THE VASCULAR EFFECTS OF HYDROGEN SULPHIDE

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Abstract

In recent years it has become apparent that hydrogen sulphide (H_2S) is an important biological mediator. In the vasculature, it produces complex responses: contraction in some blood vessels, relaxation in others, via multiple mechanisms. This thesis examined the relationship between H_2S and oxygen in determining vascular responsiveness, and was conducted using porcine splenic and mesenteric arteries. Studies were also conducted using porcine splenic veins, since few studies have examined venous function. Additionally, studies were extended to the resistance vasculature by determining responses to a H_2S donor in small arteries isolated from the rat mesentery.

Porcine vessels were set up in an isometric tension recording system and rat small mesenteric arteries were set up in a pressure myograph. Vessels were pre-contracted and responses to the H_2S donor, NaHS, were generated in the presence and absence of putative inhibitors, under either 95% O_2:5% CO_2, 95% air:5% CO_2 or 95% N_2:5% CO_2 gassing conditions.

Generally, in both porcine arteries and veins, when gassing with higher oxygen levels (95% O_2:5% CO_2 or 95% Air:5% CO_2), NaHS induced contractile responses, whereas gassing with a lower oxygen level (95% N_2:5% CO_2), NaHS induced vasorelaxation. At higher O_2 levels, removal of the endothelium or, the nitric oxide (NO) synthase inhibitor L-NAME, significantly attenuated contractile response in all porcine vessels. This suggests an interaction between endothelium-derived NO and NaHS, whereby the removal of the vasorelaxatory influence of NO resulted in

contraction. In porcine arteries, relaxation at lower O_2 levels was attenuated by glibenclamide, suggesting that NaHS activated K_{ATP} channels to cause relaxation. In porcine veins, removal of the endothelium or, L-NAME, abolished NaHS-induced relaxation, showing this relaxation occurred via the release of endothelium-derived NO. In rat mesenteric small arteries responses to NaHS did not change with different O_2 levels and NaHS-induced vasodilatation that was abolished by desensitization of sensory nerves with capsaicin or the presence of BIBN 4096. These observations suggest NaHS-induced vasodilatation is mediated via release of CGRP from sensory nerves.

Thus, responses to NaHS in large conduit arteries and veins, are sensitive to the prevailing level of O_2 the tissue is exposed to. At more physiological levels of O_2 the predominant response is a vasorelaxation, mediated by either, activation of K_{ATP} channels in arteries or, the release of NO in veins. In small arteries, the predominant response is a vasodilator response, involving the release of neuropeptides from sensory nerves. The predominance of a vasorelaxant/vasodilator response is consistent with the observation that mice which lack the capacity to generate endogenous H_2S are hypertensive.

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Declaration

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

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Chapter 1:

General Introduction

The Cardiovascular System

The cardiovascular system is responsible for transporting oxygen, after gas exchange in the lungs to different tissues around the body. It also facilitates the delivery of nutrients and removal of waste products from the body (Sherwood, 1997). The heart is responsible for pumping blood through the vasculature maintaining an appropriate cardiac output. The right side of the heart pumps blood through the pulmonary circulation, while the left side of the heart pumps blood through the aorta to the systemic vasculature. In the systemic vasculature, blood is delivered to the organs through a network of systemic arteries that become increasingly small before allowing for gas and nutrient exchange in the capillaries. Blood returns to the heart through the systemic veins.

The Anatomy of Blood Vessels

Arteries and veins contain three distinct layers (Sherwood, 1997). The outer layer of blood vessels is called the tunica adventitia. This layer is composed of collagen, connective tissue and elastic fibers. Blood flow exerts pressure on the walls of vessels and elastic fibers allow blood vessels to expand but prevent over expansion, when experiencing an increase in intraluminal pressure. The middle layer is the tunica media which contains smooth muscle cells and elastic fibers. The smooth muscle cells in this layer enable control of the diameter of arteries. The inner layer is the tunica intima, comprised of a single layer of cells referred to as the vascular endothelium. Capillaries are composed of a single layer of endothelial cells, allowing exchange of molecules between the blood and the surrounding tissue.

The Control of Arterial Blood Pressure

Blood pressure is the force blood exerts on walls of the vessel and depends upon the capacitance of the vessel and the volume of blood within it. The two major factors involved in control of blood pressure are cardiac output and total peripheral resistance. Cardiac output is determined by heart rate and stroke volume, whereas resistance is mainly controlled by the diameter of blood vessels. Changes in the diameter of arterioles, in particular, can significantly alter resistance and in turn blood pressure and regional blood flow. Higher levels of arteriolar resistance acts to dissipate pressure and cause a lower arterial blood pressure at the terminal end of these vascular trees (Davis, 2003). The venous return of blood to the heart is important in determining cardiac output. Venous pressure is much lower than arterial pressure as a significant proportion is lost by the resistance offered by the small arteries, arterioles and the capillary beds. A change in venous capacitance will alter venous return of blood to the heart and thus, stroke volume. As cardiac output is determined by stroke volume and heart rate, an increase in stroke volume, will increase the cardiac output.

The Control of Blood Vessel function

Blood vessel diameter is controlled by two sets of factors, intrinsic and extrinsic. Intrinsic control is the response to local chemical and physical stimuli, whereas, extrinsic control is the response to both neuronal and hormonal influences. Small arteries and arterioles respond to changes in the intraluminal pressure they experience by altering their diameter via the myogenic response. An increase in pressure will lead a vasoconstrictor

response and a decrease in pressure will lead to a vasodilation response to maintain myogenic tone (Meininger and Davis, 1992). Myogenic tone sets a background tone against which other mechanisms can produce vasoconstriction or vasodilatation. This mechanism acts to ensure constant blood flow to an organ under conditions were pressure is changing (Davis and Hill, 1999) and is particularly important in the cerebral vasculature.

Intrinsically, local physical and chemical changes within tissues can affect the diameter of blood vessels, and thus influence the blood flow to any particular vascular bed. For example, changes in temperature can alter vascular diameter, as can locally produced chemicals, which regulate blood flow in response to the metabolic demands of a tissue (Meininger and Davis, 1992). These chemical signals are very important in ensure blood supply is directed to meet tissue demand. The release of chemical mediators from the endothelium plays a major role in the regulation of blood vessel diameter and hence local blood flow (Hill *et al.,* 2001; Hill *et al.,* 2006).

Extrinsically, hormonal and neuronal signals can influence blood vessel diameter. The release of hormones, such as vasopressin and angiotensin II, which are important in the control of fluid balance, promote vasoconstriction (Sherwood, 1997). In addition, sympathetic nerves controlled by centres in the hypothalamus and the medulla of the brain can have neural reflex activity, influencing blood vessel diameter. Blood vessels have many sympathetic nerve fibers that when activated mainly cause vasoconstriction, acting to increase arterial blood pressure. In small arteries the main sympathetic neurotransmitters are adenosine triphosphate (ATP) and noradrenaline (Rummery *et al*., 2007).

In addition to sympathetic nerves, blood vessels also have many sensory nerves, which are mainly responsible for detecting changes in blood oxygen and carbon dioxide levels (Sherwood, 1997). However capsaicin-sensitive sensory nerves have also been shown to release vasoactive neuropeptides and can cause vasorelaxation (Kawasaki *et al.,* 1988*;* Gupta *et al.*, 2007). Calcitonin gene-related peptide (CGRP) is an important vasorelaxatory neurotransmitter released from capsaicin-sensitive sensory nerves in the rat mesenteric arterial bed (Kawasaki *et al.,* 1988). However, to date there is no evidence that capsaicin-sensitive sensory nerve mediated vasorelaxation is via the release of CGRP in either coronary or cranial arteries isolated from either pigs or humans (Gupta *et al.*, 2007).

The Vascular Endothelium

Until the early 1980s the endothelium was largely considered as the inert lining of blood vessels. However, Furchgott and Zawadzki (1980) found that rabbit aortic preparations lost their ability to produce a relaxation response to acetylcholine (ACh) when the intimal surface had been rubbed or damaged. They showed that the endothelium was releasing an endothelium-derived relaxing factor (EDRF) in response to ACh, although the chemical nature of this EDRF was not determined at that time. Subsequent studies led to the suggestion that nitric oxide (NO) was the EDRF responsible for ACh-induced vasorelaxation via increasing intracellular cyclic guanosine monophosphate (cGMP) concentrations (Palmer *et al*., 1987).

Nitric Oxide

NO is produced by the enzyme, nitric oxide synthase (NOS), requiring nicotinamide adenine dinucleotide phosphate (NADPH) and L-arginine, as well as the presence of molecular oxygen (O_2). NO is a free radical with a short half-life and is very reactive with other substances. NO readily reacts with super oxide radicals, O_2 and hydrogen peroxide (H_2O_2), to form nitrogen dioxide (NO_2) peroxynitrite ($ONOO^-$), nitrite (NO_2^-) and nitrate (NO_3^-) (Henry *et al.*, 1991). NO tends to exist in gaseous form and its small size facilitates its entry into cells. Three types of NOS isoforms have been identified, neuronal (nNOS), inducible (iNOS) and endothelial (eNOS).

nNOS (also known as, Type I) is widely expressed in a population of neurons of the brain (Forstermann *et al.*, 1990). NO produced by nNOS, acts as a neurotransmitter and has been implicated in modulation of neuronal

plasticity and pain perception (Christopherson and Bredt, 1997). In addition, nNOS is also present in nitroxidergic nerves of the periphery where it releases NO to cause vasodilatation (Toda and Okamura, 2003). iNOS (known as Type II) is not constitutively expressed but can be induced in a range of cells, especially in the immume system in response to inflammatory or pathogenic activity (MacMicking *et al.*, 1997). iNOS has been shown to be involved in host defence mechanisms and is induced by infection and chronic inflammation (Moncada and Higgs, 2006). In porcine cerebral arteries, it has also been shown that the presence of iNOS expression, as a consequence of activation of intramural nitrergic nerves, interferes with EDRF and NO induced vasodilatation (Mathewson and Wadsworth, 2004). eNOS (Type III) is expressed constitutively in the vascular endothelium and therefore, is also referred to as constitutive NOS (cNOS). eNOS regulates vascular tone by mediating vascular smooth muscle relaxation through the release of NO (Rapoport *et al.,* 1983). Endothelial expression of the eNOS isoform has been shown to mediate the role of NO as an EDRF (Palmer *et al.*, 1987).

Isoform non-specific inhibition of NOS in animals has been shown to cause an increase in blood pressure (Gardiner *et al.,* 1990; Ribeiro *et al.,* 1992; Lin *et al.,* 1996). NOS inhibitors tend to be analogues of arginine that feature substitutions of the guanidino nitrogen. These include, N^G-nitro-L-arginine (L-NNA), N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine. However, no isoform specific inhibitors of eNOS have yet been discovered. The role of eNOS has been implicated in basal control of blood pressure since disruption of the gene encoding for eNOS in mice, which produced specific suppression of NO production, increased

systemic blood pressure (Huang *et al.,* 1995). Furthermore, eNOS mediates agonist-induced endothelium dependent vasorelaxation in many blood vessels. For example, Cosentino *et al.* (1993) showed that arginine analogues inhibited relaxation to vasopressin in the canine basilar artery and L-NAME completely abolished ACh induced relaxation of noradrenaline pre-constricted isolated rabbit renal arteries (Kitagawa *et al.,* 1994).

Endothelium-Derived Hyperpolarizing Factor

It is now known that the vascular endothelium produces vasoactive substances that regulate many physiological responses (Henderson, 1991). Since the discovery of the initial EDRF by Furchgott and Zawadzki (1980), and subsequent identification of this as NO by Palmer *et al*. (1987), other endothelium-derived vasorelaxatory substances have been identified and EDRFs has now become the generic name for these. The best-characterized EDRFs are nitric oxide and prostacyclin (Busse *et al*., 2002). However, in recent years, a further EDRF responsible for causing vasodilation via hyperpolarization of smooth muscle cells has been shown. This endothelium derived hyperpolarizing factor (EDHF) was initially proposed by Taylor and Weston (1988). NO activates smooth muscle cell guanylate cyclase, increasing intracellular cGMP, thus causing relaxation. In contrast, EDHF produces membrane hyperpolarization of the smooth muscle cells to induce relaxation. It is generally accepted that EDHF-induced relaxation is NOS and cyclooxygenase (COX) independent and can be blocked by inhibition of calcium-activated potassium channel (K_{Ca2+}) (Doughty *et al*., 1999; Busse *et*

al., 2002; Gluais *et al*., 2005). Several candidates for this EDHF have been proposed.

Potassium ions have been suggested to be a possible candidate for EDHF in some vascular tissues. For example, in the rat hepatic artery, Edwards *et al*. (1998) reported that efflux of K^+ from the endothelial cells via K_{Ca2+} channels located in the endothelium, hyperpolarized vascular smooth muscle cells by opening inward rectifying K^+ channels (K_{IR}) and activating $Na^{+/}K^+$-ATPase. This hypothesis was based on the observation that ouabain and barium attenuated potassium induced hyperpolarization as well as induced EDHF-mediated responses. Campbell *et al.* (1996), provided evidence in bovine coronary arteries that epoxides derivatives of arachidonic acid can act as an EDHF, produced by the cytochrome P450 pathway. In addition to this, anandamide, an ethanolamide also derived from arachidonic acid, has also been suggested to be an EDHF released in response to stimulation by ACh and bradykinin in the rat mesenteric arterial bed (Randall *et al.*, 1996). Within the endothelium, superoxide radicals can be degraded to H_2O_2 spontaneously or via the action of the enzyme superoxide dismutase (SOD) and Matoba *et al.* (2000) suggested that H_2O_2 could act as an EDHF. In their study, Matoba *et al.* (2000) showed catalase, which dismutates H_2O_2 to O_2 and H_2O, inhibited EDHF-attributed relaxation and hyperpolarization in small mesenteric arteries obtained from eNOS knockout mice. C-type natriuretic peptide (CNP) has also been suggested to be a potential EDHF (Chauhan *et al.*, 2004), on the basis that CNP produced EDHF-like vasoactivity in rat mesenteric resistance arteries using a mechanism that activates K_{IR} and $Na^{+/}K^+$-ATPase.

Gaseous Transmitters

In addition to NO being identified as an endogenously generated gaseous transmitter, carbon monoxide (CO) has also been shown to be endogenously produced and to cause biological effects (Wu and Wang, 2005). CO is produced physiologically by the catabolism of heme to CO and iron, catalyzed by heme oxygenase (HO) with NADPH as a co-factor (Maines, 1997). The HO-1 isoform of HO is reported to be ubiquitous, and expressed under stress conditions in the nervous system, reproductive system and the cardiovascular system, whereas the HO-2 isoform is the constitutive form of the enzyme, primarily expressed in the brain and cardiovascular system (Maines, 1997). CO has been shown to cause vasorelaxation in rat aorta, tail, mesenteric, renal and pulmonary arteries (Wang *et al*., 1997). In addition to NO and CO, hydrogen sulphide (H_2S) has recently been reported to be an important endogenous gaseous transmitter (Wang, 2002). Enzymes responsible for the endogenous production of H_2S have been identified in the portal vein and thoracic aorta (Hosoki *et al*., 1997), cultured bovine aortic endothelial cells and human umbilical vein endothelial cells (Yang *et al*., 2008). It has been postulated that H_2S may be an EDRF, possibly an EDHF (Wang, 2009).

Hydrogen Sulphide

Hydrogen Sulphide is a colourless gas with a strong odour of rotten eggs and has long been known to be a toxic environmental pollutant. It is the sulphur analogue of water and can be oxidized by a variety of agents to form sulphur dioxide (SO_2), sulphates such as sulphuric acid, and elemental sulphur and these products also have toxicological implications (Reiffenstein

et al., 1992). At high concentration or, administered in the short term, H_2S becomes toxic via blocking mitochondrial oxidative phosphorylation (Reiffenstein *et al*., 1992; Dorman *et al*., 2002). However more recently, H_2S has been shown to be produced endogenously (Hosoki *et al*., 1997) and to exert a range of biological effects as a neurotransmitter in the brain (Kimura, 2000) and as a vasorelaxant in the vasculature (Wang, 2002).

Hydrogen Sulphide Biochemistry under Physiological Conditions

Under physiologically relevant conditions, in aqueous solutions and at pH 7.4, a third of H_2S is un-dissociated and two thirds dissociate into protons (H^+) and hydrosulphide ion (HS^-) (Lowicka and Beltowski, 2007). HS^- can subsequently decompose to H^+ and sulphide ion (S_2^-) however, the latter reaction occurs only at high pH, thus S_2^- does not occur in *vivo* in substantial amounts. Similarly to NO and CO, H_2S freely permeates plasma membranes as it is lipophilic although due to partial dissociation, membranes are relatively less permeable to H_2S than to both other gases (Lowicka and Beltowski, 2007). Sodium hydrosulphide (NaHS) is commonly used as an H_2S donor since it dissociates to Na^+ and HS^-, the latter then partially binding to H^+ to form un-dissociated H_2S (Lowicka and Beltowski, 2007).

Toxicology of Hydrogen Sulphide

The main mechanism of H_2S toxicity is via potent inhibition of mitochondrial cytochrome c oxidase and thus mitochondrial respiration. H_2S is a more potent inhibitor of mitochondrial respiration than cyanide (Reiffenstein *et al.,* 1992). As H_2S is a gas, inhalation can be a major cause

of H_2S associated pathology. Acute inhalation of H_2S at high concentrations has been shown to result in severe mitochondrial swelling in olfactory neurons within the olfactory epithelium in rats (Brenneman *et al*., 2000). Another study by Warenycia *et al*. (1989) provided strong evidence that cytochrome oxidase inhibition plays a critical role in H_2S-induced olfactory pathology in rats.

Chronic exposure to H_2S at lower concentrations has been shown to cause physiological changes in pulmonary function (Reiffenstein *et al.*, 1992). In the guinea-pig, H_2S provokes tachykinin-mediated neurogenic inflammatory responses in guinea-pig airways which contributes to its irritative effect in the respiratory system (Trevisani *et al.,* 2005). In the brain, changes in neurotransmitter content and release, as a result of H_2S exposure, may be related to the clinical impairment of cognition associated with H_2S exposure (Reiffenstein *et al.*, 1992). In addition the suppressive effect of H_2S on synaptic transmission in the CNS may be partly responsible for the dizziness and unconsciousness caused by acute sub-lethal H_2S exposure (Reiffenstein *et al.*, 1992).

Additionally, H_2S may play a role in mediating cerebral ischemic damage after stroke under certain conditions. Qu *et al*. (2006), reported that endogenous production of H_2S increased by up to 200% during stroke and it has been postulated that high concentrations of H_2S during stroke, may aggravate brain damage due to severe intracellular acidification (Lu *et al.*, 2010). In contrast, isoproterenol-induced myocardial injury was shown to reduce endogenous generation of H_2S and it was proposed this may contribute to the pathogenesis by increasing tissue levels of oxygen-derived

free radicals which may previously have been mopped up by interacting with H_2S (Geng *et al*., 2004).

