switch assay, because in this assay, both sulfhydrated and non-sulfhydrated thiols are blocked by IAA (iodoacetic acid). Then, the sulfhydrated thiols are reduced by DTT and then labelled with ILB (iodoacetamide linked biotin) and pulled down by streptavidin and then visualised by immunoblotting (Zhang et al., 2014). Therefore, it may be useful to investigate this method in the heart and PCA to enhance the sulfhydration role in cardiovascular functions. Thus, development of assay for measuring sulfhydration level would be useful for the monitoring H₂S levels during the cardiovascular diseases.

5.3. Comparison of Signalling Pathways of Hydrogen Sulfide Sources (Na₂S, GYY4137 and AP39) in the Relaxation Response of Porcine Coronary Artery

Most of the literature of H₂S is based on the use of salts as a source of H₂S. These salts release a large amount of H₂S in a short time and this could lead to supra-physiological concentrations. Therefore, there is a question mark about how useful and reliable these data are. Using of three different H₂S sources, which release H₂S by different ways will shed light on whether there are differences in the relaxation response and the mechanism of relaxation.

Therefore, the key aims of this chapter were to determine whether there are any differences in the relaxation responses and mechanisms of relaxation among the three different types of H₂S sources. In this chapter, we compared the effects of the three different H₂S sources on the relaxation response in PCA. The results of the present study demonstrated there was no difference in the relaxation responses though the use of three different H₂S sources, Na₂S, GYY4137 and AP39.

The results of this chapter showed that there was a significant enhancement of the relaxation responses of all the three different H₂S sources in the presence of L-NAME, TEA and glibenclamide. The results of L-NAME could suggest chemical interaction of H₂S with NO led to formation of inactive nitrosothiol. This result was in agreement with many studies, such as Ali et al., (2006) and Whiteman et al., (2006). According to Whiteman's study, the interaction of H₂S with NO leads to formation of inactive nitrosothiol molecule, which does not relax or contract blood vessels. This result is also reported by Ali's study. This observation could be due to physiological antagonism between H₂S and NO via nitrosothiol formation. Therefore, in this case NO is reducing the H₂S relaxant effect.

Some studies in the literature reported the involvement of potassium channels, in particular, K_{ATP} channels in the H₂S-mediated relaxation. In contrast, our results with TEA and glibenclamide demonstrated non-involvement of potassium channels, in particular, K_{ATP} channels in the H₂S-mediated relaxation responses in U46619-precontracted PCA. Interestingly, the blocking of potassium channels by TEA and K_{ATP} channels by glibenclamide led to an enhancement of the H₂S-mediated relaxation, and these results are in contrast with many previous studies that have been reported that H₂S relaxation was mediated via potassium channels, such as K_{ATP} channels as in the study of Zhao et al (2001). Therefore, in order to address why there is an enhanced relaxation in the presence of K⁺ channel inhibitors, the effects of K⁺ channel inhibitors on calcium-induced contractions and Bay K8644-induced contractions could be investigated further. Moreover, the effect of K⁺ channel inhibitors on angiotensin

converting enzyme (ACE) and angiotensin II activity and expression could be useful. These experiments could be helpful to clarify why the use of K⁺ channels inhibitors led to enhancing the relaxation response of the three different H₂S sources and also it could suggest alternative mechanism/s for the relaxation response of H₂S, because several studies demonstrated that H₂S could block angiotensin II activity and effects, but whether this effect is increased by K⁺ channels inhibitors needs to be clarified.

Therefore, alternative pathways for H₂S effects as a vasorelaxant could merit further exploration, because it has reported by many studies that H₂S sources, such as NaHS decreased ACE and angiotensin II activity and effect (Laggner et al., 2007; Zhao et al., 2008; Liu et al., 2014; Snijder et al., 2014; Al-Magableh et al., 2015; Snijder et al., 2015). Therefore, determination of the effects of new generation H₂S sources GYY4137, and AP39 on ACE and angiotensin II expression level and activity, and angiotensin II type1 receptor expression in PCA, human tissues, such as human cardiac tissue and mesenteric arteries (Materazzi et al., 2017) by techniques, such as using immunoblotting and ELISA (enzyme-linked immunosorbent assay) could be useful to demonstrate the effects of H₂S on vascular tone and cardiac function.

There was significant inhibition of calcium-induced contractions by all the three different H₂S sources, and these effects were maintained after 15, 30 and 60 min periods of incubation of PCA with all the three different H₂S sources, even though levels of H₂S in the buffer are likely to have dropped. This observation may be attributed to the sulfhydrylation of calcium channels, which resulted in extension of H₂S-mediated relaxation in PCA. Moreover, there was a significant inhibition of Bay K8644-precontracted PCA by all three different H₂S sources, and thus, these results suggest that H₂S acts by blocking calcium channels in agreement with the results of Zhao and Wang, (2002), Sun et al. (2008) and Zhang et al. (2012). Therefore, it would be important to determine whether calcium channels are sulfhydrated using red maleimide assay which is simple assay in PCA and human vascular smooth muscle tissue/cells. It may be useful to extend this investigation to human tissues, such as internal mammary artery (Webb et al., 2008) and mesenteric arteries (Materazzi et al., 2017) in order to determine whether H₂S could also block calcium channels via sulfhydrylation in human tissue. This would help to understand the possible translation of H₂S drugs (H₂S donors) in the treatment of cardiovascular diseases in human.

Electrophysiological studies, such as patch-clamp technique could help to determine the effects of the new generation H₂S sources, such as GYY4137 and AP39 on calcium channels currents measurements in human cardiomyocytes and vascular smooth muscle cells. Moreover, monitoring the change in intracellular calcium concentration using Fluo-4 imaging techniques. These techniques would be important to illustrate the effects of H₂S on the calcium level in the cardiovascular system at the cellular level.

Our results showed that the presence of 1 μM FCCP led to no significant effects on the response to 5 μM AP39. Therefore, in order to demonstrate the role of mitochondria in vascular tone in response to H₂S, it might be useful to repeat these experiments with a combination of different known inhibitors of the electron transport chain of the mitochondria (10 μM rotenone which inhibits complex I, 10 μM antimycin which inhibits complex III, 10 μM oligomycin which inhibits complex V of the electron transport chain) to investigate whether there is an effect for the full inhibition of the electron transport chain on the relaxation responses to AP39 in PCA. Thus, this experiment may demonstrate the role of mitochondria in vascular tone in response to H₂S.

The effect of Na₂S was decreased by the use of low oxygen level, and this might be because decrease of oxygen level may result in an increase H₂S availability with Na₂S, which releases a large amount of H₂S during a short time. This would lead to a very high H₂S level and result in decrease of the relaxation response to Na₂S. In the results of GYY4137, there was no significant difference. In contrast, the effects of AP39 were enhanced by the using of low oxygen level. This may be because the decrease of oxygen level may result in an increase H₂S availability in the PCA mitochondria and decrease mitochondrial ROS levels to physiological levels. This could enhance the relaxant effects to AP39 due to release of H₂S in the mitochondria. So the time frame of H₂S release is important.

The three H₂S sources caused some increase in the hypoxia relaxation response and decrease in the recovery response. Decreasing size of the recovery response might be beneficial to decrease the possible excessive and uncontrolled contractions induced by reoxygenation, which might lead to IRI, which is likely due to increase of ROS and calcium overload. Decrease size of the recovery response may be due to a decrease in ROS generation and excessive calcium accumulation, and maintenance of the mitochondrial function and structure integrity. These results could be similar or mimic the protective effects of H₂S against ischaemia and reperfusion injury at least in part. Therefore, H₂S sources may be considered as preconditioning agent against ischaemia reperfusion injury. This effect may be tested by application of the three H₂S sources before the reoxygenation of PCA.

It has been reported that H₂S may be toxic at high concentrations. Therefore, it might be helpful to determine at which concentrations the effects of GYY4137 and AP39 would be toxic on cell-lines, such as human cardiomyocytes and vascular smooth muscle cells. H₂S levels could be measured using the SF7-AM assay in a time-scale pattern and it may be useful to measure cell viability using the MTT assay (Szczesny et al., 2014). The use of human cells would help to understand the possible translation of H₂S drugs in the treatment of cardiovascular diseases in humans. In short, in the heart and vasculature, there is accumulating evidence about the potential contribution of H₂S pathways in the physiology, pathology and treatment of CVD. Overall, the application of exogenous H₂S via H₂S drugs and stimulation of endogenous H₂S production via L-cysteine and vitamin B6 in the wider context leads to cardiovascular protective effects and these effects emphasise the druggability of H₂S in the cardiac/vascular physiology and treatment of many CVD.

Conclusion

The development of an assay to measure CBS and MST enzyme activity in the heart, such as the SF7-AM assay is useful to understand H₂S production level, regulation and monitoring changes in H₂S level in the heart. H₂S production in the heart leads to relaxation of coronary artery, suggesting that the cardiac tissue contributes to control of vascular tone in the coronary artery in a paracrine manner.

The development of an assay to measure sulfhydrylation in the heart, such as the red maleimide assay and immunoprecipitation/immunoblotting are useful to measure sulfhydrylation, which could be considered as an index of H₂S production and potential signalling pathway for H₂S in the heart. This approach could help to understand mechanism of relaxation possibly via sulfhydrylation of calcium channels in the heart and coronary artery.

Low H₂S production has been associated with many cardiovascular pathologies. Thus, application of H₂S sources leads to a decrease in IRI in coronary artery via relaxation through calcium blockade. Therefore, the use of H₂S drugs (sources) could be used in ischaemic heart diseases, which considered as one of the common causes of death.

H₂S biology appears to have complicated and conflicting aspects, which merit further investigation. Most of the literature reports have depended on the use of inorganic H₂S salts, which highlight many disadvantages, controversies and questions about the credibility of H₂S influences, consistency and mechanism of action in the field. Although, some reports which suggested H₂S salts-mediated relaxation responses and mechanisms of relaxation may need re-evaluation with the new generation H₂S sources, such as GYY4137 and AP39, the results of present study demonstrated that there was no difference in the relaxation responses and mechanisms of relaxation among the three different sources of H₂S, Na₂S, GYY4137 and AP39.

Most H₂S studies were carried out in animal models and *in vitro*, which may not imitate the human environment. Therefore, there would be inevitable obstacles, such as the physiological and toxic concentrations, and the active form of H₂S, which may hamper the translatability of H₂S donors (drugs) to the clinic.

The development of methods for measuring of H₂S-synthesising enzyme activity, H₂S levels and H₂S-mediated signalling pathways and then, application of these methods on human tissues are important to understand the biological role of H₂S and translation of H₂S-releasing drugs to the clinical field. Intriguingly, suspended animation (hibernation-like) induced by H₂S in mice if applicable in human tissues

could help during transport of organs and buying extra-time during tissue injury and surgery as well.

Interestingly, the comparison of H₂S-releasing drugs with the classical ones suggests promising preclinical results and may provide an excellent opportunity for drug candidate discovery or as additional/alternative and prophylactic medicine based on H₂S, especially in the cardiovascular system.

Many studies have suggested that the administration of moderate amounts of natural food-derived H₂S sources, such as garlic derivatives is healthy and improves cardiovascular diseases. Therefore, in the heart and vasculature, there is accumulating evidence about the potential contribution of H₂S in the pathology and treatment of cardiovascular diseases. Finally, the heart is an important source of H₂S, and we still learning from H₂S. Hopefully, H₂S donors (drugs) could follow a similar historical and therapeutic path (fate) to NO donors (nitrates). Therefore, today research about H₂S biological profile may translate to drug tomorrow.

References

- Abe, K. and Kimura, H. (1996). The possible role of hydrogen sulfide as an endogenous neuromodulator. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 16(3), 1066–1071. <https://doi.org/10.1523/JNEUROSCI.16-03-01066.1996>.
- Agrawal, N., and Banerjee, R. (2008). Human polycomb 2 protein is a SUMO E3 ligase and alleviates substrate-induced inhibition of cystathionine β -synthase sumoylation. *PLoS ONE*, 3(12), 1–7. <https://doi.org/10.1371/journal.pone.0004032>.
- Ahmad, A., Olah, G., Szczesny, B., Wood, M., Whiteman, M., and Szabo, C. (2016). AP39, A Mitochondrially Targeted Hydrogen Sulfide Donor, Exerts Protective Effects in Renal Epithelial Cells Subjected to Oxidative Stress in Vitro and in Acute Renal Injury in Vivo. *Shock*, 45(1), 88–97. <https://doi.org/10.1097/SHK.000000000000047>.
- Ahmad, A., Sattar, M., Rathore, H., Khan, S., Lazhari, M., Afzal, S., Hashmi, F., Abdullah, N. and Johns, E. (2015). A critical review of pharmacological significance of hydrogen sulfide in hypertension. *Indian Journal of Pharmacology*, 47(3), 243–247. <https://doi.org/10.4103/0253-7613.157106>.
- Ali, M., Ping, C., Mok, Y., Ling, L., Whiteman, M., Bhatia, M., and Moore, P. (2006). Regulation of vascular nitric oxide in vitro and in vivo: a new role for endogenous hydrogen sulphide? *British Journal of Pharmacology*, 149(6), 625–634. <https://doi.org/10.1038/sj.bjp.0706906>.
- Al-Magableh, M., and Hart, J. (2011). Mechanism of vasorelaxation and role of endogenous hydrogen sulfide production in mouse aorta. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 383(4), 403–413. <https://doi.org/10.1007/s00210-011-0608-z>.
- Al-Magableh, M., Kemp-Harper, B., and Hart, J. (2015). Hydrogen sulfide treatment reduces blood pressure and oxidative stress in angiotensin II-induced hypertensive mice. *Hypertension Research*, 38(1), 13–20. <https://doi.org/10.1038/hr.2014.125>.
- Almukhtar, H., Garle, M., Smith, P., and Roberts, R. (2016). Effect of simvastatin on vascular tone in porcine coronary artery: Potential role of the mitochondria. *Toxicology and Applied Pharmacology*, 305, 176–185. <https://doi.org/10.1016/j.taap.2016.06.024>.
- Al-Taie, Y. (2011). The effects of captopril and amlodipine on the blood pressure and some renal functions tests in hypertensive patients. Master thesis in Clinical Pharmacology, the University of Mosul, Mosul, Iraq.
- Al-Taie, Y., and Al-Youzbaki, W. (2013). Serum uric acid level and renal function tests in hypertensive patients treated by captopril. *Iraqi Journal of Pharmacology*, 13 (2), 1–10.
- Andreadou, I., Iliodromitis, E., Rassaf, T., Schulz, R., Papapetropoulos, A., and Ferdinandy, P. (2015). The Role of Gasotransmitters NO, H₂S, CO in myocardial ischaemia/reperfusion injury and cardioprotection by preconditioning, postconditioning, and remote conditioning. *British Journal of Pharmacology* (6). 172 (6), 1587–1606. <http://dx.doi.org/10.1111/bph.2015.172.issue-6>.
- Ang, A., Konigstorfer, A., Giles, G., and Bhatia, M. (2012). Measuring free tissue sulfide. *Advances in Biological Chemistry*, 2012(2), 360–365. <https://doi.org/10.4236/abc.2012.24044>.
- Aracena-Parks, P., Goonasekera, S., Gilman, C., Dirksen, R., Hidalgo, C., and Hamilton, S. (2006). Identification of cysteines involved in S-nitrosylation, S-glutathionylation, and oxidation to disulfides in ryanodine receptor type 1. *Journal of Biological Chemistry*, 281(52), 40354–40368. <https://doi.org/10.1074/jbc.M600876200>.

- Asimakopoulou, A., Panopoulos, P., Chasapis, C., Coletta, C., Zhou, Z., Cirino, G., and Papapetropoulos, A. (2013). The selectivity of commonly used pharmacological inhibitors for cystathionine β synthase (CBS) and cystathionine γ -lyase (CSE). *British Journal of Pharmacology*, 169(4), 922–932. <https://doi.org/10.1111/bph.12171>.
- Avanzato, D., Merlino, A., Porrera, S., Wang, R., Munaron, L., and Mancardi, D. (2014). Role of calcium channels in the protective effect of hydrogen sulfide in rat cardiomyoblasts. *Cellular Physiology and Biochemistry*, 33(4), 1205–1214. <https://doi.org/10.1159/000358690>.
- Bae, S., Heo, C., Choi, D., Sen, D., Joe, E., Cho, B., and Kim, H. (2013). A ratiometric two-photon fluorescent probe reveals reduction in mitochondrial H₂S production in Parkinson's disease gene knockout astrocytes. *Journal of the American Chemical Society*, 135(26), 9915–9923. <https://doi.org/10.1021/ja404004v>.
- Banerjee, R., and Zou, C. (2005). Redox regulation and reaction mechanism of human cystathionine β -synthase: a PLP-dependent heme-sensor protein. *Archives of Biochemistry and Biophysics*, 433(1), 144–156. <https://doi.org/10.1016/j.abb.2004.08.037>.
- Banerjee, R., Evande, R., Kabil, O, Ojha, S., and Taoka, S. (2003). Reaction mechanism and regulation of cystathionine β -synthase. *Biochimica et Biophysica Acta*, 1647(1–2), 30–35. [https://doi.org/10.1016/S1570-9639\(03\)00044-X](https://doi.org/10.1016/S1570-9639(03)00044-X).
- Bartholomew, T., Powell, G., Dodgson, K., and Curtis, C. (1980). Oxidation of sodium sulphide by rat liver, lungs and kidney. *Biochemical Pharmacology*, 29(18), 2431–2437. [https://doi.org/10.1016/0006-2952\(80\)90346-9](https://doi.org/10.1016/0006-2952(80)90346-9).
- Baumgart, K., Wagner, F., Gröger, M., Weber, S., Barth, E., Vogt, J., Wachter, U., Huber-Lang, M., Knöferl, M., Albuszies, G., Georgieff, M., Asfar, P., Szabó, C., Calzia, E., Radermacher, P., and Simkova, V. (2010). Cardiac and metabolic effects of hypothermia and inhaled hydrogen sulfide in anesthetized and ventilated mice. *Critical Care Medicine*, 38(2):588-595. <https://doi.org/10.1097/ccm.0b013e3181b9ed2e>.
- Bazhanov, N., Escaffre, O., Freiberg, A., Garofalo, R., and Casola, A. (2017). Broad-range antiviral activity of hydrogen sulfide against highly pathogenic RNA viruses. *Scientific Reports*, 7:41029 (2017). <https://doi.org/10.1038/srep41029>.
- Bazhanov, N., Ivanciuc, T., Wu, H., Garofalo, M., Kang, J., Xian, M., and Casola, A. (2018). Thiol-activated hydrogen sulfide donors antiviral and anti-inflammatory activity in respiratory syncytial virus infection. *Viruses*, 10(249), 1-12. <https://doi.org/10.3390/v10050249>.
- Bebarta, V., Brittain, M., Chan, A., Garrett, N., Yoon, D., Burney, T., Mukai, D., Babin, M., Pilz, R., Mahon, S., Brenner, M., and Boss, G. (2017). Sodium nitrite and sodium thiosulfate are effective against acute cyanide poisoning when administered by intramuscular injection. *Annals of Emergency Medicine*, 69(6):718–725.e4. <https://doi.org/10.1016/j.annemergmed.2016.09.034>.
- Beltowski, J. (2015). Hydrogen sulfide in pharmacology and medicine- An update. *Pharmacological Reports*, 67(3), 647–658. <https://doi.org/10.1016/j.pharep.2015.01.005>.
- Beltrao, P., Bork, P., Krogan, N., and Van Noort, V. (2013). Evolution and functional cross-talk of protein post-translational modifications. *Molecular Systems Biology*, 9(1), 1–13. <https://doi.org/10.1002/msb.201304521>.