The concentrations of H_2S that are lethal are broadly similar across all mammalian species, perhaps reflecting a similar toxicological mechanism on eukaryotic cells (Reiffenstein *et al*., 1992). It is worth noting that the lethal concentration of H_2S in the rat brain is only twice the level that has been shown to be produced endogenously (Warenycia *et al*., 1989).

Endogenous Hydrogen Sulphide Levels

The level of endogenous H_2S production is variable depending on both the tissue and experimental conditions used. Endogenous levels of H_2S have been measured as; 50 - 160 µM in rat brain (Hosoki *et al.,* 1997), 46 ± 14 µM in rat plasma (Zhao *et al.,* 2001), 10 - 100 µM in human blood (Richardson et al., 2000), and 301 ± 32 µM in rat pulmonary artery VSMC (Zhang *et al.,* 2003). However, rapid oxidation of H_2S in mitochondria, which acts to prevent the intoxication of cells from accumulation of endogenously generated H_2S under normal physiological conditions may limit the capacity to measure endogenous levels of H_2S in a meaningful way (Bartholomew *et al*., 1980). For example, Whitfield *et al*. (2008) found, using a polarographic sensor to measure H_2S gas in real time in blood from several species, including lamprey, trout, mouse, rat, pig, and cow, that H_2S gas was essentially undetectable. This may be due to catabolism and oxidation of H_2S occurring very rapidly in mitochondria (Kajimura *et al.*, 2010). Rapid oxidation of sulphide, to thiosulphate and then sulphate, occurs readily in mitochondria (Bartholomew et al., 1980). In addition to not being able to

detect endogenous H_2S, Whitfield *et al.* (2008) showed exogenous sulphide was rapidly removed from blood and plasma, and by 5% bovine serum albumin in vitro (Whitfield *et al.,* 2008). This suggests that H_2S gas may not be a circulatory signal in blood although its local production undoubtedly produces important biological effects.

The situation may be even more complex. Mustafa *et al.* (2009), showed that endogenous H_2S gas S-sulphhydrates (SHY) proteins acting to convert cysteine -SH groups to -SSH. It has been proposed that this might act as a store for the release of endogenous H_2S. Furthermore, reduced sulphur atoms from the catabolism of H_2S in the mitochondria can be stored as labile sulphur species that can later be released as H_2S in response to a signal (Kajimura *et al.*, 2010). For example, the iron-sulphur (FeS) complex releases H_2S under acidic conditions (Ubuka, 2002). This suggests that H_2S may not be circulating as a gas, but may circulate as bound -SSH groups and other sulphur species. Additionally, this storage facility may explain why Whitfield *et al.* (2008) could not detect H_2S in the plasma.

Endogenous Hydrogen Sulphide Synthesis and Regulation

The endogenous production of H_2S occurs mainly from using the substrate L-cysteine via the action of two pyridoxal-5'-phosphate-dependent enzymes, cystathionine-y-lyase (CSE) and cystathionine-β-synthase (CBS) (Li and Moore, 2007). Both CBS and CSE have been studied for their activities in the liver and kidney (Stipanuk and Beck, 1982; Erickson *et al.,* 1990; Swaroop *et al*., 1992), brain (Abe and Kimura, 1996; Qu *et al*., 2006) and vasculature (Hosoki *et al*., 1997; Al-Magableh and Hart 2011).

Generation of endogenous H_2S has been shown to principally involve CBS in the brain (although more recently, CSE has also been shown to have an important role) (Abe *et al.*, 1996; Telezhkin *et al.*, 2009), while in the periphery CSE is the more widely expressed enzyme (Hosoki et al., 1997).

Abe and Kimura (1996), showed in rat brain, that CBS is highly expressed in the hippocampus. They also demonstrated that the CBS inhibitors hydroxylamine and aminooxyacetate suppressed the production of H_2S in the brain, whereas the CBS activator, S-adenosyl-L-methionine, enhanced H_2S production. In another study on the rat brain, it was shown that H_2S was synthesized in the cortex, also via CBS (Qu *et al.,* 2006). More recently, Telezhkin *et al.* (2009), showed that CSE was expressed in the rat carotid body, localized within clusters of glomus cells. Additionally, using reverse transcription-polymerase chain reactions (RT-PCR), only cystathionine g-lyase (CSE), was detected in primary cultures of rat microglia cells (Lee et al., 2006). This suggests that both CBS and CSE are present and important for the endogenous production of H_2S in the brain.

CBS has also been shown to be expressed in other tissues. For example, CBS was shown to be active in the liver and kidney of the rat (Stipanuk and Beck, 1982). In another study, CBS was show to be expressed in the liver of mice (Al-Magableh and Hart, 2011). In addition, CSE is also expressed in the liver and kidney of both the rat (Stipanuk and Beck, 1982) and the mouse (Al-Magableh and Hart, 2011). By contrast there is very little evidence for CBS expression in the vasculature (Hosoki *et al*., 1997; Al-Magableh and Hart, 2011).

The expression of CSE has been reported in the portal vein and thoracic aorta of rats (Hosoki *et al*., 1997). They showed that the CSE inhibitors, DL-propagylglycine (PPG) and β-cyano-L-alanine (BCA) significantly attenuated H_2S production in these tissues. Al-Magableh and Hart (2011), showed that CSE was also expressed and active in the aorta of mice. Additionally, in other arteries including rat mesenteric arteries (Cheng *et al.*, 2004), and human internal mammary artery (Webb *et al.*, 2008), CSE has been shown to be expressed and to actively produce H_2S from L-cysteine. Therefore, CSE is the main H_2S generating pathway identified in the vasculature. The location of the CSE enzyme in the vasculature was originally thought to be within the vascular smooth muscle (Zhao et al., 2001). However, more recent studies have suggested that CSE is more predominantly localized in the endothelium (Yang *et al*., 2008). Additionally, Al-Magableh and Hart (2011), showed strong evidence for CSE expression on the endothelium of mouse aorta since its removal abolished responses to exogenously applied L-cysteine.

More recently, it has been suggested that H_2S can also be produced from 3-mercaptopyruvate (Shibuya *et al.*, 2009). Cysteine can be converted to 3-mercaptopyruvate by cysteine aminotransferase (CAT) and then 3-mercaptopyruvate sulfurtransferase (3MST) can desulphurate 3-mercaptopyruvate producing pyruvate and H_2S (Kajimura *et al.*, 2010). This system has not yet been extensively studied. However, Shibuya *et al.* (2009), showed that CAT and 3MST are expressed in the endothelium of the rat thoracic aorta and lysates of vascular endothelial cells produced H_2S from

cysteine and α-ketoglutarate (Shibuya *et al.*, 2009). Figure 1.1 shows the known enzymatic pathways of endogenous H_2S production from L-cysteine.

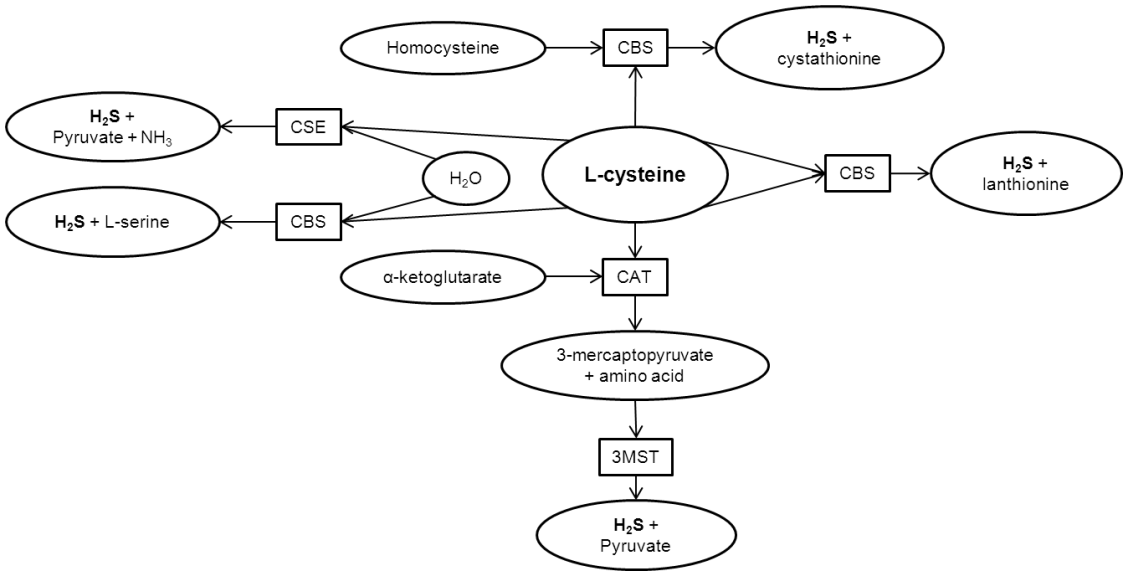

Figure 1.1 The known enzymatic pathways of endogenous H_2S production from L-cysteine. Circles indicate substrates and products, Squares indicate enzymes. CSE, cystathionine-γ-lyase; CBS, cystathionine-β-synthase; CAT, cysteine aminotransferase; 3MST, 3-mercaptopyruvate sulfurtransferase. Adapted from Olson (2011).

The endogenous level of NO has been shown to influence both the expression and activity of CSE in the vasculature. Zhao *et al.* (2001), showed that NO appears to be a physiological modulator of the endogenous production of H_2S by increasing expression of CSE and increasing its activity in several rat tissues, including aorta, tail, artery, mesenteric arteries and ileum. In another study, Zhong *et al*. (2003), showed dysfunction of vascular synthesis of H_2S through CSE was present in rats with hypertension induced by inhibition of NOS with L-NAME. This suggests that CSE is both expressed and activated by the presence of NO. In addition, Zhong *et al*. (2003), showed that exogenous H_2S effectively prevented the development of hypertension induced by L-NAME.

Evolution of Hydrogen Sulphide in Physiology

The ancestral origins of the H_2S producing enzymes have also been investigated. Although the amino acid sequence for CBS purified from rat liver has little, if any, similarity to those of CBS expressed in E. coli, rat CSE does share sequence homology with E. coli CSE (Erickson *et al*., 1990). Amino acid sequence homology between the mammalian CBS (from rat liver) and E. coli *O*-acetylserine (thiol)-lyase (cysK), suggests that mammalian CBS evolved from proteins in lower organisms which catalyze sulphur fixation to form cysteine (Swaroop et al, 1992). Dombkowski *et al.* (2005), investigated the H_2S vasoactivity in different vertebrate classes to determine whether H_2S is universally vasoactive and to identify phylogenetic and environmental trends. The results of this study by Dombkowski *et al*. (2005) suggested that H_2S is a phylogenetically ancient and versatile

vasoregulatory molecule that is opportunistically used to suit both organ specific and species-specific homeostatic requirements.

Effects of Hydrogen Sulphide in the Central Nervous System

In the central nervous system, H_2S has been shown to be involved in a number of physiological processes including neurotransmission (Abe and Kimura, 1996; Nagai *et al.,* 2004; Lee *et al*., 2006) and neuroprotection (Qu et al., 2006; Hu *et al.*,2007).

Astrocytes can release glutamate in a calcium-dependent manner and consequently signal to adjacent neurons (Parpura and Haydon, 2000). Nagai *et al.* (2004), showed in cultured astrocytes, that exogenous H_2S (using NaHS as a donor) caused an increase in intracellular calcium concentrate ($[Ca^{2+}]_i$) and induced calcium waves between astrocytes. In hippocampal slices, Nagai *et al.* (2004), also showed that, while exogenous H_2S on its own did not increase $[Ca^{2+}]_i$ in neurons H_2S may be released in response to neuronal excitation and activate Ca^{2+} channel-induced Ca^{2+} waves in neighbouring astrocytes. In another study, Lee *et al*. (2006) showed in microglia, that inhibition of CSE significantly decreased $[Ca^{2+}]_i$, suggesting that endogenous H_2S may have a positive tonic influence on $[Ca^{2+}]_i$ homeostasis. This suggests that Ca^{2+} signalling between astrocytes and glia may be enhanced by endogenous H_2S. Additionally, Nagai *et al.* (2004), showed that H_2S enhanced responses to glutamate in neurons. Methyl-D-aspartic acid (NMDA) is an amino acid derivative neurotransmitter that mimics the actions of glutamate but has specific NMDA receptors (Watkins and Jane, 2006). H_2S has also been shown to enhance NMDA receptor

function an event associated with the induction of hippocampal long-term potentiation and memory (Abe and Kimura, 1996).

H_2S has been shown to be involved in protecting neurons from apoptosis or degeneration. In a study by Hu *et al*. (2007), H_2S produced an anti-inflammatory effect in lipopolysaccharide (LPS)-stimulated microglia and astrocytes, which occurred due to inhibition of iNOS and p38 mitogen-activated protein kinases (p38 MAP kinase) signalling pathways. Western blot analysis showed that both exogenously applied, and endogenously generated H_2S significantly attenuated the stimulatory effect of LPS on iNOS expression (Hu *et al.,*2007). Lu *et al.* (2010), suggested H_2S may regulate intracellular pH (pH_i) in microglial cells, limiting the damage of activated microglia at the site of injury and providing a neuroprotective role that limits the spread of neurodegeneration. However, permanent occlusion of the middle cerebral artery caused an increase in H_2S levels in the lesioned cortex, as well as an increase in the H_2S synthesizing activity, suggesting that H_2S may have a contributory role in the cerebral ischemic damage that occurs after stroke (Qu *et al*., 2006).

Hydrogen Sulphide in the Digestive System

H_2S has been shown to be involved in the regulation of the physiology of the gastrointestinal tract (Teague *et al*., 2002; Schicho *et al.,* 2006; Matsunami *et al.,* 2009) and liver (Fiorucci *et al*., 2005).

In human and guinea-pig colon, more than 90% of guinea-pig and human submucous and myenteric neurons express CSE and CBS (Schicho *et al.,* 2006). In addition, myenteric interstitial cells of Cajal express CSE

(Schicho *et al.,* 2006). Exogenously applied NaHS produced relaxation of ileum isolated from rabbits and rats, and reduced ACh-mediated contraction of isolated guinea-pig ileum (Teague *et al.,* 2002). In this study, Teague *et al.* (2002) also showed that inhibition of CSE caused a slowly developing increase in the contraction of the guinea-pig ileum to field stimulation and suggested that, H_2S, formed as a consequence of activation of intramural nerves, may have a role in controlling the contractility of the ileum. Schicho *et al*. (2006), observed that both NaHS and L-cysteine caused concentration-dependent increases in chloride secretion in human and guinea- pig submucosal and mucosal preparations. They showed that these responses were significantly attenuated by capsaicin, suggesting that the action of H_2S involved the capsaicin sensitive, transient receptor potential vanilloid receptor 1 (TRPV1) (Schicho *et al.,* 2006). Thus, H_2S may play an important role in regulating both motility and secretory functions of the gastrointestinal tract via the release of neuropeptides from sensory nerves. In support of this mechanism, capsaicin and intracolonically administered NaHS both triggered visceral nociceptive (pain) behaviour accompanied by referred allodynia or hyperalgesia in mice (Matsunami *et al.* 2009).

Both CBS and CSE have been shown to be expressed in the liver of the rat (Stipanuk and Beck, 1982). Increased sinusoidal resistance is a factor in the pathophysiology of liver cirrhosis and depends on the contraction of hepatic stellate cells (HSC) that surround sinusoidal endothelial cell (SEC) (Fiorucci *et al*., 2005). In rats, Fiorucci *et al*. (2005) showed that H_2S induced relaxation of the noradrenaline pre-constricted hepatic microcirculation, and that CSE-derived H_2S was involved in the maintenance of portal venous

pressure in the liver. In addition, they demonstrated that CSE expression was reduced in cirrhotic livers and suggested that this may contribute to the associated development of increased intrahepatic resistance and portal hypertension.

Role of Hydrogen Sulphide in Inflammatory Processes

As mentioned above, H_2S provokes tachykinin-mediated neurogenic inflammatory responses in airways (Trevisani *et al.,* 2005). H_2S also induced anti-inflammatory effects in Lipopolysaccharide (LPS)-stimulated microglia and astrocytes (Hu *et al*., 2007). H_2S has been shown to have several other effects associated with inflammation. For example, both NaHS and L-cysteine injected into mouse paw caused oedema (di Villa Bianca *et al.,* 2010). In contrast, Zanardo *et al*. (2006) observed carrageenan-induced rat paw oedema was suppressed by exogenous H_2S, by acting at the leukocyte-endothelium interface. Furthermore, Zanardo *et al*. (2006) showed that oedema was enhanced by inhibition of H_2S synthesis, via CSE. These rather conflicting data show that endogenous H_2S may mediate inflammation or have anti-inflammatory effects depending on the experimental model or conditions employed.

Effects of Hydrogen Sulphide in the Cardiovascular System

Responses to both exogenous and endogenously produced H_2S have been extensively studied in the vasculature, showing tissue specific effects (Hosoki et al., 1997; Zhao et al., 2001; Cheng et al., 2004; Dombkowski et al 2005; di Villa Bianca *et al.,* 2011; Al-Magableh and Hart 2011).

H_2S has been shown to have cardioprotective properties. Ischemia is a restriction in blood supply which results in damage or dysfunction of tissue. Ischaemic preconditioning is a technique used to produce resistances to ischaemia by repeated but short episodes of ischaemia. This process was first identified in a study on dog coronary arteries by Murry *et al*., (1986) where ischaemic preconditioning was shown to protect the myocardium against a subsequent ischaemic insult. Bian *et al*., (2006) demonstrated that endogenous H_2S contributed to the ischemic preconditioning mechanism providing cardioprotection subsequent to an ischaemic insult. In addition, exogenous administration of H_2S, has been shown to protect myocytes and their contractile activity by directly scavenging oxygen-derived free radicals and reducing the accumulation of lipid peroxidation products (Geng *et al.*, 2004).

In the majority of vascular preparations, H_2S has been shown to cause vasorelaxation. For example, in rat aortic tissue, Zhao *et al.* (2001) showed that H_2S induced a vasorelaxation, comprising a minor endothelium-dependent component and a larger direct effect on smooth muscle cells. Similar observations were reported by Cheng *et al.* (2004), where both H_2S gas and NaHS evoke a concentration-dependent vasorelaxation of the isolated rat mesenteric arterial bed. These responses were mediated, in part, by the endothelium via an EDHF-related mechanism. Although these studies suggested a role for the endothelium in H_2S-induced vasorelaxation, Tang *et al.* (2005), suggested that H_2S directly stimulated ATP-sensitive K^+ (K_{ATP}) channels on vascular smooth muscle cells (VSMCs) isolated from small mesenteric arteries, with associated hyperpolarization and relaxation.