- Benavides, G., Squadrito, G., Mills, R., Patel, H., Isbell, T., Patel, R., and Kraus, D. (2007). Hydrogen sulfide mediates the vasoactivity of garlic. *Proceedings of the National Academy of Sciences (PNAS)*, 104(46), 17977–17982. <https://doi.org/10.1073/pnas.0705710104>.
- Berne, R. (1980). The role of adenosine in the regulation of coronary blood flow in newborn lambs. *Circulation Research*, 47(6), 807–813. <https://doi.org/10.1161/01.RES.47.6.807>.
- Bhattacharyya, S., Saha, S., Giri, K., Lanza, I., Nair, K., Jennings, N., and Mukherjee, P. (2013). Cystathionine β -Synthase (CBS) Contributes to Advanced Ovarian Cancer Progression and Drug Resistance. *PLoS ONE*, 8(11), 1–12. <https://doi.org/10.1371/journal.pone.0079167>.
- Bian, J., Yong, Q., Pan, T., Feng, Z., Ali, M., Zhou S. and Moore, P. (2006). Role of hydrogen sulfide in the cardioprotection caused by ischemic preconditioning in the rat heart and cardiac myocytes. *The Journal of Pharmacology and Experimental Therapeutics*, 316(2), 670–678. <https://doi.org/10.1124/jpet.105.092023>.
- Blackstone, E., and Roth, M. (2007). Suspended animation-like state protects mice from lethal hypoxia. *Shock*, 27(4), 370–372. <https://doi.org/10.1097/SHK.0b013e31802e27a0>.
- Blackstone, E., Morrison, M., and Roth, M. (2005). H₂S induces a suspended animation-like state in mice. *Science*, 308(5721), 518. <https://doi.org/10.1126/science.1108581>.
- Boehning, D., Patterson, R., Sedaghat, L., Glebova, N., Kurosaki, T., and Snyder, S. (2003). Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis. *Nature Cell Biology*, 5(12), 1051–1061. <https://doi.org/10.1038/ncb1063>.
- Brancaleone, V., Vellecco, V., Matassa, D., Di Villa Bianca, R., Sorrentino, R., Ianaro, A., and Cirino, G. (2015). The crucial role of androgen receptor in vascular H₂S biosynthesis induced by testosterone. *British Journal of Pharmacology*, 172(6), 1505–1515. <https://doi.org/10.1111/bph.12740>.
- Bucci, M., Mirone, V., Di Lorenzo, A., Vellecco, V., Roviezzo, F., Brancaleone, V., and Cirino, G. (2009). Hydrogen Sulphide Is Involved in Testosterone Vascular Effect. *European Urology*, 56(2), 378–384. <https://doi.org/10.1016/j.eururo.2008.05.014>.
- Bucci, M., Papapetropoulos, A., Vellecco, V., Zhou, Z., Pyriochou, A., Roussos, C., and Cirino, G. (2010). Hydrogen sulfide is an endogenous inhibitor of phosphodiesterase activity. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 30(10), 1998–2004. <https://doi.org/10.1161/ATVBAHA.110.209783>.
- Bucci, M., Papapetropoulos, A., Vellecco, V., Zhou, Z., and Zaid, A. (2012). cGMP-Dependent Protein Kinase Contributes to Hydrogen Sulfide-Stimulated Vasorelaxation. *PLoS ONE* 7(12), 1–10. <https://doi.org/10.1371/journal.pone.0053319>.
- Bucci, M., Vellecco, V., Cantalupo, A., Brancaleone, V., Zhou, Z., and Evangelista, S. (2014). Hydrogen sulfide accounts for the peripheral vascular effects of zofenopril independently of ACE inhibition. *Cardiovascular Research*, 102(1), 138–147. <https://doi.org/10.1093/cvr/cvu026>.
- Burnett, A., Lowenstein, C., Brecht, D., Chang, T., and Snyder, S. (1992). Nitric oxide: A Physiologic Mediator of Penile Erection. *Science*, 257(5068), 401–403. <https://doi.org/10.1126/science.1378650>.
- Cai, W, Wang, M., Moore, P., Jin, H., Yao, T., and Zhu, Y. (2007). The Novel Proangiogenic Effect of Hydrogen Sulfide Is Dependent on Akt Phosphorylation. *Cardiovascular Research*. 76 (1). 29–40. <https://doi.org/10.1016/j.cardiores.2007.05.026>.

- Calvert, J., Elston, M., Nicholson, C., Gundewar, S., Jha, S., Elrod, J., and Lefer, D. (2010). Genetic and pharmacologic hydrogen sulfide therapy attenuates ischaemia-induced heart failure in mice. *Circulation*, 122(1), 11–19. <https://doi.org/10.1161/CIRCULATIONAHA.109.920991>.
- Calvert, J., Jha, S., Gundewar, S., Elrod, J., Ramachandran, A., Pattillo, C., Kevil, C., and Lefer, D. (2009). Hydrogen Sulphide Mediates Cardioprotection Through Nrf2 Signalling. *Circulation Research*, 105 (4), 365–374. <https://doi.org/10.1161/CIRCRESAHA.109.199919>.
- Candela, J., Velmurugan, G., and White, C. (2016). Hydrogen Sulfide Depletion Contributes to Microvascular Remodeling in Obesity. *American Journal of Physiology-Heart and Circulatory Physiology*, <https://doi.org/10.1152/ajpheart.00062.2016>.
- Cao, X., Ding, L., Xie, Z., Yang, Y., Whiteman, M., Moore, P., and Bian, J. (2019). A Review of Hydrogen Sulfide Synthesis, Metabolism, and Measurement: Is Modulation of Hydrogen Sulfide a Novel Therapeutic for Cancer? *Antioxidants and Redox Signaling*, 31(1), 1–38. <https://doi.org/10.1089/ars.2017.7058>.
- Carretero, O., and Oparil, S. (1998). Essential Hypertension Part I: Definition and Etiology. In: Armstrong, P., and Willerson (editors), J, *Clinical cardiology: New frontiers*. *Circulation*, 97(12), 329–335. <https://doi.org/10.1161/01.CIR.101.3.329>.
- Castro-Piedras, I., and Perez-Zoghbi, J. (2013). Hydrogen sulphide inhibits Ca²⁺ release through InsP3 receptors and relaxes airway smooth muscle. *Journal of Physiology*, 591(23), 5999–6015. <https://doi.org/10.1113/jphysiol.2013.257790>.
- Chan, M. and Wallace, J. (2013). Hydrogen Sulfide-Based Therapeutics and Gastrointestinal Diseases: Translating Physiology to Treatments. *American of Journal Physiology-Gastrointestinal Liver Physiology*, 305 (52), G467–G473. <https://doi.org/10.1152/ajpgi.00169.2013>.
- Cheang, W., Wong, W., Shen, B., Lau, C., Tian, X., Tsang, S., and Huang, Y. (2010). 4-Aminopyridine-sensitive K⁺ channels contribute to NaHS-induced membrane hyperpolarization and relaxation in the rat coronary artery. *Vascular Pharmacology*, 53(3–4), 94–98. <https://doi.org/10.1016/j.vph.2010.04.004>.
- Chen, J., and Mortenson, L. (1977). Inhibition of blue methylene formation during the determination of the acid-labile sulphide of iron-sulfur protein samples containing dithionite. *Analytical Biochemistry*, 79(1-2), 157–165. [https://doi.org/10.1016/0003-2697\(77\)90390-6](https://doi.org/10.1016/0003-2697(77)90390-6).
- Chen, J., Gao, J., Sun, W., Li, L., Wang, Y., Bai, S., and Xu, C. (2016). Involvement of exogenous H₂S in recovery of cardioprotection from ischaemic post-conditioning via increase of autophagy in the aged hearts. *International Journal of Cardiology*, 220, 681–692. <https://doi.org/10.1016/j.ijcard.2016.06.200>.
- Chen, Q., Camara, A., Stowe, D., Hoppel, C., and Lesnefsky, E. (2007). Modulation of electron transport protects cardiac mitochondria and decreases myocardial injury during ischaemia and reperfusion. *American Journal of Physiology-Cell Physiology*, 292(1). <https://doi.org/10.1152/ajpcell.00270.2006>.
- Chen, X., Jhee, K., and Kruger, W. (2004). Production of the neuromodulator H₂S by cystathionine β-synthase via the condensation of cysteine and homocysteine. *Journal of Biological Chemistry*, 279(50), 52082–52086. <https://doi.org/10.1074/jbc.C400481200>.
- Cheng, Y., Ndisang, J., Tang, G., Cao, K., and Wang, R. (2004). Hydrogen sulfide-induced relaxation of resistance mesenteric artery beds of rats. *American Journal of Physiology-Heart and Circulatory Physiology*, 287(5), 2316–2323. <https://doi.org/10.1152/ajpheart.00331.2004>.

- Chitnis, M., Njie-Mbye, Y., Opere, C., Wood, M., Whiteman, M., and Ohia, S. (2013). Pharmacological actions of the slow release hydrogen sulfide donor GYY4137 on phenylephrine-induced tone in isolated bovine ciliary artery. *Experimental Eye Research*, 116, 350–354. <https://doi.org/10.1016/j.exer.2013.10.004>.
- Choi, S., Lim, M., Byeon, S., and Lee, Y. (2016). Inhibition of endoplasmic reticulum stress improves coronary artery function in the spontaneously hypertensive rats. *Scientific Reports*, 6, 1–9. <https://doi.org/10.1038/srep31925>.
- Chuah, S., Moore, P., and Zhu, Y. (2007). S-allylcysteine mediates cardioprotection in an acute myocardial infarction rat model via a hydrogen sulfide-mediated pathway. *American Journal of Physiology-Heart and Circulatory Physiology*, 293(5), H2693–H2701. <https://doi.org/10.1152/ajpheart.00853.2007>.
- Chunyo, Z., Junbao, D., Dingfang, B., Hui, Y., Xiuying, T., and Chaoshu, T. (2003). The regulatory effect of hydrogen sulfide on hypoxic pulmonary hypertension in rats. *Biochemical and Biophysical Research Communications*, 302(4), 810–816. [https://doi.org/10.1016/S0006-291X\(03\)00256-0](https://doi.org/10.1016/S0006-291X(03)00256-0).
- Cindrova-Davies, T., Herrera, E., Niu, Y., Kingdom, J., Giussani, D. and Burton, G. (2013). Reduced cystathionine γ -lyase and increased miR-21 expression are associated with increased vascular resistance in growth-restricted pregnancies: hydrogen sulfide as a placental vasodilator. *American Journal of Pathology*. 182 (4):1448-1458. <https://doi.org/10.1016/j.ajpath.2013.01.001>.
- Cirino, G., Vellecco, V., and Bucci, M. (2017). Nitric oxide and hydrogen sulfide: the gasotransmitter paradigm of the vascular system. *British Journal of Pharmacology*, 174, 4021-4031. <https://doi.org/10.1111/bph.13815>.
- Dai, L., Qian, Y., Zhou, J., Zhu, C., Jin, L., and Li, S. (2019). Hydrogen sulfide inhibited L-type calcium channels (CaV1.2) via up-regulation of the channel sulfhydration in vascular smooth muscle cells. *European Journal of Pharmacology*, 858(2019), 1-10. <https://doi.org/10.1016/j.ejphar.2019.172455>.
- De Flora, S., Balansky, R., and La Maestra, S. (2020). Rationale for the use of N-acetylcysteine in both prevention and adjuvant therapy of COVID-19. *The FASEB Journal (official publication of the Federation of American Societies for Experimental Biology)*, 34(10), 13185-13193. <https://doi.org/10.1096/fj.202001807>.
- DeRatt, B., Ralat, M., Kabil, O., Chi, Y., Banerjee, R., and Gregory, F. (2014). Vitamin B-6 restriction reduces the production of hydrogen sulfide and its biomarkers by the transsulfuration pathway in cultured human hepatoma cells. *Journal of Nutrition*. 144(10):1501-1508. <https://doi.org/10.3945/jn.114.196808>.
- Dhaese, I., Van Colen, I., and Lefebvre, R. (2010). Mechanisms of action of hydrogen sulfide in relaxation of mouse distal colonic smooth muscle. *European Journal of Pharmacology*, 628(1–3), 179–186. <https://doi.org/10.1016/j.ejphar.2009.11.024>.
- Di Villa Bianca, R., Mitidieri, E., Esposito, D., Donnarumma, E., Russo, A., Fusco, F., Ianaro, A., Mirone, V., Cirino, G., Russo, G., and Sorrentino, R. (2015). Human Cystathionine β -Synthase Phosphorylation on Serine227 Modulates Hydrogen Sulfide Production in Human Urothelium. *PLOS ONE*, 10 (9), 1-16. <http://dx.plos.org/10.1371/journal.pone.0136859>.
- Di Villa Bianca, R., Sorrentino, R., Coletta, C., Mitidieri, E., Rossi, A., Vellecco, V., and Sorrentino, R. (2011). Hydrogen sulfide-induced dual vascular effect involves arachidonic acid cascade in rat mesenteric arterial bed. *Journal of Pharmacology and Experimental Therapeutics*, 337(1), 59–64. <https://doi.org/10.1124/jpet.110.176016>.

- Doeller, J., Isbell, T., Benavides, G., Koenitzer, J., Patel, H., Patel, R., and Kraus, D. (2005). Polarographic measurement of hydrogen sulfide production and consumption by mammalian tissues. *Analytical Biochemistry*, 341(1), 40–51. <https://doi.org/10.1016/j.ab.2005.03.024>.
- Donovan, J., Wong, P. S., Garle, M., Alexander, S., Dunn, W., and Ralevic, V. (2018). Coronary artery hypoxic vasorelaxation is augmented by perivascular adipose tissue through a mechanism involving hydrogen sulfide and cystathionine β -synthase. *Acta Physiologica*, 244 (4), 1-28. <https://doi.org/10.1111/apha.13126>.
- Donovan, J., Wong, P., Roberts, R., Garle, M., Alexander, S., Dunn, W., and Ralevic, V. (2017). A critical role for cystathionine β -synthase in hydrogen sulfide-mediated hypoxic relaxation of the coronary artery. *Vascular Pharmacology*, 93-95, 20-32. <https://doi.org/10.1016/j.vph.2017.05.004>.
- Dunn, W., Alexander, S., Ralevic, V., and Roberts, R. (2016). Effects of Hydrogen Sulphide in Smooth Muscle. *Pharmacology and Therapeutics*, 158(2016), 101-113. <http://dx.doi.org/10.1016/j.pharmthera.2015.12.007>.
- Elies, J., Boyle, J., Dallas, M., MacDoughall, D., Gamber, N., Huang, D., Scragg, J., and Peers, C. (2014). Hydrogen sulfide inhibits Cav 3.2 T-type Ca^{2+} channels. *Nitric Oxide*, 39, S16-S49. <https://doi.org/10.1016/j.niox.2014.03.114>.
- Elrod, J., Calvert, J., Morrison, J., Doeller, J., Kraus, D., Tao, L., and Lefer, D. (2007). Hydrogen sulfide attenuates myocardial ischaemia-reperfusion injury by preservation of mitochondrial function. *Proceedings of the National Academy of Sciences (PNAS)*, 104(39), 15560–15565. <https://doi.org/10.1073/pnas.0705891104>.
- Enokido, Y., Suzuki, E., Iwasawa, K., Namekata, K., Okazawa, H., and Kimura, H. (2005). Cystathionine β -Synthase, a Key Enzyme for Homocysteine Metabolism, Is Preferentially Expressed in the Radial Glia/astrocyte Lineage of Developing Mouse CNS. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 19 (13), 1854–1856. <https://doi.org/10.1096/fj.05-3724fje>.
- Ereño-Orbea, J., Majtan, T., Oyenarte, I., Kraus, J., and Martínez-Cruz, L. (2014). Structural insight into the molecular mechanism of allosteric activation of human cystathionine β -synthase by S-adenosylmethionine. *Proceedings of the National Academy of Sciences (PNAS)*, 111(37), E3845-E3852. <https://doi.org/10.1073/pnas.1414545111>.
- Erickson, P., Maxwell, I., Su, L., Baumann, M., and Glode, L. (1990). Sequence of cDNA for rat cystathionine γ - lyase and comparison of deduced amino acid sequence with related *Escherichia coli* enzymes. *Biochemical Journal*, 269(2): 335–340. <http://doi.org/10.1042/bj2690335>.
- Eto, K., and Kimura, H. (2002). The production of hydrogen sulfide is regulated by testosterone and S-adenosyl-L-methionine in mouse brain. *Journal of Neurochemistry*, 83(1), 80–86. <https://doi.org/10.1046/j.1471-4159.2002.01097>.
- Evgen'ev, M. and Frenkel, A. (2020). Possible application of H₂S-producing compounds in therapy of coronavirus (COVID-19) infection and pneumonia. *Cell Stress and Chaperones*, 25:713–715. <https://doi.org/10.1007/s12192-020-01120-1>.
- Filipovic, M. (2015). Persulfidation (S-sulfhydration) and H₂S. In: Moore, P. and Whiteman, M. (editors), *Handbook of Experimental Pharmacology: Chemistry, Biochemistry and Pharmacology of Hydrogen Sulfide*, 230, 29-59. <https://doi.org/10.1007/978-3-319-18144-8>.

- Fiorucci, S., Antonelli, E., Mencarelli, A., Orlandi, S., Renga, B., Rizzo, G., Distrutti, E., Shah, V., and Morelli, A. (2005). The third gas: H₂S regulates perfusion pressure in both the isolated and perfused normal rat liver and in cirrhosis. *Hepatology*, 42(3):539-548. <http://doi.org/10.1002/hep.20817>.
- Fräsdorf, B., Radon, C., and Leimkühler, S. (2014). Characterization and interaction studies of two isoforms of the dual localized 3-mercaptopyruvate sulfurtransferase TUM1 from humans. *Journal of Biological Chemistry*, 289(50), 34543–34556. <https://doi.org/10.1074/jbc.M114.605733>.
- Fu, M., Zhang, W., Wu, L., Yang, G., Li, H., and Wang, R. (2012). Hydrogen sulfide metabolism in mitochondria and its regulatory role in energy production. *Proceedings of the National Academy of Sciences (PNAS)*, 109(8), 2943–2948. <https://doi.org/10.1073/pnas.1115634109>.
- Fukao, T., Hosono, T., Misawa, S., Seki, T., and Ariga, T. (2004). The effects of allyl sulfides on the induction of phase II detoxification enzymes and liver injury by carbon tetrachloride. *Food and Chemical Toxicology*, 42(5), 743–749. <https://doi.org/10.1016/j.fct.2003.12.010>.
- Furne, J., Saeed, A., and Levitt, M. (2008). Whole tissue hydrogen sulfide concentrations are orders of magnitude lower than presently accepted values. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 295(5), R1479–R1485. <https://doi.org/10.1152/ajpregu.90566.2008>.
- Geng, B., Chang, L., Pan, C., Qi, Y., Zhao, J., Pang, Y., and Tang, C. (2004). Endogenous hydrogen sulfide regulation of myocardial injury induced by isoproterenol. *Biochemical and Biophysical Research Communications*, 318(3), 756–763. <https://doi.org/10.1016/j.bbrc.2004.04.094>.
- Geng, B., Yang, J., Qi, Y., Zhao, J., Pang, Y., Du, J., and Tang, C. (2004). H₂S generated by heart in rat and its effects on cardiac function. *Biochemical and Biophysical Research Communications*, 313(2), 362–368. <https://doi.org/10.1016/j.bbrc.2003.11.130>.
- Geng, B., Cui, Y., Zhao, J., Yu, F., Zhu, Y., Xu, G., Zhang, Z., Tang, C., and Du, J. (2007). Hydrogen sulfide downregulates the aortic L-arginine/nitric oxide pathway in rats. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, 293(4), R1608-R1618. <https://doi.org/10.1152/ajpregu.00207.2006>.
- Geró, D., Torregrossa, R., Perry, A., Waters, A., Le-Trionnaire, S., Whatmore, J., and Whiteman, M. (2016). The novel mitochondria-targeted hydrogen sulfide donors AP123 and AP39 protect against hyperglycemic injury in microvascular endothelial cells in vitro. *Pharmacological Research*, 113, 186–198. <https://doi.org/10.1016/j.phrs.2016.08.019>.
- Gilboa-Garber, N. (1971). Direct spectrophotometric determination of inorganic sulphide in biological materials and other complex mixtures. *Analytical Biochemistry*, 43(1), 129-133. [https://doi.org/10.1016/0003-2697\(71\)90116-3](https://doi.org/10.1016/0003-2697(71)90116-3).
- Givvimani, S., Kundu, S., Narayanan, N., Armaghan, F., Qipshidze, N., Pushpakumar, S., Vacek, T., and Tyagi, S. (2013). TIMP-2 Mutant Decreases MMP-2 Activity and Augments Pressure Overload Induced LV Dysfunction and Heart Failure. *Archives of Physiology and Biochemistry*, 119(2), 65–74. <http://doi.org/10.3109/13813455.2012.755548>.
- Gollasch, M., Bychkov, R., Ried, C., Behrendt, F., Scholze, S., Luft, F., and Haller, H. (1995). Pinacidil Relaxes Porcine and Human Coronary Arteries by Activating ATP-Dependent Potassium Channels in Smooth Muscle Cells. *The Journal of Pharmacology and Experimental Therapeutics*, 257(2), 681–692.

- Gouvern, M., Andriamihaja, M., Nübel, T., Blachier, F., and Bouillaud, F. (2007). Sulfide, the first inorganic substrate for human cells. *The FASEB Journal (official publication of the Federation of American Societies for Experimental Biology)*, 21(8), 1699–1706. <https://doi.org/10.1096/fj.06-7407com>.
- Grambow, E., Mueller-Graf, F., Delyagina, E., Frank, M., Kuhla, A., and Vollmar, B. (2014). Effect of the hydrogen sulfide donor GYY4137 on platelet activation and microvascular thrombus formation in mice. *Platelets*, 25, 166–174. <https://doi.org/10.3109/09537104.2013.786823>.
- Guo, W., Cheng, Z., and Zhu, Y. (2013). Hydrogen Sulfide and Translational Medicine. *Acta Pharmacologica Sinica*, 34 (10), 1284–1291. <http://doi.org/10.1038/aps.2013.127>.
- Guo, Z., Chen, G., Zeng, G., Li, Z., Chen, A., Wang, J., and Jiang, L. (2015). Fluorescence chemosensors for hydrogen sulfide detection in biological systems. *Analyst*, 140(6), 1772–1786. <https://doi.org/10.1039/c4an01909a>.
- Gupte, S., and Wolin, M. (2006). Hypoxia promotes relaxation of bovine coronary arteries through lowering cytosolic NADPH. *American Journal of Physiology-Heart and Circulatory Physiology*, 290(6), 2228–2238. <https://doi.org/10.1152/ajpheart.00615.2005>.
- Hara, M., Agrawal, N., Kim, S., Cascio, M., Fujimuro, M., Ozeki, Y., and Sawa, A. (2005). S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. *Nature Cell Biology*, 7(7), 665–674. <https://doi.org/10.1038/ncb1268>.
- Hassan, M., Boosen, M., Schaefer, L., Kozłowska, J., Eisel, F., Von Knethen, A., and Pfeilschifter, J. (2012). Platelet-derived growth factor-BB induces cystathionine γ -lyase expression in rat mesangial cells via a redox-dependent mechanism. *British Journal of Pharmacology*, 166(8), 2231–2242. <https://doi.org/10.1111/j.1476-5381.2012.01949.x>.
- Hedegaard, E., Nielsen, B., Kun, A., Hughes, A., Krøigaard, C., Mogensen, S., and Simonsen, U. (2014). KV₇ channels are involved in hypoxia-induced vasodilatation of porcine coronary arteries. *British Journal of Pharmacology*, 171(1), 69–82. <https://doi.org/10.1111/bph.12424>.
- Hellmich, M., and Szabó, C. (2015). Hydrogen sulfide and cancer. *Experimental Pharmacology*, 230, 233–241. https://doi.org/10.1007/978-3-319-18144-8_12.
- Hosoki, R., Matsuki, N., and Kimura, H. (1997). The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochemical and Biophysical Research Communications*, 237(3), 527–531. <https://doi.org/10.1006/bbrc.1997.6878>.
- Hou, F., Cheng, J., Xi, P., Chen, F., Huang, L., Xie, G., and Zeng, Z. (2012). Recognition of copper and hydrogen sulfide in vitro using a fluorescein derivative indicator. *Dalton Transactions*, 41(19), 5799–5804. <https://doi.org/10.1039/c2dt12462a>.
- Hu, L., Li, Y., Neo, K., Yong, Q., Lee, S., Tan, B., and Bian, J. (2011). Hydrogen sulfide regulates Na⁺/H⁺ exchanger activity via stimulation of phosphoinositide 3-kinase/Akt and protein kinase G pathways. *Journal of Pharmacology and Experimental Therapeutics*, 339(2), 726–735. <https://doi.org/10.1124/jpet.111.184754>.
- Hu, L., Lu, M., Tiong, C., Dawe, G. S., Hu, G., and Bian, J. (2010). Neuroprotective effects of hydrogen sulfide on Parkinson's disease rat models. *Aging Cell*, 9(2), 135–146. <https://doi.org/10.1111/j.1474-9726.2009.00543.x>.

- Hu, L., Pan, T., Neo, K., Yong, Q., and Bian, J. (2008). Cyclooxygenase-2 mediates the delayed cardioprotection induced by hydrogen sulfide preconditioning in isolated rat cardiomyocytes. *Pflugers Archives-European Journal of Physiology*, 455(6), 971–978. <https://doi.org/10.1007/s00424-007-0346-8>.
- Hu, L., Wong, P., Moore, P., and Bian, J. (2007). Hydrogen sulfide attenuates lipopolysaccharide-induced inflammation by inhibition of p38 mitogen activated protein kinase in microglia. *Journal of Neurochemistry*, 100(4), 1121–1128. <http://doi.org/10.1111/j.1471-4159.2006.04283.x>.
- Huang, C., Feng, W., Peh, M., Peh, K., Dymock, B., and Moore, P. (2016). A novel slow-releasing hydrogen sulfide donor, FW1256, exerts anti-inflammatory effects in mouse macrophages and in vivo. *Pharmacological Research*, 113(2016), 533–546. <https://doi.org/10.1016/j.phrs.2016.09.032>.
- Hughes, M., Centelles, M., and Moore, K. (2009). Making and working with hydrogen sulfide The chemistry and generation of hydrogen sulfide in vitro and its measurement in vivo: A review. *Free Radical Biology and Medicine*, 47(10), 1346–1353. <https://doi.org/10.1016/j.freeradbiomed.2009.09.018>.
- Hullin, R., Asmus, F., Ludwig, A., Hersel, J., and Boekstegers, P. (1999). Subunit expression of the cardiac L-type calcium channel is differentially regulated in diastolic heart failure of the cardiac allograft. *Circulation*, 100(2), 155–163. <https://doi.org/10.1161/01.CIR.100.2.155>.
- Huynh, M., Russell, P., and Walsh, B. (2009). Tryptic Digestion of In-Gel Proteins for Mass Spectrometry Analysis. In: Tyther R., Sheehan D. (editors) *Two-Dimensional Electrophoresis Protocols: Methods in Molecular Biology (Methods and Protocols)*, 519, 507–513, Humana Press. http://doi.org/10.1007/978-1-59745-281-6_34.
- Hyšpler, R., Tichá, A., Indrová, M., Kzadák, Z., Hyšplerová, L., Gaspari, J., and Churáek, J. (2002). A simple, optimized method for the determination of sulphide in whole blood by GC-MS as a marker of bowel fermentation processes. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 770, 255–259. [https://doi.org/10.1016/S1570-0232\(01\)00632-8](https://doi.org/10.1016/S1570-0232(01)00632-8).
- Ichinohe, A., Kanaumi, T., Takashima, S., Enokido, Y., Nagai, Y., and Kimura, H. (2005). Cystathionine β -synthase is enriched in the brains of Down's patients. *Biochemical and Biophysical Research Communications*, 338(3), 1547–1550. <https://doi.org/10.1016/j.bbrc.2005.10.118>.
- Ikeda, K., Marutani, E., Hirai, S., Wood, M., Whiteman, M., and Ichinose, F. (2015). Mitochondria-targeted hydrogen sulfide donor AP39 improves neurological outcomes after cardiac arrest in mice. *Nitric Oxide*, 49, 90–96. <https://doi.org/10.1016/j.niox.2015.05.001>.
- Ishigami, M., Hiraki, K., Umemura, K., Ogasawara, Y., Ishii, K., and Kimura, H. (2009). A source of hydrogen sulfide and a mechanism of its release in the brain. *Antioxidants and Redox Signaling*, 11(2), 205–214. <https://doi.org/10.1089/ars.2008.2132>.
- Ishii, I., Akahoshi, N., Yamada, H., Nakano, S., Izumi, T., and Suematsu, M. (2010). Cystathionine γ -lyase-deficient mice require dietary cysteine to protect against acute lethal myopathy and oxidative injury. *Journal of Biological Chemistry*, 285(34), 26358–26368. <https://doi.org/10.1074/jbc.M110.147439>.
- Ishii, I., Akahoshi, N., Yu, X., Kobayashi, Y., Namekata, K., Komaki, G., and Kimura, H. (2004). Murine cystathionine γ -lyase: complete cDNA and genomic sequences, promoter activity, tissue distribution and developmental expression. *The Biochemical Journal*, 381, 113–123. <https://doi.org/10.1042/BJ20040243>.

- Jackson-Weaver, O., Paredes, D., Bosc, L., Walker, B., and Kanagy, N. (2011). Intermittent Hypoxia in Rats Increases Myogenic Tone Through Loss of Hydrogen Sulfide Activation of Large-Conductance Ca²⁺ Activated Potassium Channels. *Circulation Research*, 108(12), 1439–1447. <https://doi.org/10.1161/CIRCRESAHA.110.228999>.
- Janosik, M., Kery, V., Gaustadnes, M., Maclean, K., and Kraus, J. (2001). Regulation of Human Cystathionine β-Synthase by S-Adenosyl-L-Methionine: Evidence for Two Catalytically Active Conformations Involving an Autoinhibitory Domain in the C-Terminal Region. *Biochemistry*, 40 (35), 10625–10633. <http://doi.org/10.1021/bi010711p>.
- Jha, S., Calvert, J., Duranski, M., Ramachandran, A., and Lefer D. Hydrogen sulfide attenuates hepatic ischemia-reperfusion injury: role of antioxidant and antiapoptotic signaling. *American Journal of Physiology Heart-Circulatory Physiology*. 295(2):H801-H806. <http://doi.org/10.1152/ajpheart.00377.2008>.
- Jiang, B., Tang, G., Cao, K., Wu, L., and Wang, R. (2010). Molecular Mechanism for H₂S-Induced Activation of K_{ATP} Channels. *Antioxidants and Redox Signaling*, 12 (10). 1167–1178. <http://doi.org/10.1089/ars.2009.2894>.
- Jiang, H., Wu, H., Li, Z., Geng, B., and Tang, C. (2005). Changes of the new gaseous transmitter H₂S in patients with coronary heart disease. *Di Yi Jun Yi Da Xue Xue Bao = Academic Journal of the First Medical College of PLA*, 25(8), 951–954.
- Jiang, J., Chan, A., Ali, S., Saha, A., Haushalter, K., Lam, W., and Boss, G. (2016). Hydrogen Sulfide—Mechanisms of Toxicity and Development of an Antidote. *Scientific Reports*, 6 (1), 1-10. <https://doi.org/10.1038/srep20831>.
- Jin, H., Wang, Y., Wang, X., Sun, Y., Tang, C., and Du, J. (2013). Sulfur dioxide preconditioning increases antioxidative capacity in rat with myocardial ischaemia reperfusion (I/R) injury. *Nitric Oxide*, 32(2013), 56–61. <https://doi.org/10.1016/j.niox.2013.04.008>.
- Jin, Z., Zhang, Q., Wondimu, E., Verma, R., Fu, M., Shuang, T., Arif, H., Wu, L., and Wang, R. (2020). H₂S stimulated bioenergetics in chicken erythrocytes and the underlying mechanism. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. 319(1), R69–R78. <https://doi.org/10.1152/ajpregu.00348.2019>.
- Kabil, O., Motl, N., and Banerjee, R. (2014). H₂S and its Role in Redox Signaling. *Biochimica et Biophysica Acta*, 1844(8), 1355–1366. <https://doi.org/10.1016/j.bbapap.2014.01.002>.
- Kabil, O., Vitvitsky, V., Xie, P., and Banerjee, R. (2011). The quantitative significance of the transsulfuration enzymes for H₂S production in murine tissues. *Antioxidants and Redox Signaling*, 15(2), 363–372. <https://doi.org/10.1089/ars.2010.3781>.
- Kabil, O., Zhou, Y., and Banerjee, R. (2006). Human Cystathionine β-Synthase Is a Target for Sumoylation. *Biochemistry*, 45 (45), 13528–13536. <https://doi.org/10.1021/bi0615644>.
- Kajimura, M., Fukuda, R., Bateman, R., Yamamoto, T., and Suematsu, M. (2010). Interactions of Multiple Gas-Transducing Systems: Hallmarks and Uncertainties of CO, NO, and H₂S Gas Biology. *Antioxidants and redox signalling*, 13 (2), 157–192. <http://doi.org/10.1089/ars.2009.2657>.
- Kamoun, P., Belardinelli, M., Chabli, A., Lallouchi, K., and Chadeaux-Vekemans, B. (2003). Endogenous hydrogen sulfide overproduction in Down syndrome. *American Journal of Medical Genetics*, 116A(3), 310–311. <https://doi.org/10.1002/ajmg.a.10847>.