Additionally, Magableh and Hart (2011), showed both exogenous NaHS and endogenously generated H_2S, from the addition of L-cysteine caused a vasorelaxation in the mouse aorta. This led them to suggest that CSE may have a role in the regulation of vessel function and basal vascular tone.

By contrast, H_2S has also been shown to cause contraction in many preparations, in some of which there is also a vasorelaxation. For example, in both mouse aorta (Kubo *et al.,* 2007) and rat aorta (Ali *et al.*, 2006; Lim *et al.,* 2008), exogenous application of low concentrations of both H_2S gas and NaHS caused a vasocontractile response. Kubo *et al*. (2007) found in mouse aorta, the contractile activity of NaHS was attenuated by removal of the endothelium. Nonetheless, the differential nature of the response produced by H_2S requires further consideration.

The effects of the experimental conditions used to assess responses to H_2S were investigated by Dombkowski *et al.* (2005). They observed that pre-constriction of the rat aorta with noradrenaline was associated with NaHS-inducing a vasorelaxation whereas without pre-constriction, NaHS induced a contractile response. Koenitzer *et al.* (2007) showed that, in rat aorta pre-contracted with phenylephrine, H_2S produced a biphasic response, with contraction at low concentrations (5 - 100 µM) and vasorelaxation at higher levels (200 - 400 µM). These initial experiments were carried out using buffer where the O_2 level was 200 µM. However, when vessels were studied at lower (40 µM) O_2 levels H_2S elicited only vasorelaxation responses (Koenitzer *et al.*, 2007). This suggests that response to NaHS may be both dependent on H_2S concentration and O_2 level. di Villa Bianca *et al.* (2011), observed that infusion of NaHS in the isolated and perfused rat

mesentery arterial bed caused a biphasic effect, with lower concentrations of NaHS causing vasoconstriction and higher concentrations causing vasodilatation. A similar pattern of responses was obtained using L-cysteine suggesting a similar pattern with endogenously produced H_2S (di Villa Bianca *et al.,* 2011).

In *vivo*, H_2S has been shown to be involved in regulating blood pressure. Zhong *et al.* (2003), showed that dysfunction of vascular CSE was found in L-NAME-induced hypertensive rats and exogenous H_2S effectively prevented the development of hypertension induced by L-NAME. Furthermore, mice lacking the CSE gene display pronounced hypertension and diminished endothelium dependent vasorelaxation (Yang *et al.,* 2008). This suggests that endogenous generation of H_2S from CSE is important in maintaining normal blood pressure, and relaxes blood vessels to reduce blood pressure.

These data suggest that there may be multiple mechanisms involved in mediating the vascular actions of H_2S and that these may be tissue specific.

Mechanisms Mediating Hydrogen Sulphide Responses

Oxygen-Sensitive Responses

As previously stated, the main mechanism of H_2S toxicity is via potent inhibition of mitochondrial cytochrome c oxidase. These effects have been described even with relatively low concentrations of H_2S (>50 µM) (Dorman *et al.,* 2002). As cytochrome c oxidase is critically involved in ATP production

from oxygen in the mitochondria, H_2S-induced inhibition of this enzyme system can lead to a decrease in intracellular ATP concentrations, causing energy deficiency and intracellular acidosis (Kiss *et al.*, 2008). This opens the possibility that inhibition of cytochrome c oxidase may mediate some of the physiological effects of H_2S. In addition, exogenous H_2S has been shown to effectively protect myocytes and contractile activity in part by directly scavenging oxygen free radicals and reducing the accumulation of lipid peroxidations (Geng *et al*., 2004).

It has been suggested that H_2S acts as an oxygen sensor/transducer, sensing the effects of hypoxia in non-mammalian tissue (Dombkowski *et al*., 2006). Recently Olson (2008) suggested that endogenous H_2S production may contribute directly to cellular O_2 sensing in vertebrate vascular smooth muscle. The proposed mechanism of this O_2 sensing system is that a decrease in tissue O_2 levels decreases the rate of H_2S oxidation, thus increasing the concentration of biologically active H_2S (Olson, 2008). In specialized chemoreceptor organs of teleost fish it has been demonstrated that the endogenous concentration of H_2S is dependent upon the balance between its cytosolic generation and its mitochondrial oxidation (Olson *et al.,* 2008a). Furthermore, H_2S caused contraction of the dorsal aorta and efferent branchial arteries of hagfish and lampreys but not the ventral aorta or afferent branchial arteries; effects similar to those produced by hypoxia (Olson *et al*., 2008b).

In some studies on the vasculature, the O_2 concentration has been shown to directly affect responses to H_2S (Koenitzer *et al.*, 2007; Kiss *et al.*, 2008). In a study by Koenitzer *et al.* (2007) in rat aorta, H_2S causes

contraction at high O_2 (200 µM) levels but relaxation at lower O_2 levels (40 µM). In addition, Kiss *et al.* (2008) showed, in rat aortic rings, that relaxations to H_2S were dependent upon the ambient O_2 concentration, with relaxation responses to H_2S occurring at lower concentrations and contractile responses at high O_2. They proposed the higher O_2 levels resulted in oxidation of H_2S, thus removing it from the tissue or that oxygen derived H_2S products had a direct contractile effect. Thus the gassing conditions used experimentally can fundamentally affect the nature of the response to H_2S.

Interactions with Nitric Oxide

In different tissue and under different experimental conditions, H_2S and NO have been shown to interact (Zhao *et al.*, 2001; Hu *et al.*, 2007; Kubo *et al.*, 2007). Zhao *et al.* (2001), showed that in rat aorta, tail artery, mesenteric arteries and ileum, NO was a modulator of the endogenous production of H_2S by increasing CSE expression and stimulating CSE activity. In another study by, Zhong *et al.* (2003), showed found that there was dysfunction of the vascular CSE pathway in L-NAME-induced hypertensive rats, suggesting that NO may influence both the expression and activation of CSE production of endogenous H_2S in the vasculature. By contrast, Kubo *et al*. (2007) showed that exogenously applied NaHS inhibited production of NO from bovine eNOS, although this effect was only seen using high concentrations (above 300 µM NaHS). Thus H_2S and NO can have regulatory effects on each other. The physiological relevance of these effects requires further study.

H_2S has also been shown to cause the release of NO from the vascular endothelium. Both inhibition of eNOS with L-NAME, or physical

removal of the endothelium, attenuated responses to H_2S, in the rat aorta (Zhao *et al.,* 2001). In a more complicated manner, NO has been implicated in mediating contractile responses to H_2S in some preparations (Ali *et al.*, 2006; Kubo *et al*., 2007; Webb *et al.*, 2008). Ali *et al.* (2006) showed that low NaHS concentrations increased mean arterial pressure in anaesthetised rats and that this was prevented after inhibition of NOS with L-NAME. Ali *et al.* (2006) proposed that NO and H_2S were interacting to produce a pressor response in a way where H_2S reduced the biological availability of NO, preventing its usual vasodilatory influence, thus leading to a pressor action. Similarly H_2S-induced contractile responses were abolished in the presence of L-NAME in the rat aorta (Kubo et al., 2007) and human internal mammary artery (Webb *et al.*, 2008). Ali *et al.* (2006) suggested that H_2S and NO interact leading to the formation of a novel molecule that was inactive, i.e. it did not relax blood vessels either in vitro or in vivo. Whiteman *et al.* (2006), subsequently showed that NO reacts with H_2S to form a biologically inert nitrosothiol compound.

Interactions with Cyclic Nucleotide Pathways

The role of cyclic nucleotides as modulators of vascular smooth muscle tone has been widely studied and increase in either intracellular cGMP or cyclic adenosine monophosphate (cAMP) both causes vasorelaxation (Taylor *et al.*, 1999). In some instances H_2S-induced release of NO (Zhao *et al.*, 2001) leads to activation of soluble guanylate cyclase, causing an increase in the intracellular cGMP concentration within smooth muscle cells and subsequent relaxation.

In some non-vascular tissues, the effects of H_2S have been shown to be mediated via modulation of cAMP production (Kimura, 2000; Lee *et al*., 2006; Yong *et al*., 2008; Lim *et al.*, 2008). In a study by Kimura (2000), exogenous H_2S increased the production of cAMP in primary cultures of rat brain cells, neuronal and glial cell lines, and Xenopus oocytes. In addition, Lee *et al.* (2006), showed that in primary cultured microglial cells, the increase in $[Ca^{2+}]_i$ in response to exogenous H_2S involved cAMP-dependent protein kinase. In contrast, Yong *et al*. (2008) found in cardiac myocytes that exogenous application of H_2S negatively regulated β-adrenergic function via inhibition of adenylyl cyclase, reducing intracellular concentrations of cAMP. In the vasculature, Lim *et al.* (2008), showed that H_2S attenuated forskolin-induced cAMP accumulation and suggested that the contractile effect of H_2S observed in isolated rat aorta may be due, in part, to H_2S reducing intracellular cAMP levels. These differences between cardiovascular and neuronal effects of H_2S have been suggested to be due to the expression of specific isoforms of cAMP and regulatory enzymes in different tissues (Lim *et al.,* 2008).

Prostaglandin Production

The generation of prostaglandin has also been suggested to mediate some of the actions of H_2S (Koenitzer et al., 2007; di Villa Bianca *et al.,* 2011). Prostaglandin production involves enzymatic release of free arachidonic acid from membrane phospholipids, requiring phospholipases, mainly phospholipase A2 (Dennis, 1994). Two COX isoforms, COX-1 and COX-2, metabolize arachidonic acid to prostaglandin H2, which is a common

precursor to the production of thromboxane A2, prostacyclin, and other prostaglandins (Caughey et al., 2001). Prostacyclin and thromboxane A2 play an essential role in the maintenance of vascular homeostasis, where prostacyclin is a vasodilator and an inhibitor of platelet aggregation and thromboxane A2 is a vasoconstrictor and a promoter of platelet aggregation (Bunting *et al*., 1983). Koenitzer *et al.* (2007) showed that indomethacin, a COX inhibitor, enhanced the contractile effects of H_2S in rat aorta, suggesting a 'hidden' vasorelaxatory effect of H_2S involved COX-generated prostaglandins. Furthermore, in the rat mesenteric arterial bed, NaHS-induced vasorelaxatory effects were inhibited by 4-(4-octadecylphenyl)-4-oxobutenoic acid, a phospholipase A2 inhibitor (di Villa Bianca *et al.,* 2011).

Hyperpolarization Mechanism

In some tissues, vasorelaxation responses to H_2S have been shown to occur via a hyperpolarization mechanism (Zhao *et al.*, 2001; Tang *et al.,* 2005; Cheang *et al.* 2010). High concentrations of extracellular K^+ are known to inhibit membrane hyperpolarization (Edwards *et al*., 1998). In a study using rat aorta, Zhao *et al.* (2001), showed that H_2S induced vasorelaxation was abolished after tone was induced using 100 mM KCl. This hyperpolarization mechanism has also been shown in rat coronary arteries where NaHS-induced relaxation is significantly reduced in the presence of 60 mM KCl (Cheang *et al.* 2010). More direct evidence has been obtained using patch clamp techniques. Zhao *et al.* (2001) showed H_2S caused membrane hyperpolarization of aortic VSMCs. In addition, Tang *et al.* (2005), showed

that single VSMCs isolated from rat mesenteric arteries hyperpolarized in response to H_2S.

Interactions with Potassium Channels

Hyperpolarization of vascular smooth muscle can occur via modulation of K^+ channels (Edwards *et al*., 1998). Several K^+ channels have been implicated in mediating responses to H_2S, including K_{ATP} channels (Zhao *et al.*, 2001; Cheng *et al.*, 2004; Tang *et al.,* 2005; Kubo *et al*., 2007), K_{Ca2+} channels (Zhao *et al.*, 2001; di Villa Bianca *et al.,* 2011) and voltage gated potassium channels (K_V)(Cheang *et al.* 2010).

In early studies by Zhao *et al.* (2001), the K_{ATP} channel specific inhibitor, glibenclamide, inhibited H_2S-induced vasorelaxation in rat aortic rings. Similarly, Cheng *et al.* (2004) showed that the H_2S-induced vasodepressor response in rat mesenteric arterial beds was partially due to the activation of K_{ATP} channels. In isolated aortic VSMCs, Zhao *et al.* (2001), showed that H_2S directly acts on K_{ATP} channels, as is also the case in VSMCs isolated from rat mesenteric resistance (Tang *et al.,* 2005). Despite these early observations, H_2S-induced vasorelaxation in some tissues has been shown not to exclusively involve K_{ATP} channels. For example, Kubo *et al*. (2007) showed the vasorelaxatory effect of H_2S involved not only K_{ATP} channels, but also a K_{ATP} channel-independent mechanism in rat aorta. While in the mouse aorta the H_2S induced vasorelaxation was completely independent of K_{ATP} channel activation.

K_{Ca2+} channels activation has been implicated in NaHS-induced vasorelaxation in rat aorta (Zhao *et al.*, 2001) and the rat mesenteric arterial

bed (di Villa Bianca *et al.,* 2011). This conclusion was based on the fact that apamin and charybdotoxin, inhibitors of small and intermediate conductance K_{Ca2+} channels inhibited NaHS responses. Small and intermidate conductance K_{Ca2+} channels are located on endothelial cells and cause the release of K^+ into the extracellular fluid. This small increase in extracellular K^+ causes outwards current of K^+ through the inward rectifying K^+ channels in smooth muscle cells causing relaxation (Nelson and Quayle, 1995). Cheang *et al*. (2010) showed in the rat coronary artery, that NaHS-induced relaxation was significantly reduced by 4-aminopyridine at a concentration that blocks voltage-sensitive K^+ channels. Therefore, the K^+ targets of H_2S may be tissue specific and influenced by experimental conditions.

Interactions with Calcium Channels

H_2S-induced vasorelaxation have been shown to be dependent upon inhibition of extracellular calcium entry, in rat aorta (Zhao and Wang, 2002) and in mouse aorta (Al-Magableh and Hart 2011). This may be due to the opening of K_{ATP} channels leading to membrane hyperpolarization, which in turn may close voltage-gated Ca^{2+} channels or may be due to H_2S directly inhibiting voltage-gated Ca^{2+} channels (Zhao and Wang, 2002). Al-Magableh and Hart (2011), suggested the latter mechanism, albeit that they used rather high concentrations of NaHS (10mM).

In non-vascular tissues, H_2S induced modulation of Ca^{2+} channels has been shown. For example, Sun *et al*., (2008) showed that electrically-induced $[Ca^{2+}]_i$ transients and contraction of single cardiomyocytes were reduced by NaHS, suggesting that H_2S can inhibit L-type calcium channels in cardiomyocytes. In addition, Matsunami *et al*. (2009), showed that intraperitoneal NaHS facilitated intracolonic capsaicin-evoked visceral nociception in mice and this effect was abolished by the T-type channel blocker, mibefradil.

Effects of H_2S on Intracellular pH

H_2S has also been shown to have an influence on the control of internal pH (pH_i) in various cell types. For example, Lee *et al.* (2007) using A7r5 cells (derived from rat BD1X embryonic rat aortic smooth muscle) showed that NaHS decreased pHi by enhancing the activity of a 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) sensitive Cl^-/HCO_3^- exchanger. Similar observations have been made in microglial cells (Lu *et*

al., 2010). Interestingly, DIDS has been shown to inhibit NaHS-induced relaxation in rat aorta (Kiss *et al.*, 2008; Al-Magableh and Hart, 2011). Kiss *et al.* (2008) suggested that H_2S caused vascular relaxation via both the metabolic inhibition of cytochrome c oxidase and activation of the Cl^-/HCO_3^- exchanger, causing intracellular acidosis and depletion of ATP, with subsequent activation of K_{ATP} channels. However, it should be noted that as well as its effects on the Cl^-/HCO_3^- exchanger, DIDS is also a non-specific Cl^- channel inhibitor (Jentsch *et al*., 2002). Therefore, DIDS may be affecting responses to H_2S via inhibition of Cl- channels as well as the Cl^-/HCO_3^- exchanger. Nonetheless, acid loading is an effect of increased H_2S concentrations and may mediate some of its biological effects. This may be particularly important in certain metabolic situations such as acidosis where an H_2S-induced decrease in pH_i may increase K_{ATP} currents, thus causing a relaxation (Ishizaka and Kuo 1996).

Hydrogen Sulphide effects on Sensory Nerves

As well as the direct effects of H_2S on cellular function, the release of neuropeptides from sensory nerves has also been shown to mediate responses to H_2S in both the guinea-pig (Schicho *et al*., 2006; Krueger *et al*., 2010) and human (Krueger *et al*., 2010) colon. In the guinea-pig colon, NaHS was shown to evoke chloride secretion that was inhibited by capsaicin desensitization of sensory nerves and the specific TRPV1 antagonist capsazepine (Schicho *et al*., 2006). In addition, Krueger *et al*. (2010), showed that NaHS evoked mucosal secretions from guinea-pig and human colonic tissue were significantly reduced by capsazepine, suggesting that

H_2S responses were mediated via the release of substance P and activation of neurokinin-1, -2 or -3 receptors. Additionally, Trevisani *et al*. (2005) showed that NaHS provoked tachykinin-mediated neurogenic inflammatory responses in guinea-pig airways by activation of TRPV1 receptors on sensory nerves. Patacchini *et al.* (2005) also showed that H_2S stimulated capsaicin-sensitive primary afferent neurons in the rat detrusor muscle. This response was insensitive to capsazepine, the specific inhibitor of TRPV1. Patacchini *et al.* (2005), suggested that H_2S may be acting on either, a receptorial domain on the TRPV1 cation channel independent from those bound by vanilloids, or another transient receptor potential cation channel that is co-expressed with TRPV1 on primary afferent neuron terminals.

The Aims of the Current Study

In recent years it has become apparent that H_2S is an important biological mediator. In the vasculature, it produces complex responses: contraction in some blood vessels, relaxation in others via multiple mechanisms. Indeed, different responses and mechansism have been reported in the same blood vessel, particularly the rat aorta. Some of this variability may reflect the conditions employed to study the vascular responsiveness, in particular the oxygen levels used to gas tissues. The aim of this thesis was to examine the relationship between H_2S and oxygen in determining vascular responsiveness, and was conducted using porcine splenic and mesenteric arteries. Studies were also conducted using porcine splenic veins, since few studies have examined venous function. Additionally, there is little information in the small arteries that are more

important determinants of vascular resistance, and hence blood pressure, so studies were extended to look at small arteries isolated from the rat mesentery.