- Kanagy, N. L., Szabo, C., and Papapetropoulos, A. (2017). Vascular biology of hydrogen sulfide. *American Journal of Physiology-Cell Physiology*, 312(5), C537–C549. <https://doi.org/10.1152/ajpcell.00329.2016>.
- Karwi, Q., Bornbaum, J., Boengler, K., Torregrossa, R., Whiteman, M., Wood, M. E., and Baxter, G. (2017). AP39, a mitochondria-targeting hydrogen sulfide (H₂S) donor, protects against myocardial reperfusion injury independently of salvage kinase signalling. *British Journal of Pharmacology*, 174(4), 287–301. <https://doi.org/10.1111/bph.13688>.
- Karwi, Q., Whiteman, M., Wood, M., Torregrossa, R., and Baxter, G. (2016). Pharmacological postconditioning against myocardial infarction with a slow-releasing hydrogen sulfide donor, GYY4137. *Pharmacological Research*, 111, 442–451. <https://doi.org/10.1016/j.phrs.2016.06.028>.
- Kashfi, K., and Olson, K. (2013). Biology and therapeutic potential of hydrogen sulfide and hydrogen sulfide-releasing chimeras. *Biochemical Pharmacology*. <https://doi.org/10.1016/j.bcp.2012.10.019>.
- Kashiba M., Kajimura M., Goda N., Suematsu M. (2002). From O₂ to H₂S: a landscape view of gas biology, *The Keio Journal of Medicine*, 51, 1, 1-10.
- Kawasaki, H., Takasaki, K., Saito, A., and Goto, K. (1988). Calcitonin gene-related peptide acts as a novel vasodilator neurotransmitter in mesenteric resistance vessels of the rat. *Nature*, 335, 165-167. <http://doi.org/10.1038/335164a0>.
- Khan, S., Morris, G., and Hidiroglou, M. (1980). Rapid estimation of sulfide in rumen and blood with a sulfide-specific ion electrode. *Topics in Catalysis*, 25(3), 388–395. [https://doi.org/10.1016/0026-265X\(80\)90280-5](https://doi.org/10.1016/0026-265X(80)90280-5).
- Khodakhah, K., Melishchuk, A., and Armstrong, C. M. (1997). Killing K⁺ channels with TEA. *Proceedings of the National Academy of Sciences (PNAS)*, 94(24), 13335–13338. <https://doi.org/10.1073/pnas.94.24.13335>.
- Kimura, H. (2002). Hydrogen Sulfide as a Neuromodulator Hideo Kimura. *Molecular Neurobiology*, 26 (1), 13–19. <https://doi.org/10.1385/MN:26:1:013>.
- Kimura, H. (2011). Hydrogen sulfide: its production and functions. *Experimental Physiology*, 96(9), 833–835. <https://doi.org/10.1113/expphysiol.2011.057455>
- Kimura, H. (2014). The physiological role of hydrogen sulfide and beyond. *Nitric Oxide*, 41, 4–10. <https://doi.org/10.1016/j.niox.2014.01.002>
- Kimura, H. and Kimura, Y. (2004). Hydrogen Sulfide Protects Neurons from Oxidative Stress. *FASEB Journal (Official Publication of the Federation of American Societies for Experimental Biology)*, 18(10), 1165-1167. <https://doi.org/10.1096/fj.04-1815fje>.
- Kimura, Y., Goto, Y., and Kimura, H. (2010). Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. *Antioxidants and Redox Signaling*, 12(1), 1–13. <https://doi.org/10.1089/ars.2008.2282>.
- Kimura, Y., Mikami, Y., Osumi, K., Tsugane, M., Oka, J., and Kimura, H. (2013). Polysulfides are possible H₂S-derived signalling molecules in rat brain. *FASEB Journal*, 27(6), 2451–2457. <https://doi.org/10.1096/fj.12-226415>.
- Kimura, Y., Toyofuku, Y., Koike, S., Shibuya, N., Nagahara, N., Lefer, D., and Kimura, H. (2015). Identification of H₂S₃ and H₂S produced by 3-mercaptopyruvate sulfurtransferase in the brain. *Scientific Reports*, 5(1), 1–11. <https://doi.org/10.1038/srep14774>.

- Kiss, L., Deitch, E., and Szabó, C. (2008). Hydrogen sulfide decreases adenosine triphosphate levels in aortic rings and leads to vasorelaxation via metabolic inhibition. *Life Sciences*, 83(17–18), 589–594. <https://doi.org/10.1016/j.lfs.2008.08.006>.
- Klabunde, R. (2012). *Cardiovascular Physiology Concepts*. Philadelphia: Lippincott Williams and Wilkins/Wolters Kluwer.
- Koenitzer, J., Isbell, T., Patel, H., Benavides, G., Dickinson, D., Patel, R., and Kraus, D. (2007). Hydrogen sulfide mediates vasoactivity in an O₂-dependent manner. *American Journal of Physiology-Heart and Circulatory Physiology*, 292(4), 1953–1960. <https://doi.org/10.1152/ajpheart.01193.2006>.
- Kolluru, G., Shen, X., Bir, S., and Kevil, C. (2013). Hydrogen Sulfide Chemical Biology: Pathophysiological Roles and Detection. *Nitric Oxide*, 35, 5–20. <https://doi.org/10.1016/j.niox.2013.07.002>.
- Kondo, K., Bhushan, S., King, A., Prabhu, S., Hamid, T., Koenig, S., and Lefer, D. (2013). H₂S protects against pressure overload-induced heart failure via upregulation of endothelial nitric oxide synthase. *Circulation*, 127(10), 1116–1127. <https://doi.org/10.1161/CIRCULATIONAHA.112.000855>.
- Kovačić, D., Glavnik, N., Marinšek, M., Zagožen, P., Rován, K., Goslar, T., Marš, T., and Podbregar, M. (2012). Total Plasma Sulfide in Congestive Heart Failure. *Journal of Cardiac Failure*, 18 (7), 541–548. <https://doi.org/10.1016/j.cardfail.2012.04.011>.
- Kubo, S., Doe, I., Kurokawa, Y., Nishikawa, H., and Kawabata, A. (2007). Direct inhibition of endothelial nitric oxide synthase by hydrogen sulfide: Contribution to dual modulation of vascular tension. *Toxicology*, 232(1–2), 138–146. <https://doi.org/10.1016/j.tox.2006.12.023>.
- Kuo, M., Kim, D., Jandu, S., Bergman, Y., Tan, S., Wang, H., Santhanam, L. (2015). MPST but not CSE is the primary regulator of hydrogen sulfide production and function in the coronary artery. *American Journal of Physiology-Heart and Circulatory Physiology*, 310(1), H71–H79. <https://doi.org/10.1152/ajpheart.00574.2014>.
- Laggner, H., Hermann, M., Esterbauer, H., Muellner, M., Exner, M., Gmeiner, B., and Kapiotis, S. (2007). The novel gaseous vasorelaxant hydrogen sulfide inhibits angiotensin-converting enzyme activity of endothelial cells. *Journal of Hypertension*, 25(10), 2100–2104. <https://doi.org/10.1097/HJH.0b013e32829b8fd0>.
- Lavu, M., Bhushan, S., and Lefer, D. (2011). Hydrogen sulfide-mediated cardioprotection: mechanisms and therapeutic potential. *Clinical Science*, 120(6), 219–229. <https://doi.org/10.1042/CS20100462>.
- Lee, M., Schwab, C., Yu, S., McGeer, E., and McGeer, P. (2009). Astrocytes produce the antiinflammatory and neuroprotective agent hydrogen sulfide. *Neurobiology of Aging*, 30(10), 1523–1534. <https://doi.org/10.1016/j.neurobiolaging.2009.06.001>.
- Lee, S., and Lee, C. (2005). Inhibition of Na⁺-K⁺ pump and L-type Ca²⁺ channel by glibenclamide in guinea pig ventricular myocytes. *Journal of Pharmacology and Experimental Therapeutics*, 312(1), 61–68. <https://doi.org/10.1124/jpet.104.074369>.
- Lee, S., Cheng, Y., Moore, P., and Bian, J. (2007). Hydrogen sulphide regulates intracellular pH in vascular smooth muscle cells. *Biochemical and Biophysical Research Communications*, 358(4), 1142–1147. <https://doi.org/10.1016/j.bbrc.2007.05.063>.
- Lee, S., Hu, Y., Hu, L., Lu, Q., Dawe, G., Moore, P., and Bian, J. (2006). Hydrogen sulphide regulates calcium homeostasis in microglial cells. *Glia*, 54(2), 116–124. <https://doi.org/10.1002/glia.20362>.

- Lenaeus, M., Vamvouka, M., Focia, P., and Gross, A. (2005). Structural basis of TEA blockade in a model potassium channel. *Nature Structural and Molecular Biology*, 12(5), 454–459. <https://doi.org/10.1038/nsmb929>.
- Leucker, T., Nomura, Y., Kim, J., Bhatta, A., Wang, V., and Wecker, A. (2017). Cystathionine γ -lyase protects vascular endothelium: a role for inhibition of histone deacetylase 6. *American Journal of Physiology Heart-Circulatory Physiology*, 312, H711–H720. <https://doi.org/10.1152/ajpheart.00724.2016>.
- Levitt, M., Abdel-Rehim, M., and Furne, J. (2011). Free and acid-labile hydrogen sulfide concentrations in mouse tissues: Anomalously high free hydrogen sulfide in aortic tissue. *Antioxidants and Redox Signaling*, 15(2), 373–378. <https://doi.org/10.1089/ars.2010.3525>.
- Levonen, A., Lapatto, R., Saksela, M. and Raivio, K. (2000). Human cystathionine γ -lyase: developmental and in vitro expression of two isoforms. *Biochemical Journal* 347 (1), 291–295. <https://doi.org/10.1042/bj3470291>.
- Li, L., Bhatia, M., Zhu, Y., Zhu, Y., Ramnath, R., Wang, Z., Anuar, F., Whiteman, M., Salto-Tellez, M., Moore, P. (2005). Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. *FASEB Journal: Soc. Exp. Biol.* 19, 1196–1198. <https://doi.org/10.1096/fj.04-3583fje>.
- Li, L., Rose, P., and Moore, P. (2011). Hydrogen Sulfide and Cell Signaling. *Annual Reviews of Pharmacology and Toxicology*, 51, 169–187. <https://doi.org/10.1146/annurev-pharmtox-010510-100505>.
- Li, L., Salto-Tellez, M., Tan, C., Whiteman, M., and Moore, P. (2009). GYY4137, a novel hydrogen sulfide-releasing molecule, protects against endotoxic shock in the rat. *Free Radical Biology and Medicine*, 47(1), 103–113. <https://doi.org/10.1016/j.freeradbiomed.2009.04.014>.
- Li, L., Whiteman, M., Guan, Y., Neo, K., Cheng, Y., Lee, S., Zhao, Y., Baskar, R., Tan, C., and Moore, P. (2008). Characterization of a novel, water-soluble hydrogen sulfide-releasing molecule (GYY4137): New insights into the biology of hydrogen sulfide. *Circulation*, 117(18), 2351–2360. <https://doi.org/10.1161/CIRCULATIONAHA.107.753467>.
- Li, Z., Polhemus, D., and Lefer, D. (2018). Evolution of hydrogen sulfide therapeutics to treat cardiovascular disease. *Circulation Research*, 123(5), 590–600. <https://doi.org/10.1161/CIRCRESAHA.118.311134>.
- Lilyanna, S., Peh, M., Liew, O., Wang, P., Moore, P., Richards, A., and Martinez, E. (2015). GYY4137 attenuates remodeling, preserves cardiac function and modulates the natriuretic peptide response to ischaemia. *Journal of Molecular and Cellular Cardiology*, 87, 27–37. <https://doi.org/10.1016/j.yjmcc.2015.07.028>.
- Lin, V., Lippert, A., and Chang, C. (2013). Cell-trappable fluorescent probes for endogenous hydrogen sulfide signalling and imaging H_2O_2 -dependent H_2S production. *Proceedings of the National Academy of Sciences (PNAS)*, 110(18), 7131–7135. <https://doi.org/10.1073/pnas.1302193110>.
- Lin, V., Lippert, A., and Chang, C. (2015). Azide-based fluorescent probes: Imaging hydrogen sulfide in living systems. *Methods in Enzymology*, 554, 63–80. <https://doi.org/10.1016/bs.mie.2014.11.011>.
- Lippert, A., New, E. and Chang, C. (2011). Reaction-based fluorescent probes for selective imaging of hydrogen sulfide in living cells. *Journal of the American Chemical Society*, 133(26), 10078–10080. <https://doi.org/10.1021/ja203661j>.

- Liu, X., Pan, L., and Zhuo, Y. (2010). Hypoxia-inducible factor-1 α is involved in the pro-angiogenic effect of hydrogen sulfide under hypoxic stress. *Biological Pharmaceutical Bulletin*, 33 (9), 1550-1554. <https://doi.org/10.1248/bpb.33.1550>.
- Liu, Y., Lu, M., Xie, Z., Hua, F., Xie, L., Gao, J., Koh, Y., and Bian, J. (2014). Hydrogen Sulfide Prevents Heart Failure Development via Inhibition of Renin Release from Mast Cells in Isoproterenol-Treated Rats. *Antioxidants and Redox Signaling*, 20 (5), 759–769. <https://doi.org/10.1089/ars.2012.4888>.
- Longen, S., Richter, F., Köhler, Y., Wittig, I., Beck, K., and Pfeilschifter, J. (2016). Quantitative Persulfide Site Identification (qPerS-SID) Reveals Protein Targets of H₂S Releasing Donors in Mammalian Cells. *Scientific Reports*, 6, 1-12. <https://doi.org/10.1038/srep29808>.
- Lowry, O., Rosebrough, N., Farr, A. and Randall, R. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, 193(1), 265-275. [https://doi.org/10.1016/0304-3894\(92\)87011-4](https://doi.org/10.1016/0304-3894(92)87011-4).
- Lu, M., Choo, C., Hu, L., Tan, B., Hu, G., and Bian, J. (2010). Hydrogen sulfide regulates intracellular pH in rat primary cultured glia cells. *Neuroscience Research*, 66(1), 92–98. <https://doi.org/10.1016/j.neures.2009.09.1713>.
- Lu, W., Li, J., Gong, L., Xu, X., Han, T., Ye, Y., Liu, C. (2014). H₂S modulates duodenal motility in male rats via activating TRPV1 and K_{ATP} channels. *British Journal of Pharmacology*, 171(6), 1534–1550. <https://doi.org/10.1111/bph.12562>.
- Luo, X., Qu, S., Tang, Z., Zhang, Y., Liu, M., Peng, J., Tang, H., Yu, K., Zhang, C., Ren, Z., and Jiang, Z. (2014). SIRT1 in Cardiovascular Aging. *Clinica Chimica Acta*, 437, 106–114. <http://dx.doi.org/10.1016/j.cca.2014.07.019>.
- Madden, J., Ahlf, S., Dantuma, M., Olson, K., and Roerig, D. (2014). Precursors and inhibitors of hydrogen sulfide synthesis affect acute hypoxic pulmonary vasoconstriction in the intact lung. *Journal of Applied Physiology*, 112(3): 411–418. <https://doi.org/10.1152/ajpheart.00574.2014>.
- Malekova, L., Krizanova, O., and Ondrias, K. (2009). H₂S and HS⁻ donor NaHS inhibits intracellular chloride channels. *General Physiology and Biophysics*, 28, 190–194. <https://doi.org/10.4149/gpb>.
- Mancardi, D., Penna, C., Merlino, A., Del Soldato, P., Wink, D., and Pagliaro, P. (2009). Physiological and pharmacological features of the novel gasotransmitter: Hydrogen sulfide. *Biochimica et Biophysica Acta*, 1787(7), 864–872. <https://doi.org/10.1016/j.bbabi.2009.03.005>.
- Mani, S., Li, H., Untereiner, A., Wu, L., Yang, G., Austin, R., and Wang, R. (2013). Decreased endogenous production of hydrogen sulfide accelerates atherosclerosis. *Circulation*, 127(25), 2523–2534. <https://doi.org/10.1161/CIRCULATIONAHA.113.002208>.
- Manteuffel-Cymborowska, M., Chmurzynska W., Grzelakowska-Sztabert, B. (1992). Tissue-specific effects of testosterone on S-adenosylmethionine formation and utilization in the mouse. *Biochimica et Biophysica Acta*, 1116(2), 166–172. [https://doi.org/10.1016/0304-4165\(92\)90113-9](https://doi.org/10.1016/0304-4165(92)90113-9).
- Materazzi, S., Zagli, G., Nassini, R., Bartolini, I., Romagnoli, S., Chelazzi, C., and Patacchini, R. (2017). Vasodilator activity of hydrogen sulfide in human mesenteric arteries. *Microvascular Research*, 109. <https://doi.org/10.1016/j.mvr.2016.11.001>.

- Mazza, R., Pasqua, T., Cerra, M., Angelone, T., and Gattuso, A. (2013). Akt/eNOS signaling and PLN S-sulfhydration are involved in H₂S-dependent cardiac effects in frog and rat. *American Journal of Physiological-Regulatory Integrated Comparative Physiology*, 305(4), R443-R451. <https://doi.org/10.1152/ajpregu.00088.2013>.
- McCully, K. (2015). Homocysteine and the pathogenesis of atherosclerosis. *Expert Review of Clinical Pharmacology*, 8(2), 211–219. <https://doi.org/10.1586/17512433.2015.1010516>.
- Meier, M., Janosik, M., Kery, V., Kraus, J., and Burkhard, P. (2001). Structure of human cystathionine β-synthase: A unique pyridoxal 5'-phosphate-dependent heme protein. *EMBO Journal*, 20(15), 3910–3916. <https://doi.org/10.1093/emboj/20.15.3910>.
- Meng, G., Ma, Y., Xie, L., Ferro, A., and Ji, Y. (2014). Emerging Role of Hydrogen Sulfide in Hypertension and Related Cardiovascular Diseases. *British journal of pharmacology*, 172(2015), 5501-5511. <http://doi.org/10.1111/bph.12900>.
- Meng, G., Zhao, S., Xie, L., Han, Y., and Ji, Y. (2017). Protein S-sulfhydration by hydrogen sulfide in the cardiovascular system. *British Journal of Pharmacology*, 175(2017), 1146-1156. <https://doi.org/10.1111/bph.13825>.
- Mikami, Y., Shibuya, N., Kimura, Y., Nagahara, N., Ogasawara, Y., and Kimura, H. (2011). Thioredoxin and dihydrolipoic acid are required for 3-mercaptopyruvate sulfurtransferase to produce hydrogen sulfide. *Biochemistry Journal*, 439(3), 479–485. <https://doi.org/10.1042/BJ20110841>.
- Mikami, Y., Shibuya, N., Kimura, Y., Nagahara, N., Yamada, M., Kimura, H. (2011). Hydrogen sulfide protects the retina from light-induced degeneration by the modulation of Ca²⁺ influx, *Journal of Biological Chemistry*, 286, 39379–39386. <http://doi.org/10.1074/jbc.M111.298208>.
- Mikami, Y., Shibuya, N., Ogasawara, Y., and Kimura, H. (2013). Hydrogen sulfide is produced by cystathionine γ-lyase at the steady-state low intracellular Ca²⁺ concentrations. *Biochemical and Biophysical Research Communications*, 431(2), 131–135. <https://doi.org/10.1016/j.bbrc.2013.01.010>.
- Mir, S., Sen, T., and Sen, N. (2014). Cytokine-Induced GAPDH Sulfhydration Affects PSD95 Degradation and Memory. *Molecular Cell*, 56(6), 786–795. <https://doi.org/10.1016/j.molcel.2014.10.019>.
- Miyamoto, R., Otsuguro, K., Yamaguchi, S., and Ito, S. (2014). Contribution of cysteine aminotransferase and mercaptopyruvate sulfurtransferase to hydrogen sulfide production in peripheral neurons. *Journal of Neurochemistry*, 130(1), 29–40. <https://doi.org/10.1111/jnc.12698>.
- Mok, Y., Atan, M., Yoke, C., Zhong, W., Bhatia, M., Mochhala, S., and Moore, P. (2004). Role of Hydrogen Sulphide in Haemorrhagic Shock in the Rat: Protective Effect of Inhibitors of Hydrogen Sulphide Biosynthesis. *British Journal of Pharmacology*, 143 (7), 881–889. <https://doi.org/10.1038/sj.bjp.0706014>.
- Montoya, L., Pearce, T., Hansen, R., Zakharov, L., and Pluth, M. (2013). Development of selective colorimetric probes for hydrogen sulfide based on nucleophilic aromatic substitution. *Journal of Organic Chemistry*, 78(13), 6550–6557. <https://doi.org/10.1021/jo4008095>.
- Morris, S., Cumming, D., Latchman, D., and Yellon, D. (1996). Specific induction of the 70-kD heat stress proteins by the tyrosine kinase inhibitor herbimycin-A protects rat neonatal cardiomyocytes: A new pharmacological route to stress protein expression? *Journal of Clinical Investigation*, 97(3), 706–712. <https://doi.org/10.1172/JCI118468>.

- Módis, K., Bos, E., Calzia, E., Van Goor, H., Coletta, C., Papapetropoulos, A., and Szabo, C. (2014). Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part II Pathophysiological and therapeutic aspects. *British Journal of Pharmacology*, 171(8), 2123–2146. <https://doi.org/10.1111/bph.12368>.
- Módis, K., Coletta, C., Erdélyi, K., Papapetropoulos, A., and Szabo, C. (2013). Intramitochondrial hydrogen sulfide production by 3-mercaptopyruvate sulfurtransferase maintains mitochondrial electron flow and supports cellular bioenergetics. *FASEB Journal*, 27(2), 601–611. <https://doi.org/10.1096/fj.12-216507>.
- Módis, K., Ju, Y., Ahmad, A., Untereiner, A., Altaany, Z., Wu, L., and Wang, R. (2016). S-Sulfhydration of ATP synthase by hydrogen sulfide stimulates mitochondrial bioenergetics. *Pharmacological Research*, 113, 116–124. <https://doi.org/10.1016/j.phrs.2016.08.023>.
- Mulrow, C., Lawrence, V., Ackermann, R., Ramirez, G., Morbidoni, L., Aguilar, C., Arterburn, J., Block, E., Chiquette, E., Gardener, C., Harris, M., Heidenreich, P., Mullins, D., Richardson, M., Russell, N., Vickers, A., and Young, V. (2000). Garlic: effects on cardiovascular risks and disease, protective effects against cancer, and clinical adverse effects. *Evidence Reports/Technology Assessments*, (20):1-4.
- Murphy, B., Bhattacharya, R., and Mukherjee, P. (2019). Hydrogen sulfide signalling in mitochondria and disease. *The FASEB Journal*, 33(12), 13098–13125. <https://doi.org/10.1096/fj.201901304R>.
- Murray, C. and Lopez, A. (1997). Mortality by cause for eight regions of the world: Global Burden of Disease Study. *Lancet* 349(9061), 1269–1276. [https://doi.org/10.1016/S0140-6736\(96\)07493-4](https://doi.org/10.1016/S0140-6736(96)07493-4).
- Mustafa, A., Gadalla, M., Sen, N., Kim, S., Mu, W., Gazi, S., and Snyder, S. (2009). H₂S signals through protein S-Sulfhydration. *Science Signaling*, 2(96), 1-8 <https://doi.org/10.1126/scisignal.2000464>.
- Mustafa, A., Gadalla, M., and Snyder, S. (2009). Signaling by gasotransmitters. *Science Signaling*, 2(68), 1-17. <https://doi.org/10.1126/scisignal.268re2>.
- Mustafa, A., Sikka, G., Gazi, S., Stepan, J., and Snyder, S. (2011). Hydrogen Sulfide as Endothelial Derived Hyperpolarizing Factor. *Circulatory Research*, 109 (11), 1259–1268. <https://doi.org/10.1161/CIRCRESAHA.111.240242>.
- Mys, L., Budko, A., Strutnyska, N., and Sagach, V. (2017). Pyridoxal-5-phosphate restores hydrogen sulfide synthesis and redox state of heart and blood vessels tissue in old animals. *Fiziolohichnyi Zhurnal*, 63(1), 3-9. <https://doi.org/10.15407/fz63.01.003>.
- Nagahara, N. (1999). Mercaptopyruvate sulfurtransferase as a defense against cyanide toxication: Molecular properties and mode of detoxification. *Histology and Histopathology*, 14, 1277–1286. <https://doi.org/10.14670/HH-14.1277>.
- Nagahara, N. (2008). A Novel Mercaptopyruvate Sulfurtransferase Thioredoxin-Dependent Redox-Sensing Molecular Switch: A Mechanism for the Maintenance of Cellular Redox Equilibrium. *Mini-Reviews in Medicinal Chemistry*, 8, 585–589. <https://doi.org/10.2174/138955708784534409>.
- Nagahara, N., Ito, T., Kitamura, H., and Nishino, T. (1998). Tissue and subcellular distribution of mercaptopyruvate sulfurtransferase in the rat: Confocal laser fluorescence and immunoelectron microscopic studies combined with biochemical analysis. *Histochemistry and Cell Biology*, 110(3), 243–250. <https://doi.org/10.1007/s004180050286>.

- Nagahara, N., Nagano, M., Ito, T., Shimamura, K., Akimoto, T., and Suzuki, H. (2013). Antioxidant enzyme, 3-mercaptopyruvate sulfurtransferase-knockout mice exhibit increased anxiety-like behaviors: A model for human mercaptolactate-cysteine disulfiduria. *Scientific Reports*, 3, 1–7. <https://doi.org/10.1038/srep01986>.
- Nagahara, N., Sreeja, V., Li, Q., Shimizu, T., Tsuchiya, T., and Fujii-Kuriyama, Y. (2004). A Point Mutation in a Silencer Module Reduces the Promoter Activity for the Human Mercaptopyruvate Sulfurtransferase. *Biochimica et Biophysica Acta*, 1680 (3), 176–184. <http://dx.doi.org/10.1016/j.bbaexp.2004.09.007>.
- Nagpure, B., and Bian, J. (2016). Interaction of Hydrogen Sulfide with Nitric Oxide in the Cardiovascular System. *Oxidative Medicine and Cellular Longevity*, 2016, 1–16. <https://doi.org/10.1155/2016/6904327>.
- Nagy, P., Pálkás, Z., Nagy, A., Budai, B., Tóth, I., and Vasas, A. (2014). Chemical aspects of hydrogen sulfide measurements in physiological samples. *Biochimica et Biophysica Acta*, 1840(2), 876–891. <https://doi.org/10.1016/j.bbagen.2013.05.037>.
- Naik, J., Osmond, J., Walker, B., and Kanagy, N. (2016). Hydrogen sulfide-induced vasodilation mediated by endothelial TRPV4 channels. *American Journal of Physiology-Heart and Circulatory Physiology*, 311(6), H1437–H1444. <https://doi.org/10.1152/ajpheart.00465.2016>.
- Nieuwenhuijs, V., Bruijn, M., Padbury, R., and Barritt, G. (2006). Hepatic ischaemia-reperfusion injury: Roles of Ca²⁺ and other intracellular mediators of impaired bile flow and hepatocyte damage. *Digestive Diseases and Sciences*, 51(6), 1087–1102. <https://doi.org/10.1007/s10620-006-8014-y>.
- Niu, W., Guo, L., Li, Y., Shuang, S., Dong, C., and Wong, M. (2015). Highly Selective Two-Photon Fluorescent Probe for Ratiometric Sensing and Imaging Cysteine in Mitochondria. *Analytical Chemistry*, 8(3), 1908–1914. <https://doi.org/10.1021/acs.analchem.5b04329>.
- Norris, E., Culberson, C., Narasimhan, S., and Clemens, M. (2011). The liver as a central regulator of hydrogen sulfide. *Shock*, 36(3), 242–250. <https://doi.org/10.1097/SHK.0b013e3182252ee7>.
- Oh, G., Pae, H., Lee, B., Kim, B., Kim, J., Kim, H., and Chung, H. (2006). Hydrogen sulfide inhibits nitric oxide production and nuclear factor-κB via heme oxygenase-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide. *Free Radical Biology and Medicine*, 41(1), 106–119. <https://doi.org/10.1016/j.freeradbiomed.2006.03.021>.
- Olson, K. (2008). Hydrogen sulfide and oxygen sensing: implications in cardiorespiratory control. *The Journal of Experimental Biology*, 211(17), 2727–2734. <https://doi.org/10.1242/jeb.010066>.
- Olson, K. (2009). Is hydrogen sulfide a circulating “gasotransmitter” in vertebrate blood? *Biochimica et Biophysica Acta*, 1787(7), 856–863. <https://doi.org/10.1016/j.bbabi.2009.03.019>.
- Olson, K. (2011). The therapeutic potential of hydrogen sulfide: separating hype from hope. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, 301(2), R297–R312, 2011. <http://doi.org/10.1152/ajpregu.00045.2011>.
- Olson, K. (2013). Hydrogen sulfide as an oxygen sensor. *Clinical Chemistry and Laboratory Medicine*, 51(3), 623–632. <https://doi.org/10.1515/cclm-2012-0551>.
- Olson, K., DeLeon, E., and Liu, F. (2014). Controversies and conundrums in hydrogen sulfide biology. *Nitric Oxide Biology and Chemistry*, 41, 11–26. <https://doi.org/10.1016/j.niox.2014.05.012>.

- Olson, K. (2015). The role of hydrogen sulfide in evolution and the evolution of hydrogen sulfide signalling. *Nitric Oxide*, 47(2015), S6. <https://doi.org/10.1016/j.niox.2015.02.011>.
- Olson, K., Dombkowski, R., Russell, M., Doellman, M., Head, S., Whitfield, N., and Madden, J. (2006). Hydrogen sulfide as an oxygen sensor/transducer in vertebrate hypoxic vasoconstriction and hypoxic vasodilation. *The Journal of Experimental Biology*, 209(20), 4011–4023. <https://doi.org/10.1242/jeb.02480>.
- Osipov, R., Robich, M., Feng, J., Liu, Y., Clements, R., Glazer, H., and Sellke, F. (2009). Effect of hydrogen sulfide in a porcine model of myocardial ischaemia-reperfusion: Comparison of different administration regimens and characterization of the cellular mechanisms of protection. *Journal of Cardiovascular Pharmacology*, 54(4), 287–297. <https://doi.org/10.1097/FJC.0b013e3181b2b72b>.
- Ott, M., Robertson, J., Gogvadze, V., Zhivotovsky, B., and Orrenius, S. (2002). Cytochrome c release from mitochondria proceeds by a two-step process. *Proceedings of the National Academy of Sciences (PNAS)*, 99(3), 1259–1263. <https://doi.org/10.1073/pnas.241655498>.
- Pan, T., Chen, Y., and Bian, J. (2009). All in the timing: A comparison between the cardioprotection induced by H₂S preconditioning and post-infarction treatment. *European Journal of Pharmacology*, 616(1–3), 160–165. <https://doi.org/10.1016/j.ejphar.2009.05.023>.
- Papapetropoulos, A., Pyriochou, A., Altaany, Z., Yang, G., Marazioti, A., Zhou, Z., and Szabó, C. (2009). Hydrogen sulfide is an endogenous stimulator of angiogenesis. *Proceedings of the National Academy of Sciences (PNAS)*, 106(51), 21972–21977. <https://doi.org/10.1073/pnas.0908047106>.
- Papapetropoulos, A., Whiteman, M., and Giuseppe, C. (2015). Pharmacological Tools for Hydrogen Sulfide Research: A Brief, Introductory Guide for Beginners. *British journal of pharmacology*, 172(6), 1633–1637. <http://doi.org/10.1111/bph.12806>.
- Park, C., Macinkovic, I., Filipovic, M., and Xian, M. (2015). Use of the “Tag-Switch” Method for the Detection of Protein S-Sulfhydration. *Methods in Enzymology*, 555, 39–56. <https://doi.org/10.1016/bs.mie.2014.11.033>.
- Paul, B. and Snyder, S. (2012). H₂S signalling through protein sulfhydration and beyond. *Nature Reviews Molecular Cell Biology*, 13(8), 499–507. <https://doi.org/10.1038/nrm3391>.
- Paul, B., and Snyder, S. (2015). H₂S: A Novel Gasotransmitter that Signals by Sulfhydration. *Trends in Biochemical Sciences*, 40(11), 687–700. <https://doi.org/10.1016/j.tibs.2015.08.007>.
- Pazicni, S., Lukat-Rodgers, G., Oliveriusov, J., Rees, K., Parks, R., Clark, R., Rodgers, K., Kraus, J., and Burstyn, J. (2004). The Redox Behavior of the Heme in Cystathionine β-Synthase Is Sensitive to pH. *Biochemistry*, 43 (46). 14684–14695. <http://doi.org/10.1021/bi0488496>.
- Peleli, M., Bibli, S., Li, Z., Chatzianastasiou, A., Varela, A., Katsouda, A., and Papapetropoulos, A. (2020). Cardiovascular phenotype of mice lacking 3-mercaptopyruvate sulfurtransferase. *Biochemical Pharmacology*, 176, 1–12. <https://doi.org/10.1016/j.bcp.2020.113833>.
- Peng, B., Chen, W., Liu, C., Rosser, E., Pacheco, A., Zhao, Y., and Xian, M. (2014). Fluorescent probes based on nucleophilic substitution-cyclization for hydrogen sulfide detection and bioimaging. *Chemistry-European Journal*, 20(4), 1010–1016. <https://doi.org/10.1002/chem.201303757>.

- Peng, Y., Nanduri, J., Raghuraman, G., Souvannakitti, D., Gadalla, M., Kumar, G., Snyder, S., and Prabhakar, N. (2010). H₂S mediates O₂ sensing in the carotid body. *Proceedings of the National Academy of Sciences of the United States of America (PNAS)*, 107 (23), 10719-10724. <https://doi.org/10.1073/pnas.1005866107>.
- Phan, T., Ton, H., Gulyás, H., Pórszász, R., Tóth, A., Russo, R., Kay, M., Sahibzada, N., and Ahern, G. (2020). TRPV1 expressed throughout the arterial circulation enables inflammatory vasoconstriction, *BioRxiv*, 1-36. <https://doi.org/10.1101/2020.02.04.934117>.
- Piknova, B., Park, J., Cassel, K., Gilliard, C., and Schechter, A. (2016). Measuring nitrite and nitrate, metabolites in the nitric oxide pathway, in biological materials using the chemiluminescence method. *Journal of Visualized Experiments*, 2016(118), 1-7. <https://doi.org/10.3791/54879>.
- Polhemus, D. and Lefer, D. (2014). Emergence of Hydrogen Sulfide as an Endogenous Gaseous Signaling Molecule in Cardiovascular Disease. *Circulation Research*, 114 (4), 730-737. <https://doi.org/10.1161/CIRCRESAHA.114.300505>.
- Polhemus, D., Calvert, J., Butler, J., and Lefer, D. (2014). The cardioprotective actions of hydrogen sulfide in acute myocardial infarction and heart failure. *Scientifica*, 2014, 1-8. <https://doi.org/10.1155/2014/768607>.
- Pong, W., Stouracova, R., Frank, N., Kraus, J, and Eldred, W. (2007). Comparative localization of cystathionine β-synthase and cystathionine γ-lyase in retina: differences between amphibians and mammals, *The Journal of Comparative Pharmacology*, 505:158-165. <https://doi.org/10.1002/cne.21468>.
- Powell, C., Dillon, K., and Matson, J. (2018). A review of hydrogen sulfide (H₂S) donors: Chemistry and potential therapeutic applications. *Biochemical Pharmacology*, 149, 110-123. <https://doi.org/10.1016/j.bcp.2017.11.014>.
- Prathapasinghe, G., Siow, Y., Xu, Z., and Karmin, O. (2008). Inhibition of cystathionine β-synthase activity during renal ischemia-reperfusion: Role of pH and nitric oxide. *American Journal of Physiology-Renal Physiology*, 295(4), 912-922. <https://doi.org/10.1152/ajprenal.00040.2008>.
- Predmore, B., Kondo, K., Bhushan, S., Zlatopolsky, M., King, A., Aragon, J., and Lefer, D. (2012). The polysulfide diallyl trisulfide protects the ischaemic myocardium by the preservation of endogenous hydrogen sulfide and increasing nitric oxide bioavailability. *American Journal of Physiology-Heart and Circulatory Physiology*, 302(11), 2410-2418. <https://doi.org/10.1152/ajpheart.00044.2012>.
- Prieto-Lloret, J., Snetkov, V., Shaifta, Y., Docio, I., Connolly, M., MacKay, C., and Aaronson, P. (2018). Role of reactive oxygen species and sulfide-quinone oxoreductase in hydrogen sulfide-induced contraction of rat pulmonary arteries. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 314(4), L670-L685. <https://doi.org/10.1152/ajplung.00283.2016>.
- Qian, Y., Karpus, J., Kabil, O., Zhang, S., Zhu, H., Banerjee, R., and He, C. (2011). Selective fluorescent probes for live-cell monitoring of sulphide. *Nature Communications*, 2(1), 1-7. <https://doi.org/10.1038/ncomms1506>.
- Qu, K., Chen, C., Halliwell, B., Moore, P., and Wong, P. (2006). Hydrogen sulfide is a mediator of cerebral ischaemic damage. *Stroke*, 37, 889-893. <https://doi.org/10.1161/01.STR.0000204184.34946.41>
- Quesada, I., and Soria, B. (2012). Intracellular Location of K_{ATP} Channels and Sulphonylurea Receptors in the Pancreatic β-cell: New Targets for Oral Antidiabetic Agents. *Current Medicinal Chemistry*, 11(20), 2707-2716. <https://doi.org/10.2174/0929867043364379>