Chapter 2:

Materials and Method

Isometric Tension Recording

Porcine tissue was obtained from Wood's and Son Abattoir (Clipston, Nottingham, UK) from breeds of modern hybrid pig but the sex and exact age of each animal was unknown. Porcine spleens and mesenteric beds were isolated at the abattoir and stored in PSS at 4˚C during transport to the laboratory. On arrival, gross dissection of 1st order mesenteric arteries, splenic arteries and splenic veins were carried out leaving the adipose and connective tissue directly surrounding the vessels intact. Vessels were stored overnight in PSS at 4˚C pre-gassed with a 95% O_2: 5% CO_2 gas mixture. The next day vessels were pinned in a dissection dish and the remaining adipose and connective tissue was removed whilst ensuring vessels were not stretched or damaged. The length of the vessel segments was approximately 5 millimetres. Tissue was kept in PSS and allowed to normalize to room temperature before being setup in the isometric tension recording system.

Vessels were attached to two metal hooks placed through the lumen, ensuring that the hooks were not overlapping. One hook was attached to a glass rod and the other was attached to a non-stretchable silk thread. The vessel was then transfer to an organ bath where the glass rod was anchored into the system (figure 2.1). The thread attached to one of the hooks was attached to a force transducer (AD Instruments, UK) on a rack and pinion, enabling adjustment of tension. The organ bath contained 20ml PSS and was kept at a constant temperature of 37 ˚C using a thermal circulator pump. The PSS was constantly gassed with the required gas mixture for each experiment.

The force transducer was attached to a Bridge Amplifier (AD Instruments, UK) to measure alterations in voltage recorded by the transducer due to changes in the tension applied to the transducer. The Bridge Amplifier was in turn connected to a PowerLab unit (AD Instruments, UK), which connected the transducer to a computer where data was recorded using Lab Chart software (AD Instruments, UK). Transducers were calibrated with a 10g weight daily to ensure consistency of measurement.

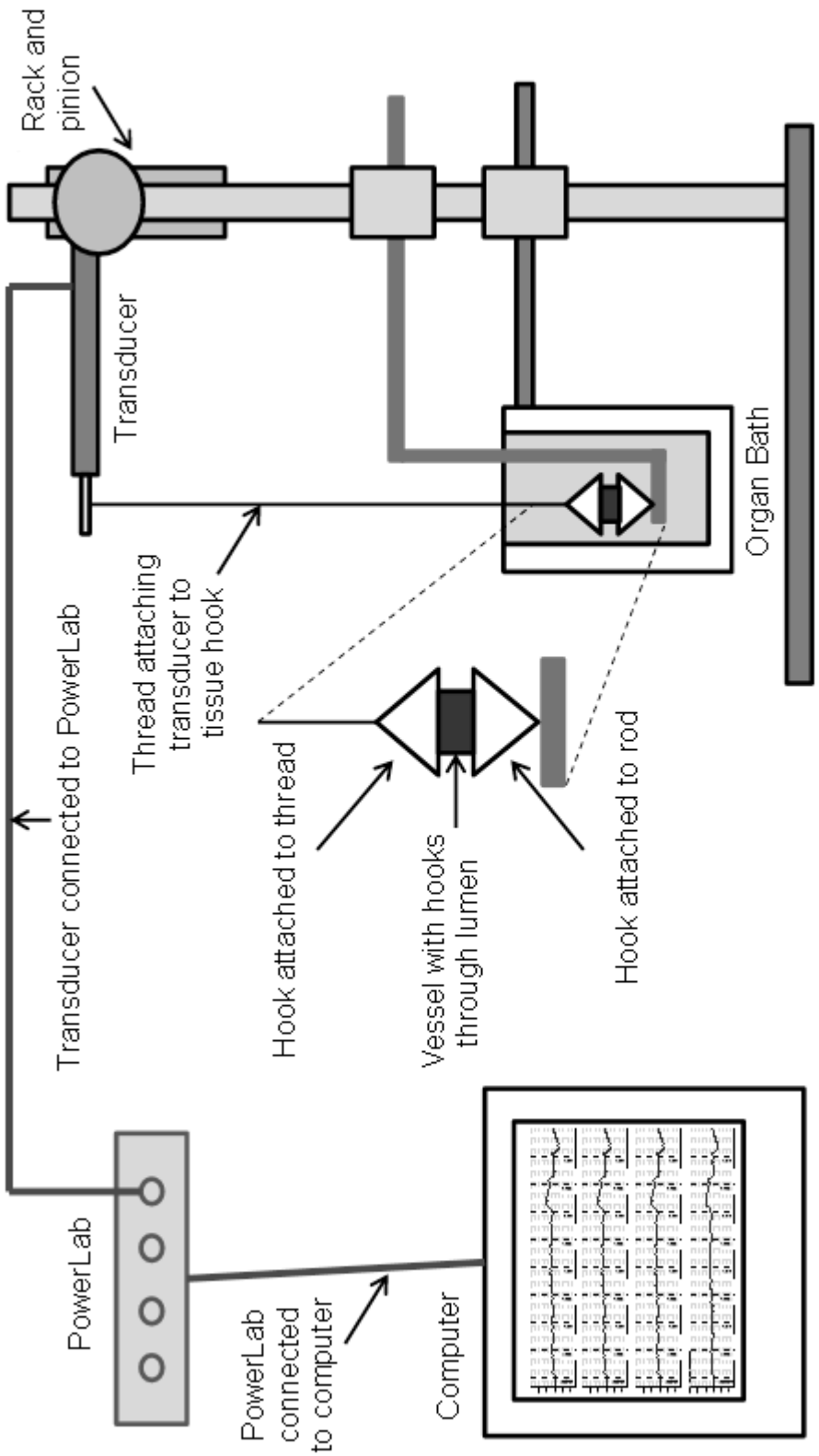

Figure 2.1 Schematic showing the isometric tension system and the set up of a vessel in an organ bath. Changes in vessel tension were measured by a transducer as changes in volts. These measurements were recorded on a computer via a PowerLab and converted to tension (g).

Pressure Myography

Male Wistar rats, approximately 225 - 275 g in weight were obtained from Charles River UK. Rats were killed using the Code of Practice for the Humane Killing of Animals under Schedule 1 of the Animals (Scientific Procedures) Act 1986, by stunning and bleeding. The mesenteric bed was dissected out of the abdomen and placed in a dissection dish that contained physiological salt solution (PSS) at 4˚C. Second order mesenteric arteries were carefully dissected out of the bed removing the surrounding adipose tissue with the aid of a dissection microscope (Nikon) using surgical forceps, ensuring vessels were not stretched or damaged. The length of the vessels was usually between 3 and 6 millimetres. The tissue was kept in PSS at 4˚C until vessels were set up in the pressure myograph.

Pressure myography was carried out using the Halpern pressure myograph (Living Systems Instrumentation, USA) which measure changes in the diameter of small vessels. Under a dissection microscope, small arteries were visualized and dissected before the vessels were transferred into a single vessel organ bath and cannulated between two micro cannulae (Figure 2.2). One of the cannulae was attached to a micrometer that allowed manipulation of the length of the vessel segment. Vessels were tied onto the cannulae on both ends using single strands of fine nylon thread. One end of the cannulated vessel system was closed off and the other was connected to a pump enabling pressurization of the vessel to a set pressure that was monitored by a pressure transducer connected in series (Living Systems Instrumentation, USA).

The bath was placed on to an inverted microscope (Olympus-CK2), which was connected to a CCTV camera (Panasonic) that transferred the image to a television monitor (Burle). A video dimension analyzer (Living Systems Instrumentation, USA) was connected to the imaging equipment to allow monitoring of vessel diameter. Data was recorded through a Power Lab (AD Instruments, UK) and PC computer, using Lab Chart software (AD Instruments, UK). The tissue was perfused with PSS, gassed with 95% air: 5% CO_2 (unless otherwise stated) and maintained at a temperature of 37 ˚C. A peristaltic pump controller (MasterFlex) (Cole Parmer Instruments) allowed perfusion of PSS at a rate of approximately 50 ml/min.

Any leaks in the vessel, or at the ties, were identified when the servo unit failed to hold pressure when the unit was switched to manual. Only Leak-free vessels were used in experiments. Vessels were left at least 30 minutes to equilibrate in the system before exposure to any drugs. All experimental substances were added to the PSS, which was recirculated.

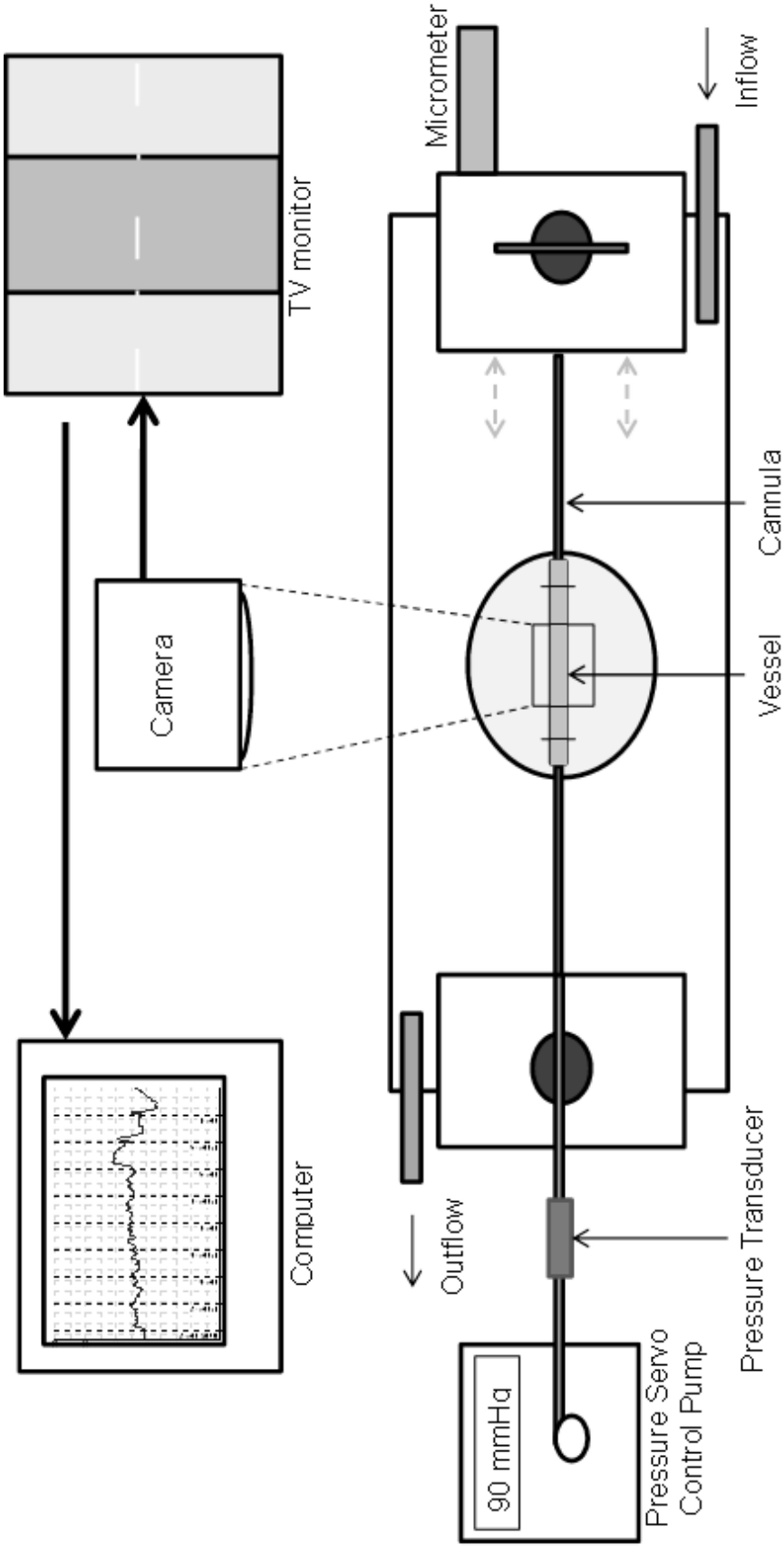

Figure 2.2 Schematic showing the experimental set up and organ bath set up used for pressure myography. Cannulated vessels were pressure in an organ bath and imaged using a camera connected to a television monitor. A video dimension analyzer determines the vessel diameter and this was recorded on a computer.

Statistics and Data Analysis

Data analysis was carried out using Prism (Graph Pad Software). Data was expressed as mean ± S.E.M. where n = the number of vessels used. The difference between means was considered statistically significant at a value of $P < 0.05$. Differences between concentration response curves were assessed using two-way ANOVA in conjunction with the Bonferroni post-hoc test to assess possible difference at individual concentrations. When data series were not normally distributed differences between the maximum relaxation and maximum contraction were assess using the Mann-Whitney U test. To compare the concentrations and levels of induced tone of pre-constriction agents, the Student's t-test was used.

Gas Mixtures

In several chapters the effect of changing the oxygen concentration in the gas mixture used to gas the PSS was examined. The gas mixtures used were 95% O_2: 5% CO_2, 95% Air: 5% CO_2 and 95% N_2: 5% CO_2. The PSS was pre-gassed with each mixture for at least 30 minutes before set up of the vessel to ensure gaseous equilibration.

Direct measurement of the partial pressure of oxygen was taken in sample experiments using an O_2 sensing electrode. The probe was calibrated using a two point calibration method. The first calibration point value was obtained by the addition of dithionate (0.5mM) to 20ml dH_2O to remove all O_2 from the solution. The second value was obtained from data

tables of O_2 concentration in stirred dH_2O under standard conditions and specific temperatures.

Drugs and Solutions

The following solutions were used:

Physiological salt solution (PSS) contained (in mM/L): NaCl 118; KCl 4.8; $NaHCO_3$ 25; KH_2PO_4 1.25; $MgSO_4$ 1.2; Glucose 11.1; and $CaCl_2$ 1.25. In raised extracellular K+ PSS equimolar replacement of NaCl with KCl was used to maintain osmolality.

The following compounds were used:

From Axxora (UK) Ltd, Nottingham, UK: endothelin-1

From Pharmnova AB, Gothenburg, Sweden: N-[2-[5-Amino-1(S)-[4-(4-pyridinyl)piperazin-1-ylcarbonyl]pentylamino]-1(R)-(3,5-dibromo-4-hydroxybenzyl)-2-oxoethyl]-4-(2-oxo-1,2,3,4-tetrahydroquinazolin-3-yl)piperidine-1-carboxamide (BIBN 4096)

From Sigma-Aldrich Ltd, Dorset, UK: sodium hydrosulphide hydrate (NaHS); L-cysteine; DL-propargylglycine (PPG); aminooxyacetic acid (AOAA); tetraethylammonium (TEA); glibenclamide; N-nitro-L-arginine methyl ester hydrochloride (L-NAME); indomethacin; 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS); 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB); anthracene 9 carboxylic acid (A9C); capsaicin; methoxamine; noradrenaline

From Tocris Bioscience, Bristol, UK: 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619)

Chapter 3:

Differential Effect of NaHS under Low and High Oxygen Levels in Porcine Mesenteric Arteries

Introduction

Over the last 10 years it has been shown that H_2S is produced endogenously and is a biologically important molecule. For example, in the central nervous system, H_2S has neuro-protective effects that directly promote cell survival of both glia and neurons (Tan *et al.*, 2010). In the kidneys, H_2S can stimulate natriuresis and diuresis (Beltowski, 2010) and H_2S may have important immune functions (Li *et al.*, 2010). H_2S is mainly generated from L-cysteine by two pyridoxal-5'-phosphate-dependent enzymes, cystathionine-y-lyase (CSE) and cystathionine-β-synthase (CBS) (Li and Moore, 2007). Generation of endogenous H_2S has been shown to principally involve CBS in the brain (Abe *et al.*, 1996) while in the periphery, CSE may be the more important enzyme (Hosoki et al., 1997).

H_2S has also been shown to have important effects in the vasculature. In studies using large conduit arteries, H_2S has been shown to cause concentration-dependent vasorelaxation. This has been shown in aortic rings isolated from rats (Zhao *et al.*, 2001; Zhao and Wang, 2002) and human internal mammary arteries (Webb *et al.*, 2008). NaHS is commonly used as an H_2S donor since it dissociates to Na^+ and HS^-; the latter then partially binds H^+ to form un-dissociated H_2S (Lowicka and Beltowski, 2007). This is a useful way of getting H_2S into solution without having to use the toxic gas. In the majority of studies the reported mechanism of H_2S and NaHS-induced vasorelaxation has been shown to be via activation of ATP sensitive K^+ channels (K_{ATP}) located in the smooth muscle, since the specific K_{ATP} channel inhibitor, glibenclamide, attenuated responses to either H_2S or

NaHS, e.g. in rat aorta (Zhao *et al.*, 2001). Patch-clamp techniques have also shown direct activation of K_{ATP} channels by H_2S, leading to hyperpolarization of smooth muscle cells (SMCs) isolated from rat mesenteric arteries (Zhao *et al.*, 2001; Cheng *et al.*, 2004; Tang *et al.,* 2005).

Although K_{ATP} channels have been shown to be a target for H_2S, more recent evidence suggests the vasoactive effects of H_2S are more complex and tissue specific. For example, Zhao *et al.* (2001), found that the NOS inhibitor, nitro-L-arginine methyl ester (L-NAME) (100 mM) attenuated H_2S-induced relaxation in rat aorta, suggesting the involvement of NO. In addition to vasorelaxation, it has been shown H_2S can induce vasoconstriction in some circumstances. For example, in the rat aorta H_2S caused concentration-dependent constrictions at lower concentrations than those required to produce a vasorelaxation (Ali *et al.,* 2006; Koenitzer *et al.*, 2007). Observations by Ali *et al.* (2006), showed that the NaHS-induced contractile response was sensitive to L-NAME and suggested that H_2S was interacting with NO, to inactivate the latter. Similarly, Lim *et al.* (2008), also suggested that interactions with NO, contributed to the H_2S-induced contractile responses in rat aorta. Subsequent studies suggested a chemical interaction between H_2S and NO, with the formation of an inactive nitrosothiol compound (Whiteman et al., 2006), with the contractile activity of H_2S occurring by the removal of basal NO and obviating its usual vasorelaxatory influence. At concentrations above 100 µM, NaHS has also been reported to directly inhibit recombinant bovine endothelial NOS activity which could contribute to the vasoconstrictor response observed (Kubo et al., 2007). Furthermore, the responses to H_2S may also be sensitive to O_2. For

example, in rat aorta, Koenitzer *et al.* (2007), found that at higher O_2 levels (200 µM), H_2S caused contractile responses, while at lower O_2 levels (40 µM), H_2S produced relaxation responses.

The aim of the present study was to further investigate the effects of NaHS on arteries using a different species, namely the pig. The present chapter examined the effects of NaHS on porcine mesenteric arteries.