- Rashid, S., Heer, J., Garle, M., Alexander, S., and Roberts, R. (2013). Hydrogen sulphide-induced relaxation of porcine peripheral bronchioles. *British Journal of Pharmacology*, 168, 1902–1910. <https://doi.org/10.1111/bph.12084>.
- Ratnam, S. (2002). Hormonal Regulation of Cystathionine β -Synthase Expression in Liver. *Journal of Biological Chemistry*, 277(45), 42912–42918. <https://doi.org/10.1074/jbc.M206588200>.
- Ratz, P., Berg, K., Urban, N., and Miner, A. (2005). Regulation of smooth muscle calcium sensitivity: KCl as a calcium-sensitizing stimulus. *American Journal of Physiology-Cell Physiology*, 288(4) C769-C783. <https://doi.org/10.1152/ajpcell.00529.2004>.
- Ravi, K., Brennan, L., Levic, S., Ross, P., and Black, S. (2004). S-nitrosylation of endothelial nitric oxide synthase is associated with monomerization and decreased enzyme activity. *Proceedings of the National Academy of Sciences (PNAS)*, 101(8), 2619–2624. <https://doi.org/10.1073/pnas.0300464101>.
- Reiffenstein, R., Hulbert, W., and Roth, SH. (1992). Toxicology of hydrogen sulfide. *Annual Review of Pharmacology and Toxicology*, 32, 109-134. <https://doi.org/10.1146/annurev.pa.32.040192.000545>.
- Richardson, C., Magee, E., and Cummings, J. (2000). A new method for the determination of sulphide in gastrointestinal contents and whole blood by microdistillation and ion chromatography. *Clinica Chimica Acta*, 293(1-2), 115–125. [https://doi.org/10.1016/S0009-8981\(99\)00245-4](https://doi.org/10.1016/S0009-8981(99)00245-4).
- Rigbolt, K., Prokhorova, T., Akimov, V., Henningsen, J., Johansen, P., Kratchmarova, I., Kassem, M., Mann, M., Olsen, J. V, and Blagoev, B. (2011). System-Wide Temporal Characterization of the Proteome and Phosphoproteome of Human Embryonic Stem Cell Differentiation. *Science Signaling*, 4 (164), 1-14. <http://doi.org/10.1126/scisignal.2001570>.
- Rose, P., Moore, P., and Zhu, Y. (2017). H₂S biosynthesis and catabolism: new insights from molecular studies. *Cellular and Molecular Life Sciences*, 74(8), 1391–1412. <https://doi.org/10.1007/s00018-016-2406-8>.
- Rossoni, G., Manfredi, B., Tazzari, V., Sparatore, A., Trivulzio, S., and Del Soldato, P. (2010). Activity of a new hydrogen sulfide-releasing aspirin (ACS14) on pathological cardiovascular alterations induced by glutathione depletion in rats. *European Journal of Pharmacology*, 648,139–145. <https://doi.org/10.1016/j.ejphar.2010.08.039>
- Roth, M., and Nystul, T. (2005). Buying time in suspended animation. *Scientific American*, 292(6), 48–55. <https://doi.org/10.1038/scientificamerican0605-48>.
- Saito, J., Zhang, Q., Hui, C., MacEdo, P., Gibeon, D., Menzies-Gow, A., and Chung, K. (2013). Sputum hydrogen sulfide as a novel biomarker of obstructive neutrophilic asthma. *Journal of Allergy and Clinical Immunology*, 131(1). <https://doi.org/10.1016/j.jaci.2012.10.005>.
- Sakaguchi, M., Marutani, E., Shin, H., Chen, W., Hanaoka, K., Xian, M., and Ichinose, F. (2014). Sodium thiosulfate attenuates acute lung injury in Mice. *Anesthesiology*, 121(6), 1248–1257. <https://doi.org/10.1097/ALN.0000000000000456>.
- Salloum, F. (2015). Hydrogen Sulfide and Cardioprotection: Mechanistic Insights and Clinical Translatability. *Pharmacology and Therapeutics*, 152, 11–17. <http://dx.doi.org/10.1016/j.pharmthera.2015.04.004>.
- Sasakura, K., Hanaoka, K., Shibuya, N., Mikami, Y., Kimura, Y., Komatsu, T., Ueno, T., Terai, T., Kimura, H., and Nagano, T. (2011). Development of a Highly Selective Fluorescence Probe for Hydrogen Sulfide. *Journal of American Chemical Society*, (133), 18003–18005. <https://doi.org/10.1016/B978-0-12-386454-3.00513-3>.

- Sbodio, J., Snyder, S., and Paul, B. (2019). Regulators of the transsulfuration pathway. *British Journal of Pharmacology*, 176(4), 583–593. <https://doi.org/10.1111/bph.14446>.
- Schwanstecher, M., Schwanstecher, C., Dickel, C., Chudziak, F., Moshiri, A., and Panten, U. (1994). Location of the sulphonylurea receptor at the cytoplasmic face of the β -cell membrane. *British Journal of Pharmacology*, 113(3), 903–911. <https://doi.org/10.1111/j.1476-5381.1994.tb17078.x>.
- Searcy, D., and Peterson, M. (2004). Hydrogen sulfide consumption measured at low steady state concentrations using a sulfidostat. *Analytical Biochemistry*, 324(2), 269–275. <https://doi.org/10.1016/j.ab.2003.09.037>.
- Sen, N. (2017). Functional and Molecular Insights of Hydrogen Sulfide Signaling and Protein Sulfhydration. *Journal of Molecular Biology*, 429(4), 543–561. <https://doi.org/10.1016/j.jmb.2016.12.015>.
- Sen, N., Hara, M., Kornberg, M., Cascio, M., Bae, I., Shahani, N., and Sawa, A. (2009). Nitric oxide-induced nuclear GAPDH activates p300/CBP and mediates apoptosis. *Natural Cell Biology*, 10(7), 866–873. <https://doi.org/10.1038/ncb1747>.
- Sen, N., Paul, B., Gadalla, M., Mustafa, A., Sen, T., Xu, R., and Snyder, S. (2012). Hydrogen sulfide-linked sulfhydration of NF- κ B mediates its antiapoptotic actions. *Molecular Cell*, 45(1), 13–24. <https://doi.org/10.1016/j.molcel.2011.10.021>.
- Sen, U., Vacek, T., Hughes, W., Kumar, M., Moshal, K., Tyagi, N., Metreveli, N., Hayden, M., and Tyagi, S. (2008). Cardioprotective role of sodium thiosulfate on chronic heart failure by modulating endogenous H₂S generation. *Pharmacology*, 82(3):201-13. <https://doi.org/10.1159/000156486>.
- Sharma, K., and Yuan, Z. (2010). Kinetics of chemical sulfide oxidation under high dissolved oxygen levels. 6th International Conference on Sewer Processes and Networks, 2010, 1-3.
- Shatalin, K., Shatalina, E., Mironov, and Evgeny, N. (2011). H₂S: A Universal Defense Against Antibiotics in Bacteria. *SCIENCE*, 334, 986–990. <https://doi.org/10.7551/mitpress/8053.003.0075>.
- Shen, X., Kolluru, G., Yuan, S., and Kevil, C. (2015). Measurement of H₂S in vivo and in vitro by the monobromobimane method. *Methods in Enzymology*, 554, 31–45. <http://doi.org/10.1016/bs.mie.2014.11.039>.
- Shen, X., Pattillo, C., Pardue, S., Bir, S., Wang, R., and Kevil, C. (2011). Free Radical Biology and Medicine Measurement of Plasma Hydrogen Sulfide in Vivo and in Vitro. *Free Radical Biology and Medicine* 50(9), 1021–1031. <http://dx.doi.org/10.1016/j.freeradbiomed.2011.01.025>.
- Shen, X., Peter, E., Bir, S., Wang, R., and Kevil, C. (2012). Analytical measurement of discrete hydrogen sulfide pools in biological specimens. *Free Radical Biology and Medicine*, 52(11–12), 2276–2283. <http://doi.org/10.1016/j.freeradbiomed.2012.04.007>.
- Shen, Y., Shen, Z., Luo, S., Guo, W., and Zhu, Y. (2015). The Cardioprotective Effects of Hydrogen Sulfide in Heart Diseases: From Molecular Mechanisms to Therapeutic Potential. *Oxidative Medicine and Cellular Longevity*, 2015, 1-13. <http://doi.org/10.1155/2015/925167>.
- Shibuya, N., Koike, S., Tanaka, M., Ishigami-Yuasa, M., Kimura, Y., Ogasawara, Y., and Kimura, H. (2013). A novel pathway for the production of hydrogen sulfide from D-cysteine in mammalian cells. *Nature Communications*, 4, 1–7. <http://doi.org/10.1038/ncomms2371>.

- Shibuya, N., Mikami, Y., Kimura, Y., Nagahara, N., and Kimura, H. (2009). Vascular endothelium expresses 3-mercaptopyruvate sulfurtransferase and produces hydrogen sulfide. *Journal of Biochemistry*, 146(5), 623–626. <http://doi.org/10.1093/jb/mvp111>.
- Shibuya, N., Tanaka, M., Yoshida, M., Ogasawara, Y., Togawa, T., Ishii, K., and Kimura, H. (2009). 3-Mercaptopyruvate Sulfurtransferase Produces Hydrogen Sulfide and Bound Sulfane Sulfur in the Brain. *Antioxidants and Redox Signaling*, 11 (4). 703–714. <http://doi.org/10.1089/ARS.2008.2253>.
- Sidhu, R., Singh, M., Samir, G., Carson, R. (2001). L-cysteine and sodium hydrosulphide inhibit spontaneous contractility in isolated pregnant rat uterine strips in vitro. *Pharmacological Toxicology*. 88(4):198-203. <http://doi.org/10.1034/j.1600-0773.2001.d01-104.x>.
- Simon, F., Giudici, R., Duy, C., Schelzig, H., Öter, S., Gröger, M., and Calzia, E. (2008). Hemodynamic and metabolic effects of hydrogen sulfide during porcine ischaemia-reperfusion injury. *Shock*, 30(4), 359–364. <https://doi.org/10.1097/SHK.0b013e3181674185>.
- Singh, S., Padovani, D., Leslie, R., Chiku, T., and Banerjee, R. (2009). Relative contributions of cystathionine β -synthase and γ -cystathionase to H₂S biogenesis via alternative trans-sulfuration reactions. *Journal of Biological Chemistry*, 284(33), 22457–22466. <https://doi.org/10.1074/jbc.M109.010868>.
- Sitdikova, G., Weiger, T., and Hermann, A. (2010). Hydrogen sulfide increases calcium-activated potassium (BK) channel activity of rat pituitary tumor cells. *Pflugers Archives-European Journal of Physiology*, 459(3), 389–397. <https://doi.org/10.1007/s00424-009-0737-0>.
- Sivarajah, A., Collino, M., Yasin, M., Benetti, E., Gallicchio, M., Mazzon, E., and Thiernemann, C. (2009). Anti-apoptotic and anti-inflammatory effects of hydrogen sulfide in a rat model of regional myocardial ischaemia/reperfusion. *Shock*, 31(3), 267–274. <https://doi.org/10.1097/SHK.0b013e318180ff89>.
- Skovby, F., Kraus, J., and Rosenberg, L. (1984). Biosynthesis and proteolytic activation of cystathionine β -synthase in rat liver. *The Journal of Biological Chemistry*, 259(1), 588–593.
- Smani, T., Hernández, A., Ureña, J., Castellano, A., Franco-Obregón, A., Ordoñez, A., and López-Barneo, J. (2002). Reduction of Ca²⁺ channel activity by hypoxia in human and porcine coronary myocytes. *Cardiovascular Research*, 53(1), 97–104. [https://doi.org/10.1016/S0008-6363\(01\)00422-9](https://doi.org/10.1016/S0008-6363(01)00422-9).
- Snijder, P., Frenay, A., de Boer, R., Pasch, A., Hillebrands, J., Leuvenink, H, and van Goor, H. (2015). Exogenous administration of thiosulfate, a donor of hydrogen sulfide, attenuates angiotensin II-induced hypertensive heart disease in rats. *British Journal of Pharmacology*, 172(6):1494-1504. <http://doi.org/10.1111/bph.12825>.
- Snijder, P., Frenay, A., Koning, A., Bachtler, M., Pasch, A., Kwakernaak, A., and Van Goor, H. (2014). Sodium thiosulfate attenuates angiotensin II-induced hypertension, proteinuria and renal damage. *Nitric Oxide Biology and Chemistry*, 42, 87–98. <https://doi.org/10.1016/j.niox.2014.10.002>.
- Sobenin, I., Pryanishnikov, V., Kunnova, L., Rabinovich, Y., Martirosyan, D., and Orekhov, A. (2010). The effects of time-released garlic powder tablets on multifunctional cardiovascular risk in patients with coronary artery disease. *Lipids in Health and Disease*, 9(1), 1-6. <https://doi.org/10.1186/1476-511X-9-119>.

- Sodha, N., Clements, R., Feng, J., Liu, Y., Bianchi, C., Horvath, E., and Sellke, F. (2009). Hydrogen sulfide therapy attenuates the inflammatory response in a porcine model of myocardial ischaemia/reperfusion injury. *Journal of Thoracic and Cardiovascular Surgery*, 138(4), 977–984. <https://doi.org/10.1016/j.jtcvs.2008.08.074>.
- Sordi, R., Fernandes, D., Heckert, B., and Assreuy, J. (2011). Early potassium channel blockade improves sepsis-induced organ damage and cardiovascular dysfunction. *British Journal of Pharmacology*, 163(6), 1289–1301. <https://doi.org/10.1111/j.1476-5381.2011.01324.x>.
- Stein, A., and Bailey, S. (2013). Redox biology of hydrogen sulfide: Implications for physiology, pathophysiology, and pharmacology. *Redox Biology*, 1(1), 32–39. <https://doi.org/10.1016/j.redox.2012.11.006>.
- Stipanuk M., and Beck P. (1982). Characterization of the enzymic capacity for cysteine desulfhydration in liver and kidney of the rat. *The Journal of Biochemistry*, 206, 267–277.
- Streeter, E., Hart, J., and Badoer, E. (2012). An investigation of the mechanisms of hydrogen sulfide-induced vasorelaxation in rat middle cerebral arteries. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 385(10), 991–1002. <https://doi.org/10.1007/s00210-012-0779-2>.
- Sun, W., Liu, F., Chen, Y., and Zhu, Y. (2012). Hydrogen sulfide decreases the levels of ROS by inhibiting mitochondrial complex IV and increasing SOD activities in cardiomyocytes under ischaemia/reperfusion. *Biochemical and Biophysical Research Communications*, 421(2), 164–169. <https://doi.org/10.1016/j.bbrc.2012.03.121>.
- Sun, Y., Cao, Y., Wang, W., Ma, S., Yao, T., and Zhu, Y. (2008). Hydrogen sulphide is an inhibitor of L-type calcium channels and mechanical contraction in rat cardiomyocytes. *Cardiovascular Research*, 79(4), 632–641. <https://doi.org/10.1093/cvr/cvn140>.
- Suwanai, Y., Nagahara, N., Naito, Z. and Orimo, H. (2016). Functional Analysis of 3-Mercaptopyruvate Sulfurtransferase Using Knockout Mice. *Advanced Techniques in Biology and Medicine*, 4(167), 1-4. <https://doi.org/10.4172/2379-1764.1000167>.
- Suzuki, K., Olah, G., Modis, K., Coletta, C., Kulp, G., Gero, D., and Szabo, C. (2011). Hydrogen sulfide replacement therapy protects the vascular endothelium in hyperglycemia by preserving mitochondrial function. *Proceedings of the National Academy of Sciences (PNAS)*, 108(33), 13829–13834. <https://doi.org/10.1073/pnas.1105121108>.
- Suzuki, Y., Saito, J., Uematsu, M., Fukuhara, A., Sato, S., Togawa, R., and Liu, S. (2016). Hydrogen Sulfide (H₂S)-Releasing Compounds: Therapeutic Potential in Cardiovascular Diseases. *American Journal of Physiology-Heart and Circulatory Physiology*, 310(1), H71–H79. <https://doi.org/10.1183/13993003.congress-2016.pa3379>.
- Swaroop, M., Bradley, K., Ohura, T., Tahara, T., Roper, M., Rosenberg, L., and Kraus, J. (1992). Rat cystathionine β- synthase. Gene organization and alternative splicing. *Journal of Biological Chemistry*, 267(16), 11455–11461. [https://doi.org/10.1016/S0021-9258\(19\)49931-6](https://doi.org/10.1016/S0021-9258(19)49931-6).
- Rosenberg, L.E., and Kraus, J.D. 1995. Rat cystathionine β- synthase. Gene organization
- Szabó, C. (2007). Hydrogen sulphide and its therapeutic potential. *Nature Reviews Drug Discovery*, 6(11), 917–935. <https://doi.org/10.1038/nrd2425>.