Materials and Methods

Set up and General Protocol

Porcine mesenteric arteries were isolated and set up in an isometric tension recording system as described in the methods chapter. Vessels were set up in baths containing 20 ml PSS at 37˚C and gassed with either 95% O_2:5% CO_2, 95% Air:5% CO_2 or 95% N_2:5% CO_2. The PSS was pre-gassed with each mixture for at least 30 minutes to ensure gaseous equilibrium of the PSS. Vessels were pre-tensioned to approximately 10-12 g and allowed to relax for 30-40 minutes to achieve a stable baseline tone. Thereafter KCl (60 mM) was added to the bath and vessels were allowed to contract. After the response plateaued, the KCl was washed out with fresh PSS at least 2 times at 5 min intervals. Following the return to a stable baseline and, after a further 20 minutes, exposure to KCl was repeated.

After three repeats of responses to KCl, any test compounds used in the experiment were added and allowed at least 30 minutes incubation time. The following test compounds were used: indomethacin (1 µM) to inhibit the cyclooxygenase enzyme (Iwatani *et al.*, 2008); the NOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME) (100 µM) (Zhao *et al.*, 2001); the non-specific K^+ channel blocker, tetraethylammonium (TEA) (1 mM) (White and Hiley, 1997) and the selective K_{ATP} channel inhibitor, glibenclamide (10 µM) (Zhao *et al.*, 2001). After this period vessels were pre-contracted to a level of 30-70% of the maximum KCl response. In most experiments, this was achieved using either, the thromboxane A2 receptor agonist, U46619 or the endothelin receptor agonist, endothelin-1. Once vessel tension had

stabilized, a concentration-response curve to NaHS was obtained by adding it in a cumulative manner, in 3-fold increments between 100 nM and 3 mM, with a period of 5 minutes between additions.

In all experiments a second segment from the same tissue was set up and used as a control i.e. only one concentration response curve to NaHS was obtained in each preparation.

In the initial experiments examining responses to NaHS and L-cysteine, the thromboxane A2 agonist U46619 was used to pre-constrict the vessels. This was added to the PSS starting at a concentration of 1 nM and increased in small increments to achieve the appropriate level of pre-constriction. In instances where U46619 was unable to produce a stable tone, for example when using the 95% N_2:5% CO_2 gas mixture, the endothelin receptor agonist, endothelin-1, was used, raising the concentration from 1 nM. In some experiments a PSS containing raised extracellular KCl was used to contract vessels. The high K^+ PSS was made by substituting NaCl with KCl to achieve a concentration of 30 mM K^+ in the PSS.

Endogenous Production of H_2S

Some experiments were carried out to investigate the potential generation of endogenous H_2S from L-cysteine in mesenteric arteries. To do this, vessels were pre-contracted to 30-70% of the maximum KCl response using U46619. Thereafter, responses to L-cysteine were obtained by adding

it in 3-fold increments over the concentration range 10 µM to 30 mM, with a period of at least 5 minutes between additions. Some experiments were conducted the presence of the following test compounds; dl-Propargylglycine (PPG) (10 µM; Cheng *et al.*, 2004) an inhibitor of CSE and Aminooxyacetic acid (AOAA) (10 µM; Abe *et al.*, 1996) an inhibitor of CBS.

Results

Initial experiments investigated responses to exogenous NaHS in porcine 1st order mesenteric arteries gassed with a 95% air:5% CO_2 gas mixture after pre-constriction with U46619. NaHS had little effect at lower concentrations, but produced a contraction at concentrations of 100 µM and above (Figure 3.1a and 3.1b).

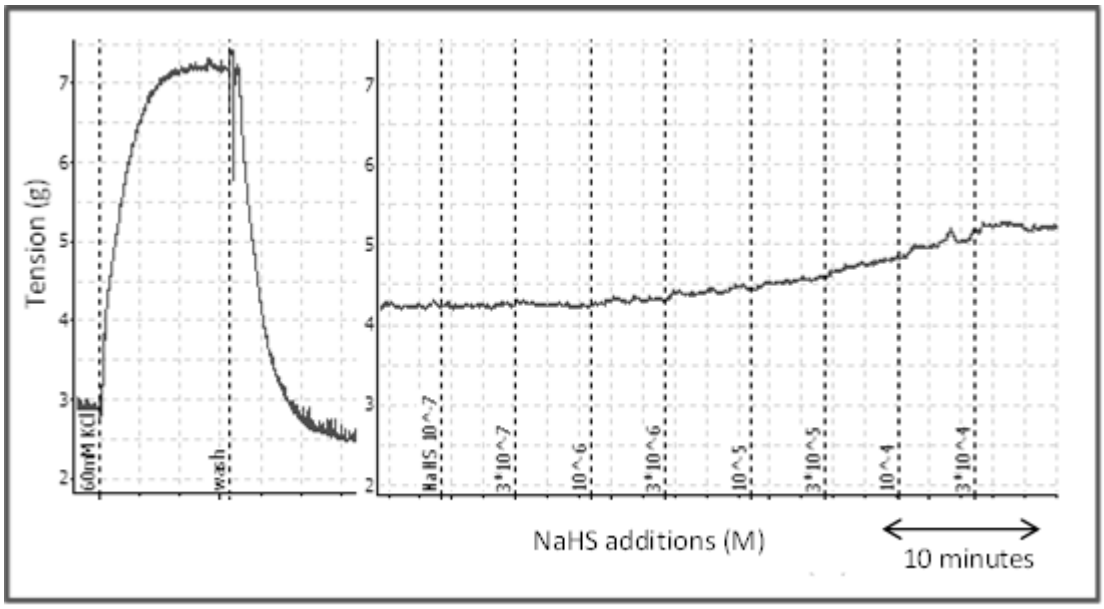

Figure 3.1a Representative trace showing responses to NaHS in porcine mesenteric arteries gassed with the 95% air:5% CO_2 gas mixture. Responses to 60 mM KCl were obtained (left panel) before U46619 was used to pre-constrict vessels by 35% of the 60 mM KCl response, after which concentration response curves to NaHS were obtained. The interval between additions was approximately 5 minutes. NaHS caused a contractile response at a concentration greater than 100 µM.

In these of experiments, the level of pre-constriction obtained with U46619 was 38 ± 4% of the 60 mM KCl responses using a U46619 concentration of 5 X 10^{-8} ± 1 X 10^-8 M (n = 7). Figure 3.1b shows the summary data of these experiments.

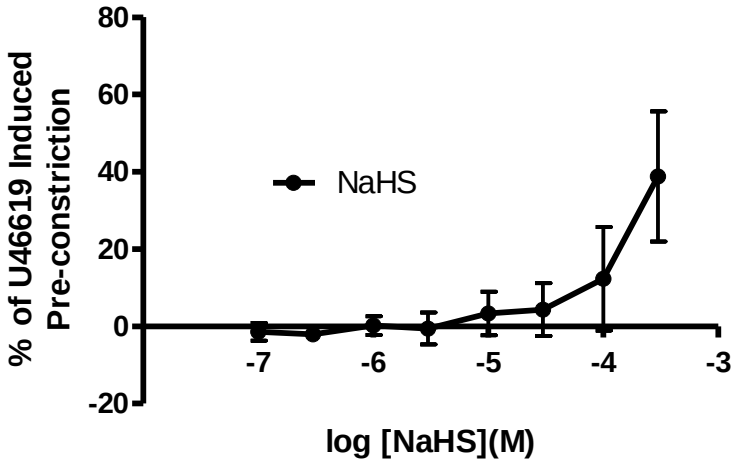

Figure 3.1b Concentration response curves to NaHS in porcine mesenteric arteries pre-constricted with U46619 and gassed with 95% air:5% CO_2. Each point represents the mean ± S.E.M. (n = 7).

Concentration response curves to L-cysteine were carried out in the 95% air:5% CO_2 gas mixture after pre-constricting the vessels with U46619. In these experiments, the level of pre-constriction obtained with U46619 was 44 ± 10% of the maximum KCl response using an average U46619 concentration of 4 X 10^{-8} ± 1 X 10^{-8} M (n=8). L-cysteine caused small contractile responses between 1 and 100 µM followed by further contractions above 100 µM that were much larger than those produced by NaHS (Figure 3.2a and 3.2b).

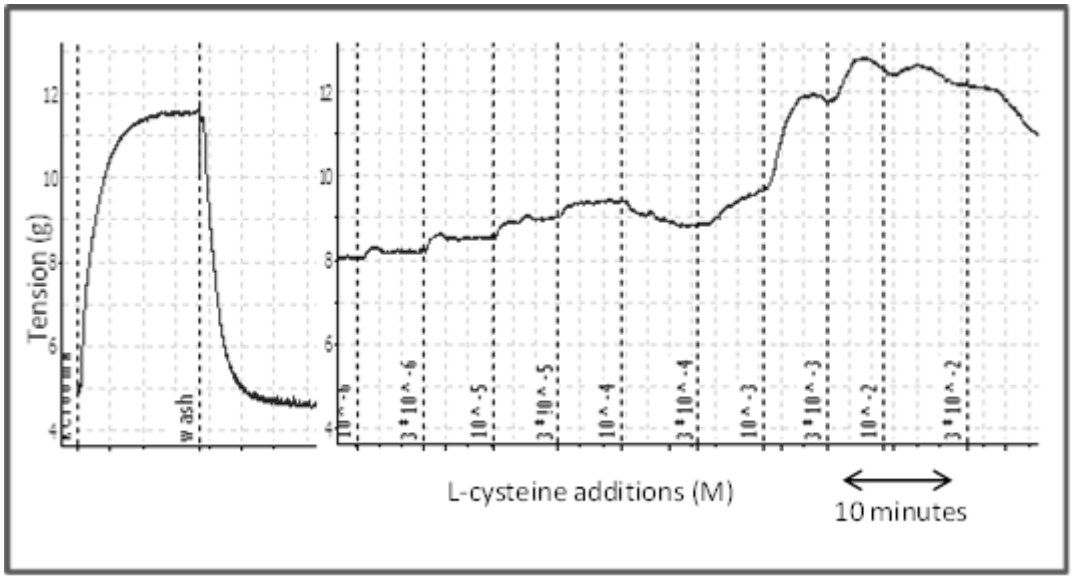

Figure 3.2a Representative trace showing responses to L-cysteine in porcine mesenteric arteries gassed with 95% air:5% CO_2. Responses to 60 mM KCl were obtained (left panel) before U46619 was used to pre-constrict vessels by 49% of the 60 mM KCl response, after which concentration response curves to L-cysteine were obtained. L-cysteine caused small contractile responses between 1 µM and 300 µM followed by a larger contractile response concentrations greater than 1 mM. The interval between additions was approximately 5 minutes.

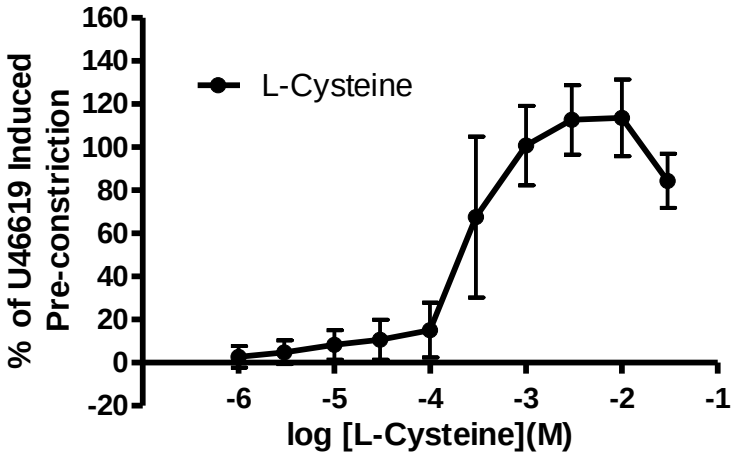

Figure 3.2b Concentration response curves to L-cysteine in porcine mesenteric arteries pre-constricted with U46619 and gassed with 95% air:5% CO_2. Each point represents the mean ± S.E.M. (n = 8).

To investigate whether the L-cysteine-induced contractile response was due to the endogenous production of H_2S, L-cysteine concentration response curves were carried out in the presence and absence of the CSE inhibitor, PPG (10 µM) (Figure 3.3). In these experiments, a similar level of pre-constriction was obtained in the presence and absence of PPG which also had no effect on the U46619 concentration required for pre-constriction. PPG did not significantly affect the contractile response to L-cysteine.

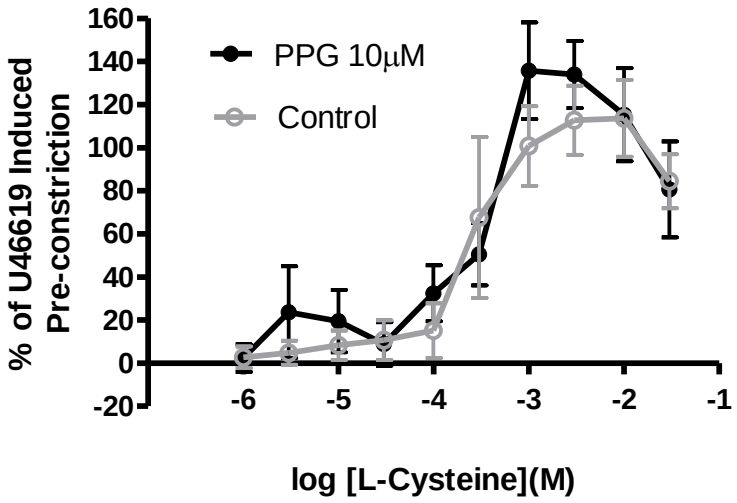

Figure 3.3 Concentration response curves to L-cysteine in the presence (closed circles) and absence (open circles) of PPG (10 µM) in porcine mesenteric arteries, pre-constricted with U46619, and gassed with a 95% air:5% CO_2 gas mixture. Each point represents the mean ± S.E.M. (n = 8). There was no statistical difference between the curves ($p>0.05$; 2 way ANOVA).

L-cysteine concentration response curves were obtained in the presence and absence of the CBS inhibitor, AOAA (10 μM). AOAA had no significant effect on the U46619 concentration required for pre-constriction. AOAA did not affect L-cysteine responses (Figure 3.4).

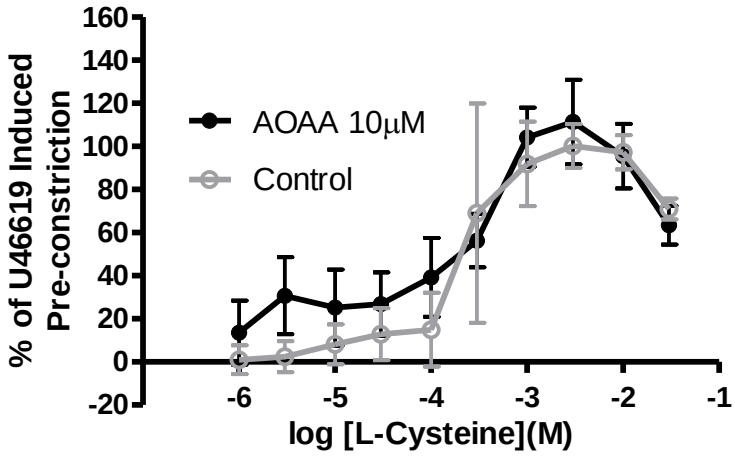

Figure 3.4 Concentration response curves to L-cysteine in the presence (closed circles) or absence (open circles) of AOAA (10 μM) in porcine mesenteric arteries pre-constricted with U46619 and gassed with the 95% air:5% CO_2 gas mixture. Each point represents the mean ± S.E.M. (n = 8). There was no statistical difference between the curves ($p>0.05$; 2 way ANOVA).

To investigate the influence of O_2 concentration on responses to NaHS, vessels were gassed with 95% O_2:5% CO_2, 95% Air:5% CO_2 or 95% N_2:5% CO_2. In the 95% N_2:5% CO_2 gas mixture, U46619-induced tone was unstable and not maintained. For this reason experiments investigating the different gassing mixtures were all carried out using endothelin-1 to pre-constrict the vessels. Using an oxygen sensing electrode, measurements of the partial pressure of O_2 were taken from 20ml PSS when gassing with different mixtures. In 95% O_2:5% CO_2 gassed PSS the partial pressure of O_2 was 619 ± 17 mmHg (n = 3); in PSS gassed with 95% Air:5% CO_2 the partial pressure of O_2 was 140 ± 4 mmHg (n = 3); in 95% N_2:5% CO_2 gassed PSS the partial pressure was 33 ± 6 mmHg O_2 (n = 3).

KCl responses were 9.8 ± 5.0g in O_2:5% CO_2 (n = 9), 7.7 ± 3.1g in air:5% CO_2 (n = 7), 6.6 ± 1.9g in N_2:5% CO_2 (n = 7). For this series of experiments vessels were pre-constricted by 45 ± 9% of the KCl responses (60 mM) response in O_2:5% CO_2 (n = 9) 43 ± 10% in 95% Air:5% CO_2 (n = 7) and 38 ± 4% in 95% N_2:5% CO_2 (n = 7). There was no significant differences between the levels of tone ($p>0.05$; Student's t-test). The average concentration of endothelin-1 required for pre-constriction was $4 \times 10^{-9} \pm 1 \times 10^{-9}$ M in both 95% O_2:5% CO_2 (n = 9) and 95% Air:5% CO_2 (n = 7). In the 95% N_2:5% CO_2 gas mixture, the average concentration of endothelin-1 used was more variable, requiring between 3×10^{-9} and 1×10^{-8} M (n = 7).

In 95% O_2:5% CO_2, NaHS caused a contractile response with a threshold concentration of 1 µM. In 95% air:5% CO_2, NaHS caused a modest contractile response at concentrations above 100 µM, but had little effect at

lower concentration. In 95% N_2:5% CO_2, NaHS caused relaxation responses at concentrations above 10 µM. Figure 3.5 shows a summary of this data.

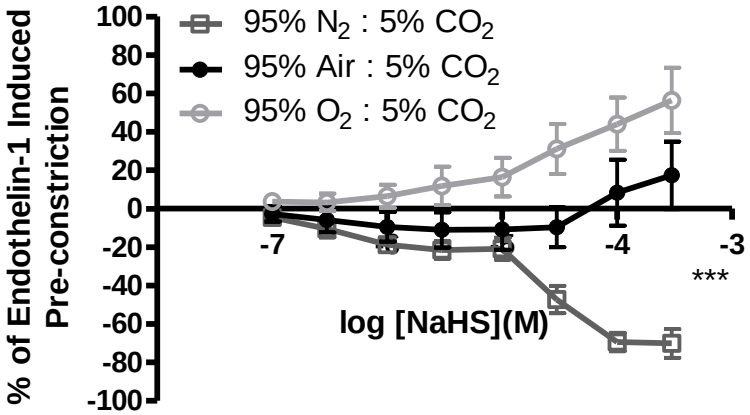

Figure 3.5 Concentration response curves to NaHS gassed with 95% O_2:5% CO_2 (open circles), 95% air:5% CO_2 (closed circles) or 95% N_2:5% CO_2 (open squares) in porcine mesenteric arteries, pre-constricted with endothelin-1. Each point represents the mean ± S.E.M. (n = 9 for 95% O_2:5% CO_2, n = 7 for 95% air:5% CO_2 and n = 7 for 95% O_2:5% CO_2). *** represents a significant difference between the curves ($p<0.001$; 2 way ANOVA).

To investigate the potential role of NO interactions in both the contraction and relaxation responses of NaHS, NaHS concentration responses curves were carried out in the presence and absence of the NOS inhibitor, L-NAME (100 µM) under all three gassing conditions.

In 95% O_2:5% CO_2, vessels were pre-constricted by 46 ± 15% in the absence of L-NAME and 48 ± 11% in the presence of L-NAME (n = 5). L-NAME did not change the concentration of endothelin-1 required to cause this level of vasoconstriction, however on additions, L-NAME did increase the basal tone by 26 ± 12%. L-NAME had a small, but significant, effect on the NaHS response, slightly decreasing the magnitude of contractions observed (Figure 3.6a).

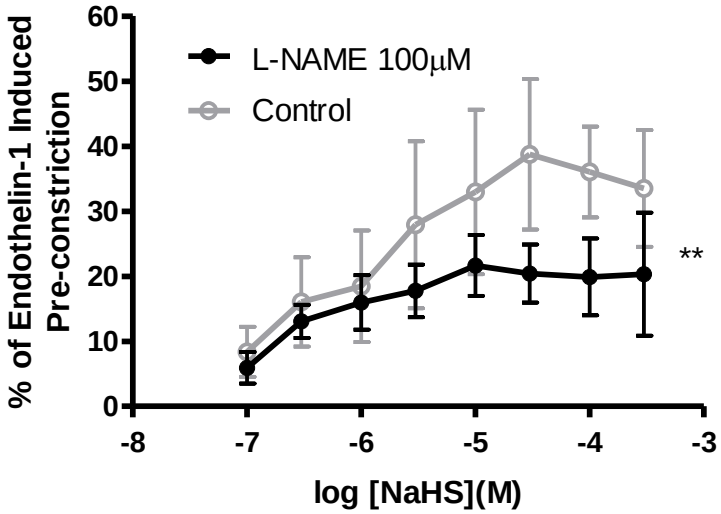

Figure 3.6a Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of L-NAME (100 μM) in porcine mesenteric arteries pre-constricted with endothelin-1 and gassed with 95% O_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 5). ** represents a significant difference between the curves ($p<0.05$; 2 way ANOVA).

In 95% air:5% CO_2, vessels were pre-constricted by 47 ± 11% in the absence and 47 ± 13% in the presence of L-NAME (n = 6), using an average endothelin-1 concentration of 4 X 10^{-9} M under both conditions. Although there was only a small response to NaHS under this gassing condition, L-NAME significantly attenuated the maximum contractile response to NaHS ($p<0.05$; Mann Whitney test) (Figure 3.6b).

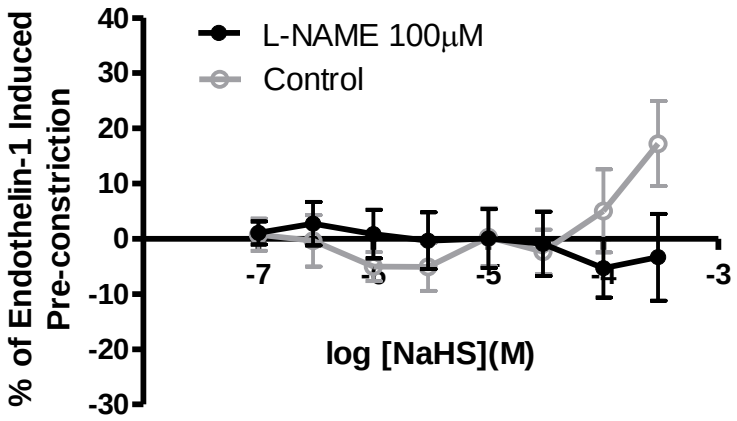

Figure 3.6b Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of L-NAME (100 μM) in porcine mesenteric arteries pre-constricted with endothelin-1 and gassed with 95% air:5% CO_2. Each point represents the mean ± S.E.M. (n = 6). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

In 95% N_2:5% CO_2, the level of pre-constriction was similar with or without L-NAME using the same mean concentration of endothelin-1 ($3 \times 10^{-9} \pm 1 \times 10^{-9}$ M) (n = 6). L-NAME had no effect on NaHS-induced vasorelaxant responses, under these conditions (Figure 3.6c).

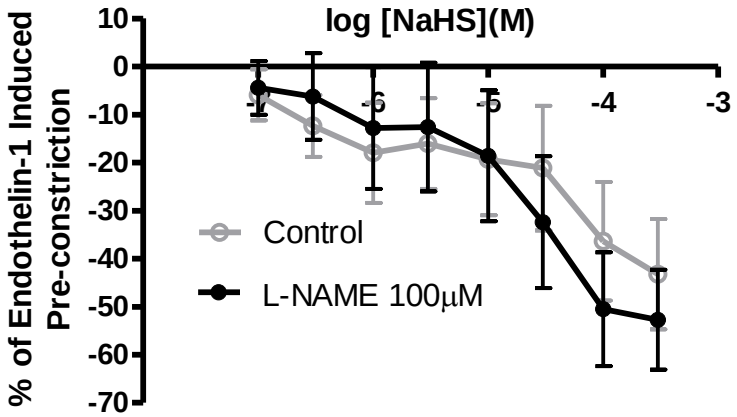

Figure 3.6c Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of L-NAME (100 µM) in porcine mesenteric arteries pre-constricted with endothelin-1 and gassed with 95% N_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 6). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

To investigate the potential involvement of cyclooxygenase in mediating NaHS responses under different gassing conditions, indomethacin (1 µM) was used. In 95% O_2:5% CO_2, indomethacin had no significant effect on NaHS responses (Figure 3.7a). The level of pre-constriction in the 95% O_2:5% CO_2 experiments was 35 ± 3% in the presence of indomethacin and 56 ± 13% in its absence. Under both conditions, the same endothelin-1 concentration of 1 X 10^{-9} ± 8 X 10^{-10} M was used (n = 8).

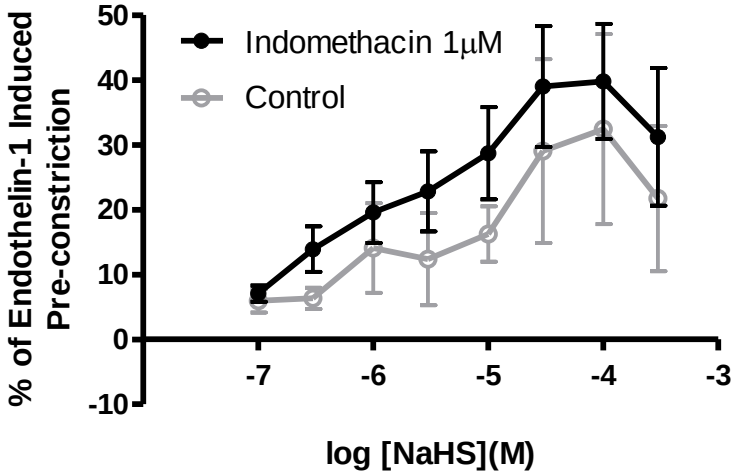

Figure 3.7a Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of indomethacin (1 µM) in porcine mesenteric arteries pre-constricted with endothelin-1 and gassed with 95% O_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 8). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

The level of pre-constriction in the 95% air:5% CO_2 experiments was 42 ± 10% in the presence and 51 ± 7% in the absence of indomethacin. Although NaHS responses were small under the 95% air:5% CO_2 conditions indomethacin had a significant effect on NaHS responses, tending to abolish relaxation responses (Figure 3.7b), although these remained relatively small in magnitude.

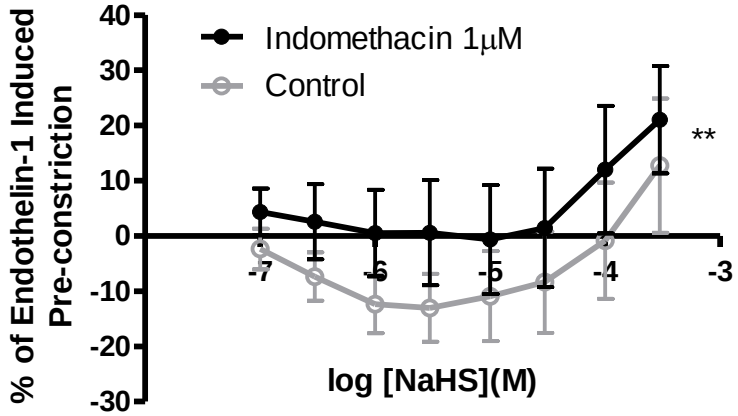

Figure 3.7b Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of indomethacin (1 μM) in porcine mesenteric arteries pre-constricted with endothelin-1 and gassed with 95% air:5% CO_2. Each point represents the mean ± S.E.M. (n = 7). ** represents a significant difference between the curves ($p<0.05$; 2 way ANOVA).

In 95% N_2:5% CO_2, indomethacin had no significant effects on NaHS-induced relaxation responses (Figure 3.7c). The level of pre-constriction in 95% N_2:5% CO_2 data was 40 ± 9% in the presence of indomethacin and 42 ± 6% in its absence, using the same average endothelin-1 concentration of 4 X 10^{-9} ± 2 X 10^{-9} M under both conditions (n = 7).

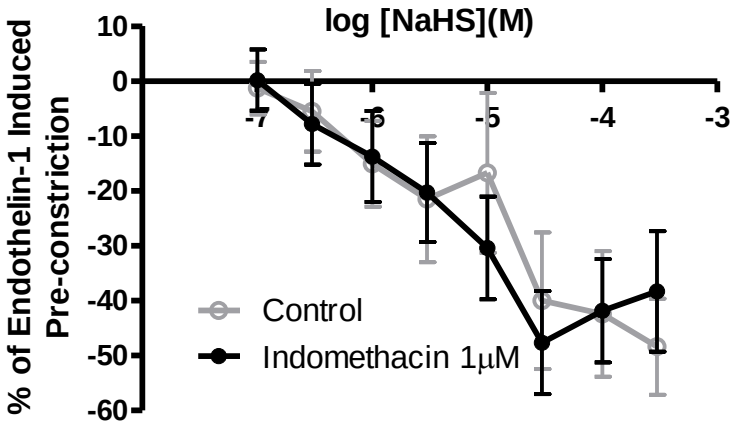

Figure 3.7c Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of indomethacin (1 μM) in porcine mesenteric arteries pre-constricted with endothelin-1 and gassed with 95% N_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 7). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

To investigate the potential involvement of K^+ channels in the relaxation responses to NaHS observed when pre-constricting with endothelin-1 and gassing with 95% N_2:5% CO_2, experiments were conducted using raised extracellular K^+ PSS to induce pre-constriction. The level of pre-constriction induced by substituting the standard PSS with a raised extracellular K^+ PSS (30 mM K^+) was 41 ± 6% compared to the level of pre-constriction induced by endothelin-1 of 38 ± 4% (n = 6). In raised extracellular K^+, NaHS had a tendency to cause contraction in contrast to when endothelin-1 was used to induce tone where the overall effect of NaHS was to cause a relaxation. (Figure 3.8a)

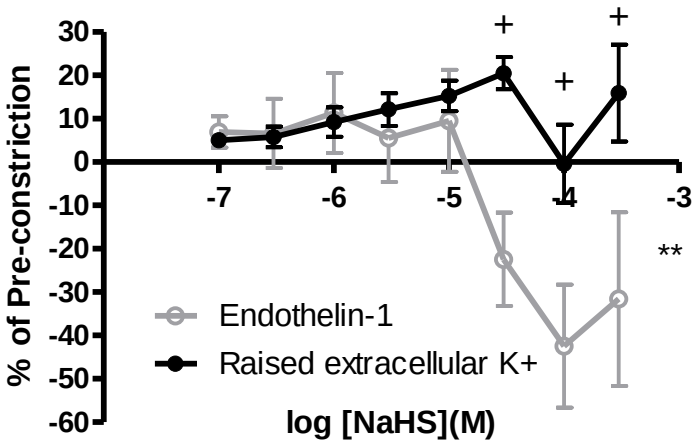

Figure 3.8a Concentration response curves to NaHS pre-constricted with either raised extracellular K+ PSS (closed circles) or endothelin-1 (open circles) in porcine mesenteric arteries pre-constricted with endothelin-1 and gassed with 95% N_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 6). ** represents a significant difference between the curves ($p<0.05$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

To investigate the potential involvement of K^+ channels in mediating the NaHS-relaxation responses in 95%N_2: 5% CO_2, the non-specific K+ channel blocker TEA (1 mM) was used. TEA modestly attenuated the NaHS relaxant response (Figure 3.8b). The level of pre-constriction of these experiments data was 42 ± 8% in the presence of TEA and 46 ± 7% in its absence (n = 9), both using an average endothelin-1 concentration of 5 X 10^{-9} ± 2 X 10^{-9} M.

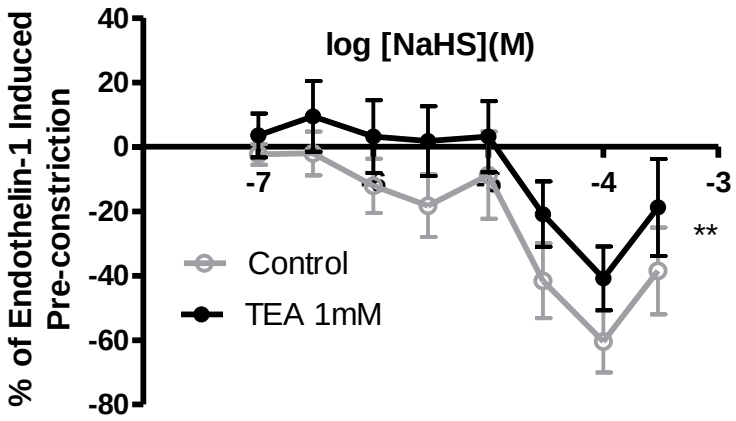

Figure 3.8b Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of TEA (1 mM) in porcine mesenteric arteries, pre-constricted with endothelin-1, and gassed with 95% N_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 9). ** represents a significant difference between the curves ($p<0.05$; 2 way ANOVA).

In parallel to using TEA, the effects of glibenclamide (the specific K_{ATP} channel blocker) were assessed. A similar level of pre-constriction in these experiments was achieved using a mean endothelin-1 concentration of 4 X 10^{-9} ± 2 X 10^{-9} M in the presence of glibenclamide and 6 X 10^{-9} ± 3 X 10^{-9} M in its absence (n = 6). In these experiments glibenclamide attenuated the NaHS relaxation response (Figure 3.8c).

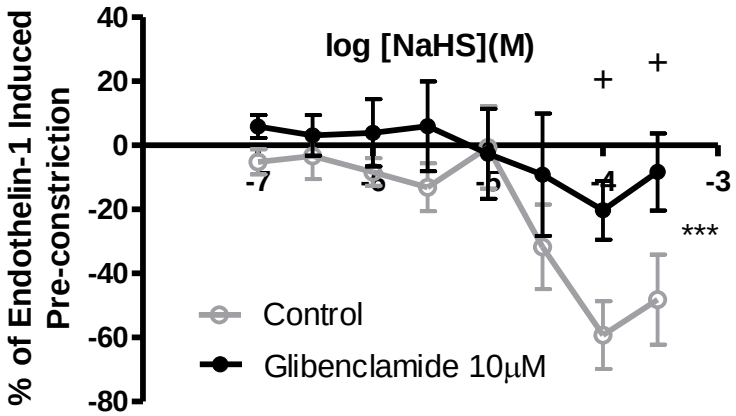

Figure 3.8c Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of glibenclamide (10 μM) in porcine mesenteric arteries pre-constricted with endothelin-1 and gassed with 95% N_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 6). *** represents a significant difference between the curves ($p<0.01$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

Discussion

In the present study it has been shown that when pre-constricting porcine 1st order mesenteric arteries with endothelin-1, NaHS causes varied responses depending on the O_2 level used to buffer the PSS. When gassing with 95% O_2:5% CO_2, NaHS caused a contractile response. In 95% air:5% CO_2, NaHS caused a smaller contractile response. The contractile responses, under either gassing condition were attenuated in the presence of L-NAME, suggesting the removal of the vasorelaxatory effects of NO was contributing to contractile responses produced by NaHS. In contrast, when gassing with 95% N_2:5% CO_2, NaHS caused a relaxation response, that was attenuated by raising extracellular K^+ or by glibenclamide, suggesting a role for K_{ATP} channels.

Experiments were carried out to assess the potential generation of H_2S from its biochemical substrate, L-cysteine in porcine mesenteric arteries. L-cysteine caused a large contractile response under gassing conditions of 95% air:5% CO_2. This is consistent with H_2S generation since NaHS induced contractile responses under the same gassing conditions. Contractile responses have also been observed in rat aortic rings pre-constricted with phenylephrine, where L-cysteine reversed acetylcholine-induced vasorelaxation (Ali *et al.*, 2006). However, this may depend on the preparation or experimental conditions as relaxation response to L-cysteine have also been observed. Al-Magableh and Hart (2011), observed that mouse aorta relaxed in response to L-cysteine at a concentration of 1 mM. Relaxations have also been observed in rat mesentery arterial beds with high

concentrations of L-cysteine (Cheng *et al.*, 2004). However, there was no evidence of H_2S production via the CSE or CBS enzymes, since neither PPG nor AOAA inhibited the contractile responses to L-cysteine. In contrast, Ali *et al.,* (2006) found that the L-cysteine effects in the rat aorta were partially inhibited by the presence of PPG, although they used a much higher concentration of PPG (1 mM). This suggests the concentration of PPG used in the current study (10 µM) may not have been sufficient. However, Cheng *et al.* (2004), found l-cysteine (1 mM) caused a relaxation response in the rat mesenteric arterial bed that was completely abolished using the same concentration of PPG (10 µM) that was used in this study. Future studies could use a higher concentration of PPG to further assess the role of the CSE enzyme in porcine mesenteric arteries. It is possible that H_2S is being produced from L-cysteine via another mechanism of H_2S production. For example, Shibuya *et al.* (2009), showed that 3-Mercaptopyruvate Sulfurtransferase (3-MST) is expressed in the vascular endothelium of rat thoracic aorta and produces H_2S from l-cysteine via generation of 3-mercaptopyruvate.