- Szabó, C. (2018). A timeline of hydrogen sulfide (H₂S) research: From environmental toxin to biological mediator. *Biochemical Pharmacology*, 149(2018), 5–19. <https://doi.org/10.1016/j.bcp.2017.09.010>.
- Szabó, C. and Hellmich, M. (2013). Endogenously Produced Hydrogen Sulfide Supports Tumor Cell Growth and Proliferation. *Cell Cycle*, 12 (18), 2915–2916. <https://doi.org/10.4161/cc.26064>.
- Szabó, C., Coletta, C., Chao, C., Módis, K., Szczesny, B., Papapetropoulos, A., and Hellmich, M. (2013). Tumor-derived hydrogen sulfide, produced by cystathionine β-synthase, stimulates bioenergetics, cell proliferation, and angiogenesis in colon cancer. *Proceedings of the National Academy of Sciences (PNAS)*, 110(30), 12474–12479. <https://doi.org/10.1073/pnas.1306241110>.
- Szabó, C., Ransy, C., Módis, K., Andriamihaja, M., Murghes, B., Coletta, C., and Bouillaud, F. (2014). Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I: Biochemical and physiological mechanisms. *British Journal of Pharmacology*, 171(2014), 2099–2122. <https://doi.org/10.1111/bph.12369>.
- Szabó, G., Veres, G., Radovits, T., Gerő, D., Módis, K., Miesel-Gröschel, C., Horkay, F., Karck, M., and Szabó, C. (2011). Cardioprotective Effects of Hydrogen Sulfide. *Nitric Oxide*, 25 (2), 201–210. <https://doi.org/10.1016/j.niox.2010.11.001>.
- Szczesny, B., Módis, K., Yanagi, K., Coletta, C., Le Trionnaire, S., Perry, A., and Szabo, C. (2014). AP39, a novel mitochondria-targeted hydrogen sulfide donor, stimulates cellular bioenergetics, exerts cytoprotective effects and protects against the loss of mitochondrial DNA integrity in oxidatively stressed endothelial cells in vitro. *Nitric Oxide Biology and Chemistry*, 41, 120–130. <https://doi.org/10.1016/j.niox.2014.04.008>.
- Takano, Y., Echizen, H., and Hanaoka, K. (2017). Fluorescent Probes and Selective Inhibitors for Biological Studies of Hydrogen Sulfide-and Polysulfide-Mediated Signaling. *Antioxidants and Redox Signaling*, 27(10), 669–683. <https://doi.org/10.1089/ars.2017.7070>.
- Tamaki, J., Yamada, Y., Yamamoto, Y., Matsuoka, M., and Ota, I. (2000). Sensing properties to dilute hydrogen sulfide of ZnSb₂O₆ thick-film prepared by dip-coating method. *Sensors and Actuators B: Chemical*, 66(1-3), 70–73. [https://doi.org/10.1016/S0925-4005\(99\)00408-6](https://doi.org/10.1016/S0925-4005(99)00408-6).
- Tanase, S., and Morino, Y. (1976). Irreversible inactivation of aspartate aminotransferases during transamination with L-propargylglycine. *Biochemical and Biophysical Research Communications*, 68(4), 1301–1308. [https://doi.org/10.1016/0006-291X\(76\)90338-7](https://doi.org/10.1016/0006-291X(76)90338-7).
- Tang, G., Wu, L., Liang, W., and Wang, R. (2005). Direct Stimulation of K_{ATP} Channels by Exogenous and Endogenous Hydrogen Sulfide in Vascular Smooth Muscle Cells. *Molecular Pharmacology*, 68(6), 1757–1764. <https://doi.org/10.1124/mol.105.017467>.
- Tangerman, A. (2009). Measurement and biological significance of the volatile sulfur compound hydrogen sulfide, methanethiol and dimethyl sulfide in various biological matrices. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 877(28), 3366–3377. <https://doi.org/10.1016/j.jchromb.2009.05.026>.
- Taoka, S., and Banerjee, R. (2001). Characterization of NO binding to human cystathionine β-synthase: Possible implications of the effects of CO and NO binding to the human enzyme. *Journal of Inorganic Biochemistry*, 87(4), 245–251. [https://doi.org/10.1016/S0162-0134\(01\)00335-X](https://doi.org/10.1016/S0162-0134(01)00335-X).

- Taoka, S., Lepore, B., Kabil, Ö., Ojha, S., Ringe, D., and Banerjee, R. (2002). Human cystathionine β -synthase is a heme sensor protein. Evidence that the redox sensor is heme and not the vicinal cysteines in the CXXC motif seen in the crystal structure of the truncated enzyme. *Biochemistry*, 41(33), 10454–10461. <https://doi.org/10.1021/bi026052d>.
- Taoka, S., Ohja, S., Shan, X., Kruger, W., and Banerjee, R. (1998). Evidence for Heme-Mediated Redox Regulation of Human Cystathionine β -Synthase Activity. *The Journal of biological chemistry*, 273 (39), 25179–25184. <https://doi.org/10.1074/jbc.273.39.25179>.
- Telezhkin, V., Brazier, S., Cayzac, S., Müller, C., Riccardi, D., and Kemp, P. (2009). Hydrogen sulfide inhibits human B_{KCa} channels. *Advances in Experimental Medicine and Biology*, 648, 65–72. https://doi.org/10.1007/978-90-481-2259-2_7.
- Teng, H., Wu, B., Zhao, K., Yang, G., Wu, L., and Wang, R. (2013). Oxygen-sensitive mitochondrial accumulation of cystathionine β -synthase mediated by Lon protease. *Proceedings of the National Academy of Sciences (PNAS)*, 110(31), 12679–12684. <https://doi.org/10.1073/pnas.1308487110>.
- Thomas, G., Chung, M., and Cohen, C. (1985). A dihydropyridine (Bay k 8644) that enhances calcium currents in guinea pig and calf myocardial cells. A new type of positive inotropic agent. *Circulation Research*, 56(1), 87–96. <https://doi.org/10.1161/01.RES.56.1.87>.
- Tian, X., Wong, W., Sayed, N., Luo, J., Tsang, S., Bian, Z., and Huang, Y. (2012). NaHS relaxes rat cerebral artery in vitro via inhibition of L-type voltage-sensitive Ca^{2+} channels. *Pharmacological Research*, 65(2), 239–246. <https://doi.org/10.1016/j.phrs.2011.11.006>.
- Togawa, T., Ogawa, M., Nawata, M., Ogasawara, Y., and Kawanabe, K. (1992). High Performance Liquid Chromatographic Determination of Bound Sulfide and Sulfite and Thiosulfate at Their Low Levels in Human Serum by Pre-column Fluorescence Derivatization with Monobromo-mobimane. *Chemical and Pharmaceutical Bulletin*, 40, 3000–3004. <https://doi.org/10.1248/cpb.40.3000>.
- Tomasova, L., Pavlovicova, M., Malekova, L., Misak, A., Kristek, F., Grman, M., and Whiteman, M. (2015). Effects of AP39, a novel triphenylphosphonium derivatised anethole dithiolethione hydrogen sulfide donor, on rat haemodynamic parameters and chloride and calcium Ca_v3 and $RyR2$ channels. *Nitric Oxide*, 46, 131–144. <https://doi.org/10.1016/j.niox.2014.12.012>.
- Trevisani, M., Patacchini, R., Nicoletti, P., Gatti, R., Gazzieri, D., Lissi, N., and Harrison, S. (2005). Hydrogen sulfide causes vanilloid receptor 1-mediated neurogenic inflammation in the airways. *British Journal of Pharmacology*, 145(8), 1123–1131. <https://doi.org/10.1038/sj.bjp.0706277>.
- Ubuka, T. (2002). Assay methods and biological roles of labile sulfur in animal tissues. *Journal of Chromatography B*, 781(1–2), 227–249. [https://doi.org/10.1016/S1570-0232\(02\)00623-2](https://doi.org/10.1016/S1570-0232(02)00623-2).
- Untereiner, A., Fu, M., Módis, K., Wang, R., Ju, Y., and Wu, L. (2016). Stimulatory effect of CSE-generated H_2S on hepatic mitochondrial biogenesis and the underlying mechanisms. *Nitric Oxide*, 58, 67–76. <https://doi.org/10.1016/j.niox.2016.06.005>.
- Vandiver, M. and Snyder, S. (2012). Hydrogen Sulfide: A Gasotransmitter of Clinical Relevance. *Journal of Molecular Medicine*, 90 (3), 255–263. <https://doi.org/10.1007/s00109-012-0873-4>.

- Verma, S., Rajeevan, V., Jain, P., and Bordia, A. (2005). Effect of garlic (*Allium Sativum*) oil on exercise tolerance in patients with coronary artery disease. *Indian Journal of Physiology and Pharmacology*, 49, 115–118.
- Vitvitsky, V., Yadav, P., Kurthen, A., and Banerjee, R. (2015). Sulfide oxidation by a noncanonical pathway in red blood cells generates thiosulfate and polysulfides. *Journal of Biological Chemistry*, 290(13), 8310–8320. <https://doi.org/10.1074/jbc.M115.639831>.
- Wagner, F., Asfar, P., Calzia, E., Radermacher, P., and Szabó, C. (2009). Bench-to-Bedside Review: Hydrogen Sulfide the Third Gaseous Transmitter: Applications for Critical Care. *Critical Care* 13 (3), 1-9. <http://doi.org/10.1186/cc7700>.
- Wallace, J. and Wang, R. (2015). Hydrogen sulfide-based therapeutics: exploiting a unique but ubiquitous gasotransmitter. *Nature Reviews Drug Discovery*, 14(5):329-345. <https://doi.org/10.1038/nrd4433>.
- Wallace, J., Caliendo, G., Santagada, V., Cirino, G., and Fiorucci, S. (2007). Gastrointestinal Safety and Anti-Inflammatory Effects of a Hydrogen Sulfide-Releasing Diclofenac Derivative in the Rat. *Gastroenterology*, 132 (1), 261–271. <http://doi.org/10.1053/j.gastro.2006.11.042>.
- Wallace, J., Vaughan, D., Dickey, M., MacNaughton, W., and de Nucci, G. (2018). Hydrogen Sulfide-Releasing Therapeutics: Translation to the Clinic. *Antioxidant Redox Signaling*, 28(16):1533-1540. <https://doi.org/10.1089/ars.2017.7068>.
- Wang, M., Guo, Z., and Wang, S. (2014). Regulation of cystathionine γ -lyase in mammalian cells by hypoxia, *Biochemical Genetics*, 52, 29–37, <http://doi.org/10.1007/s10528-013-9624-7>.
- Wang, Q., Liu, H., Mu, Q., Rose, P., and Zhu, Y. (2009). S-Propargyl-Cysteine Protects Both Adult Rat Hearts and Neonatal Cardiomyocytes from Ischemia/hypoxia Injury: The Contribution of the Hydrogen Sulfide-Mediated Pathway. *Journal of Cardiovascular Pharmacology*, 54 (2), 139–146. <http://doi.org/10.1097/FJC.0b013e3181ac8e12>.
- Wang, Q., Wang, X., Liu, H., Rose, P., and Zhu, Y. (2010). Protective effects of cysteine analogues on acute myocardial ischaemia: Novel modulators of endogenous H₂S production. *Antioxidants and Redox Signaling*, 12(10), 1155–1165. <https://doi.org/10.1089/ars.2009.2947>.
- Wang, R. (2012). Physiological Implications of Hydrogen Sulfide: A Whiff Exploration That Blossomed. *Physiological Reviews*, 92(2), 791–896. <https://doi.org/10.1152/physrev.00017.2011>.
- Warenycia M, Goodwin L, Benishin C, Reiffenstein R, Francom D, Taylor J, and Dieken F (1989). Acute hydrogen sulfide poisoning: demonstration of selective uptake of sulfide by the brainstem by measurement of brain sulfide levels, *Biochemical Pharmacology*, 38, 6, 973- 981. [https://doi.org/10.1016/0006-2952\(89\)90288-8](https://doi.org/10.1016/0006-2952(89)90288-8).
- Warenycia, M., Goodwin, L., Francom, D., Dieken, F., Kombianl, S., and Reiffensteinl, R. (1990). Dithiothreitol liberates non-acid labile sulfide from brain tissue of H₂S-poisoned animals. *Archives of Toxicology*, 64(8), 650–655. <http://doi.org/10.1007/BF01974693>.
- Watanabe, M., Osada, J., Aratani, Y., Kluckman, K., Reddick, R., Malinow, M., and Maeda, N. (1995). Mice deficient in cystathionine β -synthase: animal models for mild and severe homocysteinemia. *Proceedings of the National Academy of Sciences (PNAS)*, 92(5), 1585–1589. <https://doi.org/10.1073/pnas.92.5.1585>.

- Webb, G., Lim, L., Oh, V., Yeo, S., Cheong Y., and Ali, M. (2008). Contractile and vasorelaxant effects of hydrogen sulphide and its biosynthesis in the human internal mammary artery. *The Journal of Pharmacology and Experimental Therapeutics*, 324(2), 876–882. <https://doi.org/10.1124/jpet.107.133538>.
- Wedmann, R., Bertlein, S., Macinkovic, I., Böltz, S., Miljkovic, J., Muñoz, L., and Filipovic, M. (2014). Working with "H₂S": Facts and apparent artefacts. *Nitric Oxide Biology and Chemistry*, 41, 85–96. <https://doi.org/10.1016/j.niox.2014.06.003>.
- Wedmann, R., Onderka, C., Wei, S., Szijártó, I., Miljkovic, J., Mitrovic, A., Filipovic, M. (2016). Improved tag-switch method reveals that thioredoxin acts as depersulfidase and controls the intracellular levels of protein persulfidation. *Chemical Science*, 7(5), 3414–3426. <https://doi.org/10.1039/c5sc04818d>.
- Wei, W., Hu, X., Zhuang, X., Liao, L., and Li, W. (2014). GYY4137, a novel hydrogen sulfide-releasing molecule, likely protects against high glucose-induced cytotoxicity by activation of the AMPK/mTOR signal pathway in H9c2 cells. *Molecular and Cellular Biochemistry*, 389(1–2), 249–256. <https://doi.org/10.1007/s11010-013-1946-6>.
- Wen, Y., Wang, H., and Zhu, Y. (2018). The Drug Developments of Hydrogen Sulfide on Cardiovascular Disease. *Oxidative Medicine and Cellular Longevity*, 2018, 1-21. <https://doi.org/10.1155/2018/4010395>.
- White, B., Smith, P., and Dunn, W. (2013). Hydrogen sulphide mediated vasodilatation involves the release of neurotransmitters from sensory nerves in pressurized mesenteric small arteries isolated from rats. *British Journal of Pharmacology*, 168(4), 785–793. <https://doi.org/10.1111/j.1476-5381.2012.02187>.
- Whiteman, M. and Moore, P. (2009). Hydrogen Sulfide and the Vasculature: A Novel Vasculoprotective Entity and Regulator of Nitric Oxide Bioavailability? *Journal of Cellular and Molecular Medicine*, 13 (3), 488–507. <https://doi.org/10.1111/j.1582-4934.2009.00645.x>.
- Whiteman, M., Cheung, N., Zhu, Y., Chu, S., Siau, J., Wong, B., Armstrong, J, and Moore, P. (2005). Hydrogen sulphide: a novel inhibitor of hypochlorous acid-mediated oxidative damage in the brain? *Biochemical Biophysical Research Communications*. 326 (4), 794-798. <https://doi.org/10.1016/j.bbrc.2004.11.110>.
- Whiteman, M., Le Trionnaire, S., Chopra, M., Fox, B., and Whatmore, J. (2011). Emerging role of hydrogen sulfide in health and disease: critical appraisal of biomarkers and pharmacological tools. *Clinical Science*, 121(11), 459–488. <https://doi.org/10.1042/CS20110267>.
- Whiteman, M., Li, L., Kostetski, I., Chu, S., Siau, J., Bhatia, M., and Moore, P. (2006). Evidence for the formation of a novel nitrosothiol from the gaseous mediators nitric oxide and hydrogen sulphide. *Biochemical and Biophysical Research Communications*, 343(1), 303–310. <https://doi.org/10.1016/j.bbrc.2006.02.154>.
- Whiteman, M., Li, L., Rose, P., Tan, C., Parkinson, D., and Moore, P. (2010). The Effect of Hydrogen Sulfide Donors on Lipopolysaccharide-Induced Formation of Inflammatory Mediators in Macrophages. *Antioxidants and redox signalling*, 12 (10), 1147–54. <https://doi.org/10.1089/ars.2009.2899>.
- Whitfield, N., Kreimier, E., Verdial, F., Skovgaard, N., and Olson, K. (2008). Reappraisal of H₂S/sulfide concentration in vertebrate blood and its potential significance in ischemic preconditioning and vascular signalling. *American Journal of Physiology, Regulatory, Integrative and Comparative Physiology*, 294(6), R1930–R1937. <https://doi.org/10.1152/ajpregu.00025.2008>.

- Wills, M., Johns, R., Stone, D., Moscicki, J., and Difazio, C. (1989). Vascular effects of 2-chloroprocaine and sodium metabisulfite on isolated rat aortic rings. *American Society of Regional Anesthesia*, 14(60), 271-273.
- Wing, D., and Baskin, S. (1992). Modifiers of mercaptopyruvate sulfurtransferase catalyzed conversion of cyanide to thiocyanate in vitro. *Journal of Biochemical Toxicology*, 7(2), 65-72. <https://doi.org/10.1002/jbt.2570070203>.
- Wintner, E., Deckwerth, T., Langston, W., Bengtsson, A., Leviten, D., Hill, P., and Szabo, C. (2010). A monobromobimane-based assay to measure the pharmacokinetic profile of reactive sulphide species in blood. *British Journal of Pharmacology*, 160(4), 941-957. <https://doi.org/10.1111/j.1476-5381.2010.00704.x>.
- Wu, C., Li, C., Tsai, S., and Sheen, L. (2004). Diallyl Trisulfide Modulates Cell Viability and the Antioxidation and Detoxification Systems of Rat Primary Hepatocytes. *The Journal of Nutrition*, 134(4), 724-728. <https://doi.org/10.1093/jn/134.4.724>.
- Wu, D., Hu, Q., Liu, X., Pan, L., Xiong, Q., and Zhu, Y. (2015). Hydrogen sulfide protects against apoptosis under oxidative stress through SIRT1 pathway in H9c2 cardiomyocytes. *Nitric Oxide*, 46(2015), 204-212. <https://doi.org/10.1016/j.niox.2014.11.006>.
- Wu, X., Zhang, W., Wu, D., Sammynaiken, R., Wang, R. and Yang, Q. (2006). Using carbon nanotubes to absorb low-concentration hydrogen sulfide in the fluid. *NanoBioscience*, 5(3), 204-209. <https://doi.org/10.1109/TNB.2006.880843>.
- Xie, L., Gu, Y., Wen, M., Zhao, S., Wang, W., Ma, Y., and Ji, Y. (2016). Hydrogen sulfide induces Keap1 S-sulfhydration and suppresses diabetes-accelerated atherosclerosis via Nrf2 activation. *Diabetes*, 65(10), 3171-3184. <https://doi.org/10.2337/db16-0020>.
- Xu, M., Wu, Y., Li, Q., Liu, S., Li, Q., and He, R. (2011). Electrophysiological effects of hydrogen sulfide on human atrial fibers. *Chinese Medical Journal*, 124, 3455-3459. <https://doi.org/10.3760/cma.j.issn.0366-6999.2011.21.004>.
- Yan, H., Du, J., and Tang, C. (2004). The possible role of hydrogen sulfide on the pathogenesis of spontaneous hypertension in rats. *Biochemical and Biophysical Research Communications*, 313(1), 22-27. <https://doi.org/10.1016/j.bbrc.2003.11.081>.
- Yang, G., Wu, L., Bryan, S., Khaper, N., Mani, S., and Wang, R. (2010). Cystathionine γ -lyase deficiency and overproliferation of smooth muscle cells. *Cardiovascular Research*, 86(3), 487-495. <https://doi.org/10.1093/cvr/cvp420>.
- Yang, G., Pei, Y., Teng, H., Cao, Q., and Wang, R. (2011). Specificity protein-1 as a critical regulator of human cystathionine gamma-lyase in smooth muscle cells. *Journal of Biological Chemistry*, 286(30):26450-26460. <https://doi.org/10.1074/jbc.M111.266643>.
- Yang, G., Wu, L., Jiang, B., Yang, W., Qi, J., Cao, K., and Wang, R. (2008). H₂S as a physiologic vasorelaxant: Hypertension in mice with deletion of cystathionine γ -lyase. *Science*, 322(5901), 587-590. <https://doi.org/10.1126/science.1162667>.
- Yang, M., Chadwick, A., Dart, C., Kamishima, T., and Quayle, J. (2017). Bioenergetic profile of human coronary artery smooth muscle cells and the effect of the metabolic intervention. *PLOS ONE*, 12(5), 1-18. <https://doi.org/10.1371/journal.pone.0177951>.
- Yong, Q., Pan, T., Hu, L., and Bian, J. (2008). Negative regulation of β -adrenergic function by hydrogen sulphide in the rat hearts, *Journal of Molecular and Cellular Cardiology*, 44(4), 701-710. <https://doi.org/10.1016/j.yjmcc.2008.01.007>.