The effect of changing the oxygen concentration used to gas the PSS on NaHS responses was investigated. U46619 could not maintain a stable contraction when gassed with 95% N_2:5% CO_2, therefore, endothelin-1 was used as the pre-contractile agent. Altering oxygen levels changed the nature of the response observed with NaHS. In 95% O_2:5% CO_2 contractile responses were observed, while under gassing conditions of 95% N_2:5% CO_2, NaHS produced a vasorelaxation. This is similar to observations in the rat aorta made by Koenitzer *et al*. (2007), where H_2S promoted

vasoconstriction at 200 µM O_2 (equivalent to PSS gassed with 95% air:5% CO_2) and promoted vasorelaxation effects at 40 µM O_2 (equivalent to PSS gassed with 95% N_2:5% CO_2). Koenitzer *et al*., (2007), found that at 200 µM O_2, exogenous H_2S was rapidly oxidized and suggested that the H_2S-induced contractile response was due to reduced levels of H_2S available for vasorelaxation. However, they also suggested that generation of H_2S oxidation products at higher oxygen may have contractile activity.

In previous studies, several mechanisms have been proposed to explain H_2S-induced vasoconstriction. H_2S has been shown to inhibit cAMP accumulation in smooth muscle cells promoting a contractile response in the rat aorta (Lim *et al.,* 2008). Ali *et al.* (2006) observed a contraction produced by NaHS at relatively low concentrations in rat aorta, and suggested it was due to an interaction between H_2S and NO, producing an inactive nitrosothiol, thus removing the vasorelaxatory effect of NO. Reinforcing this suggestion, Whiteman *et al.* (2006), used different biochemical techniques and showed that NO interacts with H_2S chemically, lowering biologically available NO concentrations. In porcine mesenteric arteries L-NAME, slightly attenuated the contractile response to NaHS, consistent with observations in rat aorta (Lim *et al.,* 2008; Ali *et al.,* 2006). However, L-NAME did not abolish the contractile responses to NaHS at higher oxygen levels and therefore it is possible that this is mediated via another mechanism, such as the generation of contractile H_2S oxidation products as suggested by Koenitzer *et al*., (2007).

Indomethacin attenuated NaHS-induced vasorelaxation responses observed when preparations were gassed with the 95% air:5% CO_2 mixture,

but had no effect on experiments in PSS gassed with the other mixtures, suggests a small H_2S interaction with cyclooxygenase products causing a relaxation specifically under the 95% air: 5% CO_2 gassing conditions. This was similar to observations by Koenitzer *et al.* (2007), who found in rat aorta, that indomethacin (5 µM) caused a small enhancement of contractile responses to NaHS at 200 µM (equivalent to gassing with 95% air:5% CO_2) but not at lower O2 levels (equivalent to gassing with 95% N_2:5% CO_2).

In the present study, at lower oxygen levels (95% N_2:5% CO_2), NaHS-induced a vasorelaxation. When gassing with 95% N_2:5% CO_2, the presence of L-NAME had no effect on NaHS-mediated vasorelaxation, suggesting that NO was not responsible for producing this response. In contrast, Zhao *et al*. (2001), showed NaHS vasodilator responses in rat aorta were significantly reduced in the presence of the L-NAME. When vessels were pre-constricted with raised extracellular K^+, relaxation responses to NaHS were abolished, suggested they involved a hyperpolarization mechanism. TEA also caused attenuation of NaHS relaxation responses, although to a lesser extent than raised extracellular K^+. Furthermore, the K_{ATP} channel specific blocker glibenclamide significantly attenuated NaHS-induced vasorelaxation. Collectively, these data indicate some involvement of K_{ATP} channels in the NaHS-induced vasorelaxation. Similarly, in a study using the rat perfused mesenteric arterial bed, NaHS-induced relaxation responses were also inhibited by glibenclamide (Cheng *et al.*, 2004).

Under 95% O_2:5% CO_2, 95% air:5% CO_2 or 95% N_2:5% CO_2, the partial O_2 pressures was shown to be 619 ± 17 mmHg, 140 ± 4 mmHg and 33 ± 6 mmHg, respectively. In mammals, the partial O_2 pressure generally

ranges from 50 to 110 mmHg in arterial blood (Erecińska and Silver, 2001; Tsai *et al*., 2003). In addition, In a study specifically on porcine tissue showed that awake swine under normal conditions and minimum stress had an arterial partial O_2 pressure of 90.65 ± 6.95 mmHg (Gianotti *et al.,* 2010). This suggests that the most physiologically relevant conditions in the current study were achieved by gassing with 95% air:5% CO_2. Although under these conditions NaHS did not cause a very significant response, higher or lower oxygen levels, caused vasocontraction or vasorelaxation, respectively. Olson (2008), proposed that H_2S may act as an endogenous oxygen sensor by an increase in endogenous levels of H_2S under hypoxia inducing relaxation of blood vessels thus increasing the supply of oxygenated blood to tissue. Although the present study shows no evidence for endogenous H_2S production from exogenous additions of L-cysteine, the fact that at low oxygen levels (95% N_2:5% CO_2) NaHS causes vasorelaxation would fit with this theory. As under 95% N_2:5% CO_2 conditions there does not appear to be an interaction between NO and NaHS suggests that this phenomenon may be dependent on ambient oxygen concentration, potentially stopping H_2S from causing vasorelaxation at higher oxygen levels. In the presence of 95% O_2:5% CO_2, NO has been suggested to readily reacts with super oxide radicals, forming among others, $ONOO^-$ (henry *et al.*, 1991). In addition directly interacting with NO, Whiteman *et al.* (2006) also showed that $ONOO^-$ interacts with H_2S to form nitrosothiol. This may be an important mediating step in NO/H_2S interactions. Therefore, at lower oxygen levels without the creation of oxidation productions of NO and H_2S, they may not react to a significant level.

In summary, NaHS caused small contractile responses in vessels gassed with 95% air:5% CO_2 and these contractile responses were larger in 95% N_2:5% CO_2. Since these responses were partly sensitive to L-NAME the contraction may be due, in part, to an interaction between NaHS and NO, such that the latter is made inactive. When gassing with 95% O_2:5% CO_2, the responses to NaHS was a relaxation. The presence of raised extracellular K^+ PSS attenuated this response as did TEA and glibenclamide, indicating an involvement of K_{ATP} channels.

Chapter 4:

NaHS Causes Endothelium-Dependent Vasoconstriction in Porcine Splenic Arteries

Introduction

In the preceding chapter the effects of different gassing conditions were examined on NaHS concentration response curves in porcine mesenteric arteries. It was shown that different oxygen levels in the gassing mixtures changed the nature of NaHS-induced responses. Overall, NaHS caused contractile responses in vessels gassed with 95% air:5% CO_2 and these contractile responses were larger in 95% O_2:5% CO_2. Since these responses were partly sensitive to L-NAME the contraction may be due, in part, to an interaction between NaHS and NO, removing the vasodilator effects of NO. Ali *et al.* (2006) observed similar interactions of NaHS with NO that caused a contraction in rat aorta. In contrast, when gassing with 95% N_2:5% CO_2, NaHS-induced a relaxation. This response was sensitive to glibenclamide, indicating an involvement of K_{ATP} channels. K_{ATP} channels have also been shown to be involved in mediating responses to H_2S in isolated rat aortic rings (Zhao *et al.*, 2001). The present chapter was designed to test how consistent the observations made using porcine mesenteric arteries are across the vasculature and so was conducted using porcine splenic arteries.

Materials and Methods

Set up and General Protocol

Porcine splenic arteries were isolated and set up in an isometric tension recording system as described in the methods chapter. Vessels were set up in organ baths containing 20ml PSS at 37˚C and gassed with either 95% O_2:5% CO_2, 95% Air:5% CO_2 or 95% N_2:5% CO_2. The PSS was pre-gassed with the appropriate gas mixture for at least 30 minutes to ensure gaseous equilibrium of the PSS. Vessels were pre-tensioned to approximately 10-12 g and allowed to relax for 30-40 minutes to achieve a stable baseline tone. Thereafter KCl (60 mM) was added to the bath and vessels were allowed to contract. After the response plateaued, the KCl was washed out with fresh PSS at least 2 times at 5 min intervals. Following the return to a stable baseline, and after a further 20 minutes, exposure to KCl, followed by the washout process, was repeated.

After three repeats of responses to KCl, any test compounds used in the experiment were added and allowed at least 30 minutes incubation time. The following test compounds were used: indomethacin (1 µM) to inhibit the cyclooxygenase enzyme; the NOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME) (100 µM); to desensitize sensory nerves, capsaicin (10 µM) (Gupta *et al.*, 2007); the non-specific K^+ channel blocker, tetraethylammonium (TEA) (1 mM) and the selective K_{ATP} channel inhibitor, glibenclamide (10 µM). After this period vessels were pre-contracted to a level of 30-70% of the maximum KCl response, achieved using the thromboxane A2 receptor agonist, U46619. Once vessel tension had

stabilized, a concentration-response curve to NaHS was obtained by adding it in a cumulative manner in 3-fold increments, between 100 nM and 3 mM, with a period of 5 minutes between additions.

In all experiments a second segment of the same vessel was set up and used as a control.

Endothelium Denudation

Some experiments were designed to investigate the role of the endothelium in mediating responses to NaHS. In these experiments, the endothelium was removed mechanically from vessel segments prior to set up in the organ bath. Endothelium denudation was achieved by rubbing the luminal surface of the vessel segment with forceps, while rolling the segment along a paper towel soaked with PSS. To keep this process consistent, vessels were rolled 15 - 20 times applying a modest level of pressure.

In these experiments, substance P (1 μM) was used to assess endothelial function. To do this, vessels were pre-constricted with noradrenaline after the third KCl response. Once responses to noradrenaline plateaued, substance P was added. After 5-10 minutes, noradrenaline and substance P were washed out and 15 minutes later pre-constriction with U46619 was carried out. The criterion was set that only vessels that did not respond to substance P were accepted as successfully denuded, in comparison to their paired segment that produced a relaxation of at least 25%.

Results

Experiments were carried out to investigate NaHS responses in porcine splenic arteries when gassing the PSS with gas mixtures containing different levels of O_2. Representative traces of responses are shown in Figure 4.1, and a summary of the data is shown in Figure 4.2. In the majority of experiments when gassing with the 95% O_2:5% CO_2 gas mixture, NaHS caused relatively small contractions rising to a maximal contraction of around 30-40% of the maximum KCl response at 1 mM NaHS. When gassing with 95% air:5% CO_2, responses were biphasic. In the majority of preparations NaHS produced concentration dependent contractions between 10 and 100 µM followed by the loss of this contraction on exposure to 300 µM and some relaxation at 1 mM. Unfortunately it was impossible to maintain U46619-induced tone in the 95% N_2:5% CO_2 mixture and therefore this series was discontinued. Perhaps surprisingly, KCl responses in the 95% N_2:5% CO_2 mixture were not significantly different to the other gassing conditions, yielding an increase in tension of 8.2 ± 2.7 g (n = 7) compared with 9.2 ± 2.7 g gassing with 95% air:5% CO_2 (n = 25) and 8.8 ± 2.9 g gassing with 95% O_2:5% CO_2 (n = 23).

The level of pre-constriction in the 95% O_2:5% CO_2 data was 56 ± 8% of the 60 mM KCl response using an average U46619 concentration of 3 x 10^{-8} 1*10^{-8} M (n = 23) and in the 95% air:5% CO_2 data the level of pre-constriction was 40 ± 9% although this required a higher average U46619 concentration of 2 x 10^{-7} ± 3*10^{-7}M (n = 25). There was a significant increase in the concentration of U46619 required to induce less tone under

95% air:5% CO_2 gassing conditions compared with 95% O_2:5% CO_2 ($p>0.05$; Student's t-test).

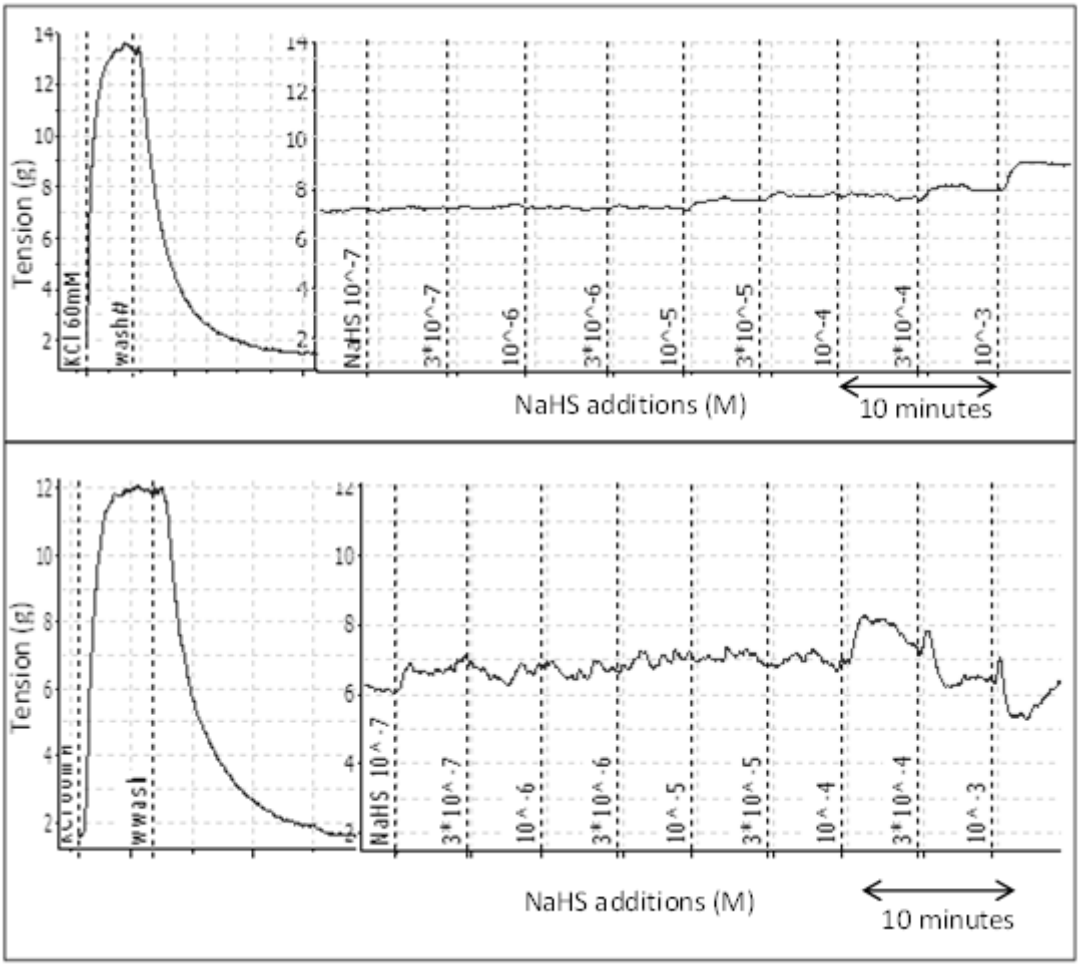

Figure 4.1 Representative traces showing responses to NaHS in porcine splenic arteries under different gassing conditions. Responses to 60 mM KCl were obtained (left panels) before U46619 was used to pre-constrict vessels by 30-70% of 60 mM KCl response after which concentration response curves to NaHS were obtained. The interval between additions of NaHS was 5 minutes. The top example (a), shows responses to NaHS when gassing with 95% O_2:5% CO_2. The bottom example (b) shows an example of standard responses when gassing with 95% air:5% CO_2.

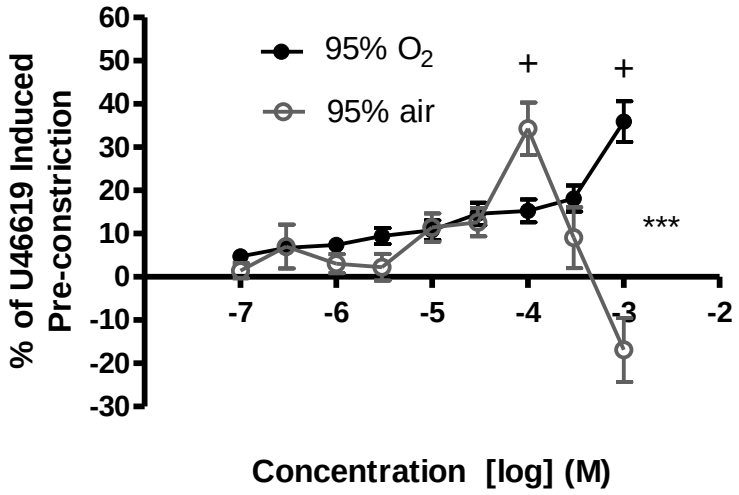

Figure 4.2 Concentration response curves to NaHS gassed with 95% O_2:5% CO_2 (closed circles) or 95% air:5% CO_2 (open circles) in porcine splenic arteries pre-constricted with U46619. Each point represents the mean ± S.E.M. (n = 23 for 95% O_2:5% CO_2 and n = 25 for 95% air:5% CO_2). *** represents a significant difference between the curves ($p<0.001$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

Experiments were carried out to assess the involvement of K^+ channels in mediating responses to NaHS under different gassing conditions. Experiments were carried out in the presence and absence of TEA (1 mM), a non-selective K+ channel blocker. In 95% O_2:5% CO_2, TEA had no significant effect on NaHS contractile responses (Figure 4.3a). In these experiments, there was no difference in the level of pre-constriction using similar concentrations of U46619.

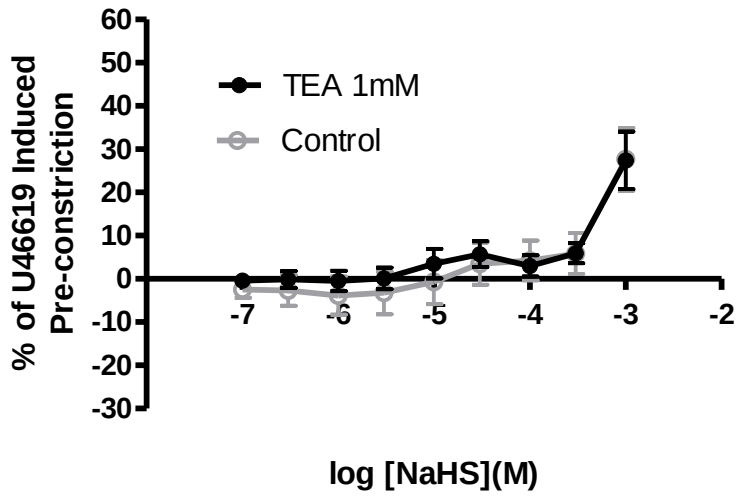

Figure 4.3a Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of TEA (1 mM) in porcine splenic arteries pre-constricted with U46619 and gassed with 95% O_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 7). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

In the 95% air:5% CO_2 mixture, TEA had no significant effect on NaHS-induced contractile responses (Figure 4.3b).