- Yu, F., Zhao, J., Tang, C., and Geng, B. (2010). Effect of synthesized GYY4137, a slowly releasing hydrogen sulfide donor, on cell viability and distribution of hydrogen sulfide in mice. *Beijing Da Xue Bao Yi Xue Ban, Journal of Beijing University (Health Sciences)*, 42 (5), 493–497.
- Yu, W., Jin, H., Tang, C., Du, J., and Zhang, Z. (2018). Sulfur-containing gaseous signal molecules, ion channels and cardiovascular diseases. *British Journal of Pharmacology*, 175(8), 1114–1125. <https://doi.org/10.1111/bph.13829>.
- Yu, X., Cui, L., Wu, K., Zheng, X., Cayabyab, F., Chen, Z., and Tang, C. (2014). Hydrogen Sulfide as a Potent Cardiovascular Protective Agent. *Clinica Chimica Acta*, 437, 78–87. <https://doi.org/10.1016/j.cca.2014.07.012>.
- Yusuf, M., Huat, B., Hsu, A., Whiteman, M., Bhatia, M., and Moore, P. (2005). Streptozotocin-induced diabetes in the rat is associated with enhanced tissue hydrogen sulfide biosynthesis, *Biochemical Biophysical Research Communications*. 333 (4), 1146–1152, <http://doi.org/10.1016/j.bbrc.2005.06.021>.
- Zagli, G., Patacchini, R., Trevisani, M., Abbate, R., Cinotti, S., Gensini, G., Masotti, G., and Geppetti, P (2007). Hydrogen sulfide inhibits human platelet aggregation. *European Journal of Pharmacology*, 559(1), 65-68. <https://doi.org/10.1016/j.ejphar.2006.12.011>.
- Zanardo, R., Brancaleone, V., Distrutti, E., Fiorucci, S., Cirino, G., and Wallace, J. (2006). Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. *The FASEB Journal*, 20, E1411-E1418. <https://doi.org/10.1096/fj.06-6270fje>.
- Zeng, T., Zhang, C., Zhu, Z., Yu, L., Zhao, X., and Xie, K. (2008). Diallyl trisulfide (DATS) effectively attenuated oxidative stress-mediated liver injury and hepatic mitochondrial dysfunction in acute ethanol-exposed mice. *Toxicology*, 252(1–3), 86–91. <https://doi.org/10.1016/j.tox.2008.07.062>.
- Zhang, D., Macinkovic, I., Devarie-Baez, N., Pan, J., Park, C., Carroll, K, and Xian, M. (2014). Detection of protein S-sulphydration by a tag-switch technique. *Angewandte Chemie-International Edition*, 53(2), 575–581. <https://doi.org/10.1002/anie.201305876>.
- Zhang, Da, Du, J., Tang, C., Huang, Y., and Jin, H. (2017). H₂S-induced sulphydration: Biological Function and Detection Methodology. *Frontiers in Pharmacology*, 8(2017), 1–13. <https://doi.org/10.3389/fphar.2017.00608>.
- Zhang, L., Wang, Y., Li, Y., Li, L., Xu, S., Feng, X., and Liu, S. (2018). Hydrogen Sulfide (H₂S)-Releasing Compounds: Therapeutic Potential in Cardiovascular Diseases. *Frontiers in Pharmacology*, 9, 1-17. <https://doi.org/10.3389/fphar.2018.01066>.
- Zhang, R., Sun, Y., Tsai, H., Tang, C., Jin, H., and Du, J. (2012). Hydrogen sulfide inhibits L-type calcium currents depending upon the protein sulfhydryl state in rat cardiomyocytes. *PLoS ONE*, 7(5), 1-11. <https://doi.org/10.1371/journal.pone.0037073>.
- Zhang, T., Mubeen, S., Myung, N., and Deshusses, M. (2008). Recent progress in carbon nanotube-based gas sensors. *Nanotechnology*, 19(33), 1-14. <https://doi.org/10.1088/0957-4484/19/33/332001>.
- Zhang, W., Xu, C., Yang, G., Wu, L., and Wang, R. (2015). Interaction of H₂S with calcium-permeable channels and transporters. *Oxidative Medicine and Cellular Longevity*, 2015, 1–7. <https://doi.org/10.1155/2015/323269>.
- Zhang, Y., Tang, Z., Ren, Z., Qu, S., Liu, M., Liu, L., and Jiang, Z. (2013). Hydrogen Sulfide, the next Potent Preventive and Therapeutic Agent in Aging and Age-Associated Diseases. *Molecular and cellular biology*, 33 (6), 1104–1113. <http://doi.org/10.1128/MCB.01215-12>.

- Zhang, Z., Huang, H., Liu, P., Tang, C., and Wang, J. (2007). Hydrogen Sulfide Contributes to Cardioprotection during Ischaemia-Reperfusion Injury by Opening K_{ATP} Channels. *Canadian Journal of Physiology and Pharmacology*, 85(12), 1248–1253. <http://doi.org/10.1139/Y07-120>.
- Zhang, Z., Wang, J., Xu, G., Ding, M., Liu, F., Yuan, L., Wang, T., Xu, J., Xie, X., Deng, B., Sun, D., and Lu, W. (2019). Inhalation of sodium hydrosulfide (NaHS) alleviates NO_2 -induced pulmonary function and hematological impairment in rats. *Life Sciences*, 232, 116650. <https://doi.org/10.1016/j.lfs.2019.116650>
- Zhao, K., Ju, Y., Li, S., Altaany, Z., Wang, R., and Yang, G. (2014). S-sulfhydration of MEK 1 leads to PARP-1 activation and DNA damage repair. *EMBO reports*, 15(7), 792–800. <http://doi.org/10.1002/embr.201338213>.
- Zhao, K., Li, H., Li, S., and Yang, G. (2014). Regulation of cystathionine γ -lyase/ H_2S system and its pathological implication. *Frontiers in Bioscience*, 19(8), 1355–1369. <https://doi.org/10.2741/4286>.
- Zhao, W., and Wang, R. (2002). H_2S -induced vasorelaxation and underlying cellular and molecular mechanisms. *American Journal of Physiology-Heart and Circulatory Physiology*, 283(2), H474–H480. <https://doi.org/10.1152/ajpheart.00013.2002>.
- Zhao, W., Ndisang, J., and Wang, R. (2003). Modulation of endogenous production of H_2S in rat tissues. *Canadian Journal of Physiology and Pharmacology*, 81(9), 848–853. <https://doi.org/10.1139/y03-077>.
- Zhao, W., Zhang, J., Lu, Y., and Wang, R. (2001). The vasorelaxant effect of H_2S as a novel endogenous gaseous K_{ATP} channel opener. *EMBO Journal*, 20 (21), 6008–6016. <https://doi.org/10.1093/emboj/20.21.6008>.
- Zhao, X., Zhang, L., Zhang, C., Zeng, X., Yan, H., Jin, H., Tang, C., and Du, J. (2008). Regulatory Effect of Hydrogen Sulfide on Vascular Collagen Content in Spontaneously Hypertensive Rats. *Hypertension Research*, 31 (8), 1619–1630. <http://doi.org/10.1291/hypres.31.1619>.
- Zhao, Y., Biggs, T., and Xian, M. (2014). Hydrogen sulfide (H_2S) releasing agents: chemistry and biological applications. *Chemical Communications*, 50(80), 11788–11805. <https://doi.org/10.1039/c4cc00968a>.
- Zhong, G., Chen, F., Cheng, Y., Tang, C., and Du, J. (2003). The Role of Hydrogen Sulfide Generation in the Pathogenesis of Hypertension in Rats Induced by Inhibition of Nitric Oxide Synthase. *Hypertension Journal*, 21 (10). 1879–1885. <http://doi.org/10.1097/00004872-200310000-00015>.
- Zhong, G., Li, Y., Liu, X., Guo, L., Chen, M., and Yang, X. (2010). Hydrogen Sulfide Opens the K_{ATP} Channel on Rat Atrial and Ventricular Myocytes, *Cardiology*, 115, 120–126. <http://doi.org/10.1159/000260073>.
- Zhou, Y., Yu, J., Lei, X., Wu, J., Niu, Q., Zhang, Y., and Wu, F. (2013). High-throughput tandem-microwell assay identifies inhibitors of the hydrogen sulfide signalling pathway. *Chemical Communications*, 49, 11782–11784. <https://doi.org/10.1039/c3cc46719h>.
- Zhu, X., Liu, S., Liu, Y., Wang, S., and Ni, X. (2010). Glucocorticoids suppress cystathionine γ -lyase expression and H_2S production in lipopolysaccharide-treated macrophages. *Cellular and Molecular Life Sciences*, 67(7), 1119–1132. <https://doi.org/10.1007/s00018-009-0250-9>.

- Zhu, Y., Wang, Z., Ho, P., Loke, Y., Zhu, Y., Huang, S., and Moore, P. (2007). Hydrogen sulfide and its possible roles in myocardial ischaemia in experimental rats. *The Journal of Applied Physiology*, 102(1), 261–268. <https://doi.org/10.1152/jappphysiol.00096.2006>.
- Zhuo, Y., Chen, P., Zhang, A., Zhong, H., Chen, C., and Zhu, Y. (2009). Cardioprotective effect of hydrogen sulfide in ischaemic reperfusion experimental rats and its influence on expression of survivin gene. *Biological and Pharmaceutical Bulletin*, 32(8), 1406–1410. <https://doi.org/10.1248/bpb.32.1406>.
- Zou, C., and Banerjee, R. (2003). Tumour necrosis factor- α induced targeted proteolysis of cystathionine β -synthase modulates redox homeostasis. *Journal of Biological Chemistry*, 278, 16802–16808. <https://doi.org/10.1074/jbc.M212376200>.
- Züнкler, B., Henning, B., Ott, T., Hildebrandt, A., and Fleck, E. (1997). Effects of tolbutamide on ATP-sensitive K⁺ channels from human right atrial cardiac myocytes. *Pharmacology and Toxicology*, 80(2), 69–75. <http://doi.org/10.1111/j.1600-0773.1997.tb00286.x>.

Appendices

The attended training courses and seminars

The attended training courses

1. First year:

Training course	Date	Points
1. Radiation - Safe Working with Ionising Radiations	07 October 2015	0
2. Microsoft Word: Introduction	11 November 2015	2
3. Introduction to Research Data Management	12 November 2015	1
4. Laboratory Practice 2 - basic techniques	20 November 2015	1
5. Planning your research	25 November 2015	1
6. Presentation skills for researchers (all disciplines)	08 December 2015	2
7. Creating and Managing Long Documents in Microsoft Word	18 December 2015	2
8. More Functions in Excel	06 January 2016	2
9. Nature of the doctorate and the supervision process	19 January 2016	1
10. Prism use and applications	25 January 2016	0
11. Laboratory Practice 1 - basic practice, health and safety	08 February 2016	1
12. Creating a Poster in PowerPoint	15 February 2016	1
13. Using Excel as a Database	25 February 2016	2
14. Researcher information skills for new researchers in Medicine & Health Sciences	08 March 2016	0
15. Mathematics in the Lab	18 March 2016	1
16. Microsoft PowerPoint: Introduction	31 March 2016	2
17. Preparing for your confirmation review	19 April 2016	0
18. Basic Statistics with SPSS	04 May 2016	2

2. Second year

Training course	Date	Points
1. How to be an Effective Researcher for Research Students	28/07/2016	3
2. Microsoft Powerpoint: Creating a Research Poster	23/11/2016	1
3. Demonstrating in Laboratory Practicals	18/01/2017	1
4. Writing Scientific Abstracts	18/01/2017	1
5. Foundations of Teaching in HE	03/02/2017	1
6. Public Engagement for Researchers: Community, Schools, and Colleges (SSAGC)	27/02/2017	1
7. Presentation skills for researchers (all disciplines)	28/02/2017	1
8. Good Laboratory Practice: Techniques	17/03/2017	1
9. Critical Appraisal of scientific literature 1 (non-clinical)	28/03/2017	1
10. Photographs: Working with GIMP	25/04/2017	1

3. Third year

Training course	Date	Points
1. Recruitment Interviews & Selection Exercises: Developing Confidence & Strategies	06/12/2017	1
2. Creative Problem Exploration in Research	18/01/2018	2
3. Preparing for the viva	03/02/2018	1
4. Applying for academic jobs - PhD students	02/05/2018	1
5. What do I want to get out of a conference - and how do I do it?	19/06/2018	1

The attended seminars:

1. First year:

Date	Presenter	Title
14 Jan 2016	Chris Wood	Camera choices for microscopy
19 Jan 2016	Hazel Rogers	Therapeutic targeting of the PI3K signaling pathway for the treatment of the childhood brain tumour ependymoma
20 Jan 2016	Steve Griffin	Using small molecules to understand and target the functions of virus-coded ion channels
18 Nov 2015	Julie Welburn	Mechanism and regulation of microtubule motors
29 Jan 2016	Prof. Marysia Placzek	Hypothalamic stem cells and homeostasis
9 Feb 2016	Dr. Nick Hannan	Human Foregut Stem Cells: Developmental Modelling, Regenerative Medicine, and Translational Research
23 Feb 2016	Hoang Minh	Creating Human Cellular Models to Investigate Cardioprotective Functions of GRK5 Hln41Leu using CRISPER/Cas Technology
24 Feb 2016	Nigel Minton (SoLS Research Group Presentation)	Microbial Engineering
29 Feb 2016	Maria Rosa Dominogo Sananes	Irreversible bistability in a Trypanosoma brucei developmental switch
12 Apr 2016	Sebastiaan Winkler	Post-transcriptional control of gene expression in tumour cells
15 Apr 2016	Dr. Karen Gregory (Monash Institute of Pharmaceutical Sciences)	Novel discovery strategies for modulating glutamatergic neurotransmission

2. Second year:

Date	Presenter	Title
31 Oct 2016	Stephen Knight (from Fisher Scientific)	Overview of qPCR and the Basic Principles of qPCR, Common Chemistries and the Applications of qPCR – going through DNA, RNA and protein applications
10 Nov 2016	Isabella Maiellaro (University of Wurzburg)	"cAMP Signals in Drosophila Motor Neurons Are Confined to Single Synaptic Boutons
16 Nov 2016	Peter Kolb Professor of Pharmaceutical Chemistry, Philipps- Universität Marburg, Germany.	Cell Signalling and Pharmacology Research Group Forum (Searching for (selective) GPCR ligands in chemical space
17 Nov 2016	Christopher McDevitt	Molecular mechanisms of metal ion homeostasis in Streptococcus pneumonia
30 Nov 2016	Fred Sablitzky	Molecular Cell Biology and development
7 Dec 2016	Ian Holt	Mitochondrial DNA loops the loop
11 Jan 2017	Professor Paul Harrison /University of Oxford	"Recent advances in schizophrenia genetics and their implications"
1 Feb 2017	Mark Field	Evolution of the nuclear pore complex and nuclear lamina
15 Feb 2017	Dr. Andrew Fenton (Sheffield University)	Identification of new cell wall Biogenesis factors in Steptococcus pneumoniae using TnSeq
22 Feb 2017	Dr. Derek Davies	Flow cytometry, its numerous applications, and technical advances.

3. Third year:

Date	Presenter	Title
17 Jan 2018	Professor Anthony Heagerty (Division of Cardiovascular Sciences, School of Medical Sciences, University of Manchester)	The Role of Perivascular Adipose Tissue in Health and Obesity
07 Feb 2018	Mike Benton FRS (University of Bristol)	Homology and deep homology: exploring the origin of feathers in pterosaurs, dinosaurs, and birds
14 Feb 2018	Elizabeth Cartwright (University of Manchester)	Understanding the role of the calcium extrusion pump PMCA in heart failure"
21 Feb 2018	Mike Trenell (Newcastle University)	"Lifestyle, metabolism and ageing; do we have a reverse gear?"
28 Feb 2018	Prof Andrew Fry (University of Leicester, UK)	'Microtubule organization in dividing cells: from molecular mechanisms to cancer therapies'
7 March 2018	Elisabetta Verderio Edwards (Nottingham Trent University)	Transglutaminases and kidney fibrosis
14 March 2018	Tanmay Bharat (University of Oxford)	Structural biology inside cells using electron cryotomography"
21 March 2018	Ransom Lecture Mark Caulfield (Queen Mary University of London)	Ransom Lecture: "The 100,000 Genomes Project"
20 Apr 2018	John Armour	"Human alpha-defensin variation: blood cells, kidney disease and tooth loss"
25 Apr 2018	Christian Rudolph (Brunel University)	" How colliding forks shape the landscape of the Escherichia coli chromosome"
27 Apr 2018	Jonathan Ball	"Pseudo viruses in the art of cell entry"

4. Fourth year

Date	Presenter	Title
03 Apr 2019	Donald Davidson (University of Edinburgh)	"Cathelicidin: an antimicrobial inflammomodulatory fire alarm in infectious lung disease"
14 Jun 2019	Gustavo Lazzaro Rezende	"Evolution, physiology and genetics of water relations in insect eggs"
24 Jul 2019	Lauren Sumner-Rooney	"Distributed visual systems: how do many-eyed animals see the world?"
21 Jun 2019	Simona Huwiler and Jess Tyson	"Predatory Bdellovibrio bacteriovorus: towards understanding the prey exit process and application as 'living antibiotic'"
10 Sep 2019	Professor Fernando Baquero	Individual-Health, One-Health and Global-Health in Antibiotic Resistance