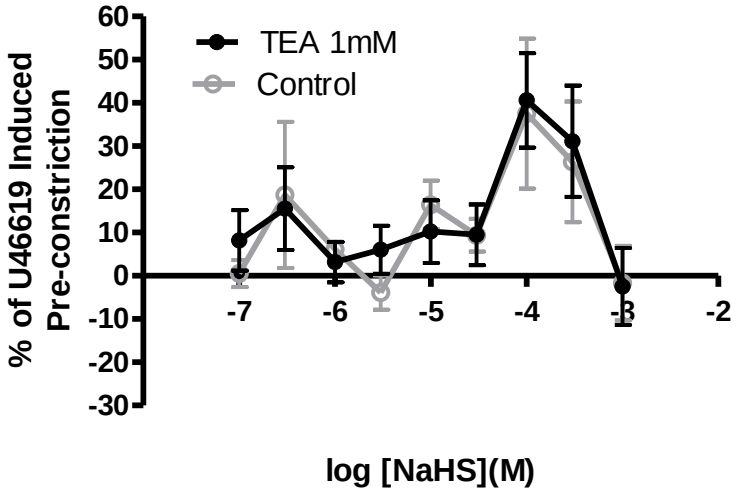

Figure 4.3b Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of TEA (1 mM) in porcine splenic arteries pre-constricted with U46619 and gassed with 95% air:5% CO_2. Each point represents the mean ± S.E.M. (n = 7). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

The specific K_{ATP} channel inhibitor, glibenclamide (10 µM) was used to investigate potential K_{ATP} channel involvement in mediating responses to NaHS. In 95% O_2:5% CO_2, glibenclamide modestly, but significantly, enhanced contraction responses produced by NaHS (Figure 4.4a). In the 95% O_2:5% CO_2 data vessels were pre-constricted by similar amounts using an average U46619 concentration of $3 \times 10^{-8} \pm 0$ M in the absence of glibenclamide and $3 \times 10^{-7} \pm 0$ M in its presence (n = 4). There was a significant difference between the concentrations of U46619 in the presence and absence of glibenclamide (Student's t-test, $p<0.05$).

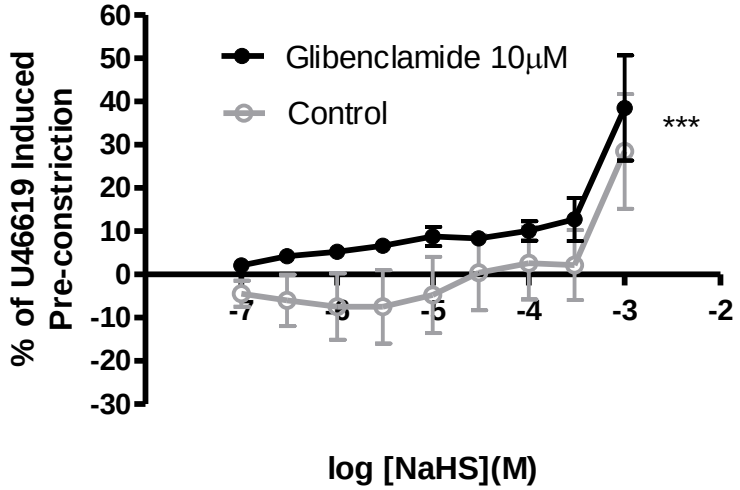

Figure 4.4a Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of glibenclamide (10 µM) in porcine splenic arteries pre-constricted with U46619 and gassed with 95% O_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 4). *** represents a significant difference between the curves ($p<0.001$; 2 way ANOVA).

In the 95% air:5% CO_2 mixture, glibenclamide significantly reduced the relaxation response produced at concentrations above 100 µM NaHS (Figure 4.4b). It should be noted that in this series of experiments the relaxation response observed in the controls was larger than were observed in other experimental sets. In 95% air:5% CO_2, vessels were pre-constricted to similar levels by an average concentration of $8 \times 10^{-7} \pm 5 \times 10^{-7}$ M U46619 in the presence of glibenclamide and $4 \times 10^{-7} \pm 6 \times 10^{-7}$ M in its absence (n= 6). There was no significant difference between the concentrations of U46619 in the presence and absence of glibenclamide (Student's t-test, $p>0.05$).

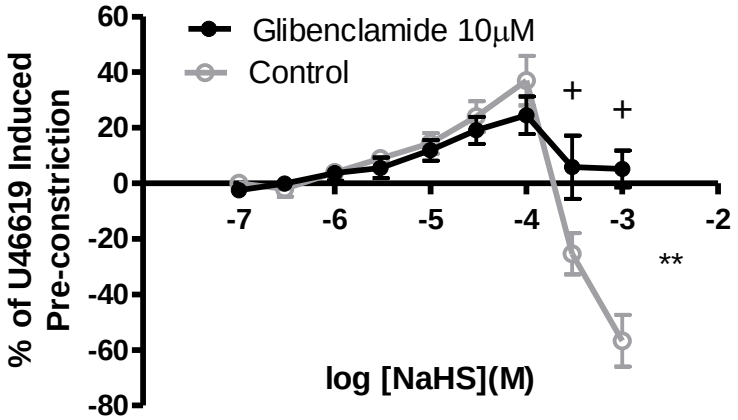

Figure 4.4b Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of glibenclamide (10 µM) in porcine splenic arteries pre-constricted with U46619 and gassed with 95% air:5% CO_2. Each point represents the mean ± S.E.M. (n = 6). ** represents a significant difference between the curves ($p<0.01$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

To assess the role of sensory nerves and vasoactive neuropeptide release from sensory nerves in modifying or contributing to responses to NaHS, experiments were carried out in the absence or after capsaicin (10 μM) pre-treatment. In 95% O_2:5% CO_2 gassed PSS, capsaicin had no significant effect on NaHS contractile responses (Figure 4.5a). In 95% O_2:5% CO_2, there was no significant difference between either the concentrations of U46619 used or level of pre-constriction.

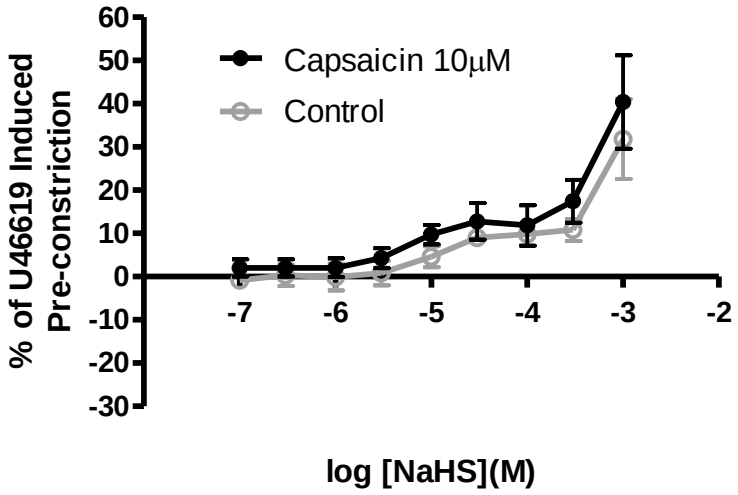

Figure 4.5a Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of capsaicin (10 μM) in porcine splenic arteries pre-constricted with U46619 and gassed with 95% O_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 6). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

Capsaicin had no significant effect on NaHS-induced responses, in the 95% air:5% CO_2 gassed PSS (Figure 4.5b). Again the level of pre-constriction or concentration of U46619 required to induce this were similar.

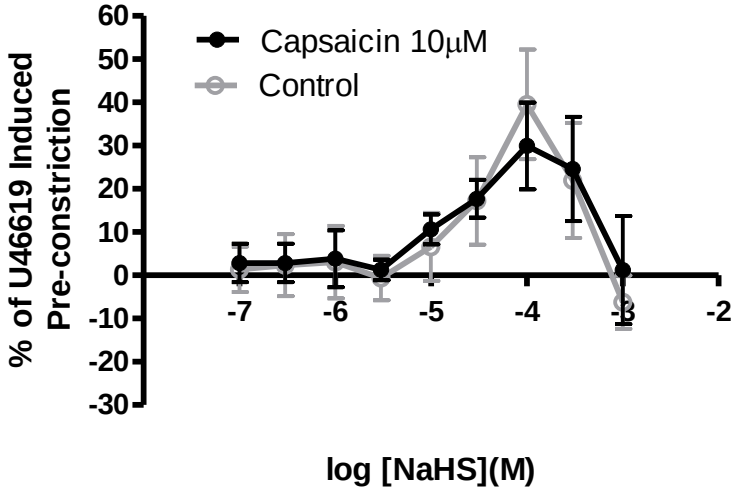

Figure 4.5b Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of capsaicin (10 μM) in porcine splenic arteries pre-contracted with U46619 and gassed with 95% air:5% CO_2. Each point represents the mean ± S.E.M. (n = 6). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

Responses to NaHS in vessels with denuded endothelium were assessed. Responses to substance P were used to confirm the presence of the endothelium and vessels that did not response to substance P were considered successfully endothelium denuded. In 95% O_2:5% CO_2, in endothelium-intact vessels, substance P caused a transient relaxation by 45 ± 15% of the noradrenaline-induced contraction compared with 0 ± 0% in endothelium-denuded vessels (n = 9). Average KCl responses were 8.9 ± 1.7 g in endothelium-intact vessels and 8.1 ± 2.9 g in endothelium-denuded vessels (n = 9). There was no significant difference between responses to KCl between the two groups ($p>0.05$; Student's t-test).

Removal of the endothelium completely abolished NaHS-induced contractile responses to NaHS in 95% O_2:5% CO_2 gassed PSS (Figure 4.6a). There was no difference in the level of pre-constriction (60 ± 7%) or concentration of U46619 ($3 \times 10^{-8} \pm 1 \times 10^{-8}$ M) used between the two groups (n = 9).

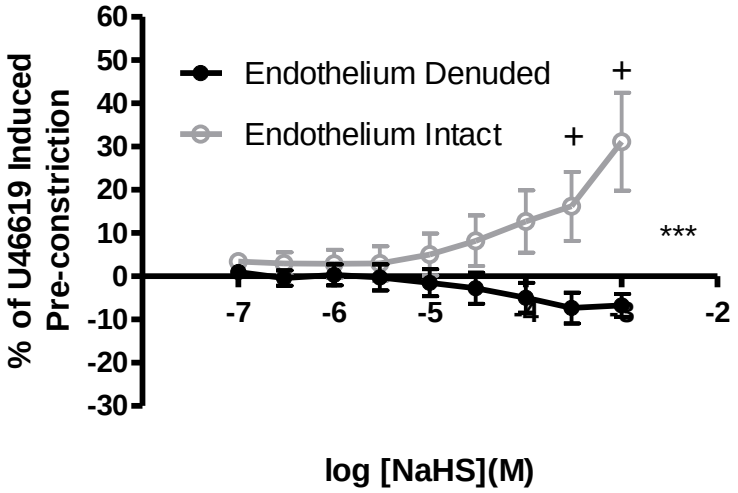

Figure 4.6a Concentration response curves to NaHS with endothelium intact (open circles) or endothelium removed (closed circles) in porcine splenic arteries pre-constricted with U46619 and gassed with 95% O_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 9). *** represents a significant difference between the curves ($p<0.001$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

In 95% air:5% CO_2, the removal of the endothelium also significantly attenuated responses to NaHS, completely abolishing the contractile responses uncovering a small relaxation response at 1 mM NaHS (Figure 4.6b). Average KCl responses were 8.9 ± 3.4 g in endothelium-intact vessels and 7.9 ± 2.9 g in endothelium-denuded vessels (n = 9). There was no significant difference between responses to KCl between the two groups ($p>0.05$; Student's t-test). In endothelium-intact vessels, substance P caused a transient relaxation by 42 ± 4% of noradrenaline pre-constriction compared with no relaxation (0 ± 0%) in the endothelium-denuded vessels. The same average concentration of U46619 ($1 \times 10^{-8} \pm 3 \times 10^{-9}$ M) was used. However, there was a significant difference in the level of pre-constriction induced, increasing from 43 ± 10% in the endothelium-intact vessels to 60 ± 9% in endothelium-denuded vessels ($p<0.05$; Student's t-test; n = 4).

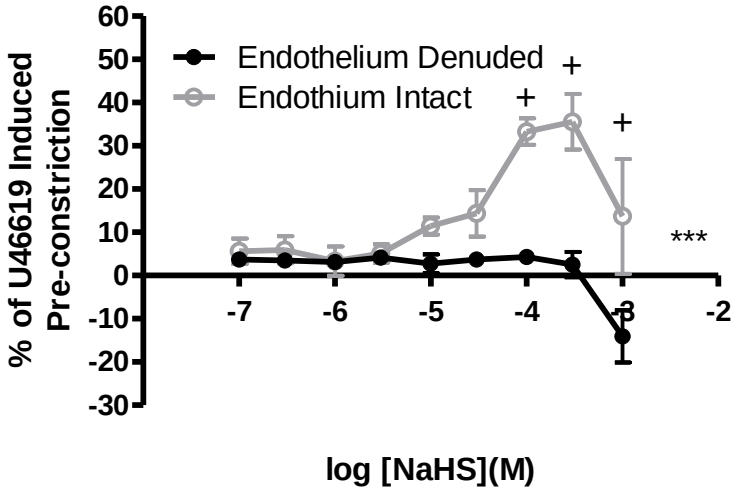

Figure 4.6b Concentration response curves to NaHS in endothelium intact (open circles) or endothelium-denuded (closed circles) porcine splenic arteries, pre-constricted with U46619 and gassed with 95% air:5% CO_2. Each point represents the mean ± S.E.M. (n = 4). *** represents a significant difference between the curves ($p<0.001$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

To investigate the potential role of cyclooxygenase and its products in influencing responses to NaHS, responses were obtained in the presence or absence of indomethacin (1 μM). In 95% O_2:5% CO_2, indomethacin had no effect on NaHS responses (Figure 4.7a).

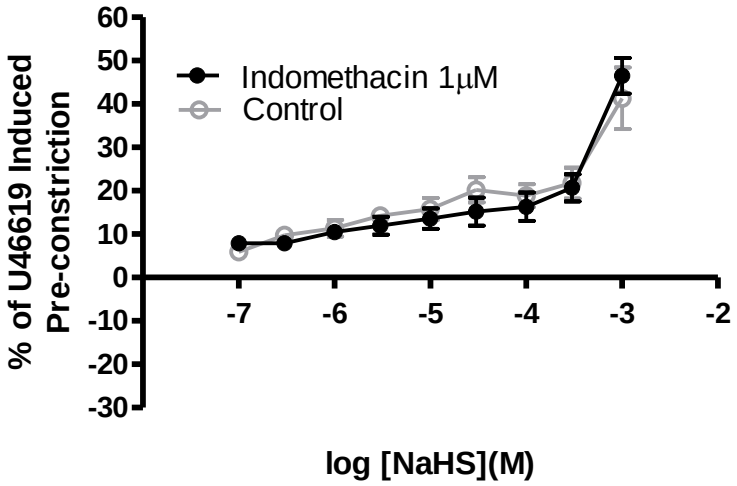

Figure 4.7a Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of indomethacin (1 μM) in porcine splenic arteries pre-constricted with U46619 and gassed with 95% O_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 7). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

In 95% air:5% CO_2, indomethacin had no significant effect on NaHS responses (Figure 4.7b).

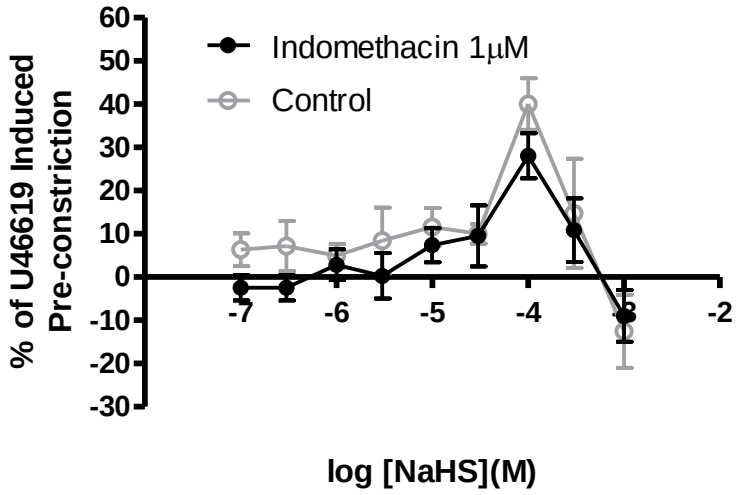

Figure 4.7b Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of indomethacin (1 μM) in porcine splenic arteries pre-constricted with U46619 and gassed with 95% air:5% CO_2. Each point represents the mean ± S.E.M. (n = 5). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

To investigate if the attenuation of NaHS-induced contractile after denudation of the endothelium was due to an interaction with NO, responses were carried out in the presence and absence of the NOS inhibitor, L-NAME (100 µM). In 95% O_2:5% CO_2, L-NAME attenuated the NaHS contractile responses (Figure 4.8a). The concentration of U46619 required to pre-constriction the vessels to similar levels was not affected in the presence of L-NAME.

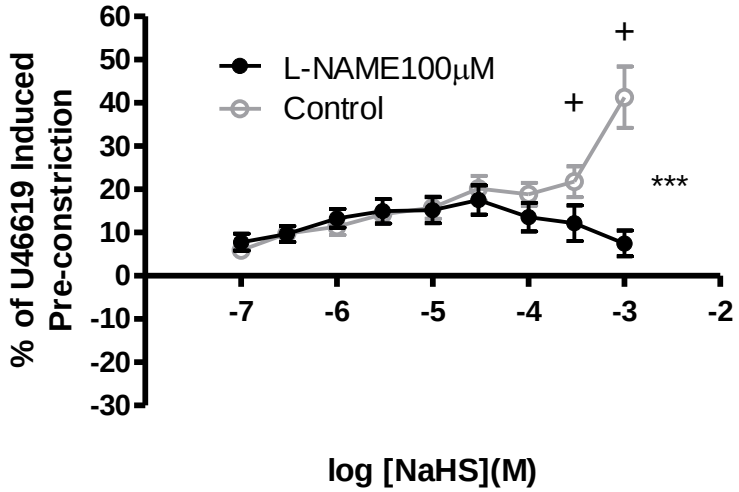

Figure 4.8a Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of L-NAME (100 µM) in porcine splenic arteries pre-constricted with U46619 and gassed with 95% O_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 7). *** represents a significant difference between the curves ($p<0.001$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

In 95% air:5% CO_2 gassed PSS, L-NAME also abolished the contractile response to NaHS uncovering a small relaxation response at concentrations of NaHS above 100 µM (Figure 4.8b). The concentration of U46619 required to induce pre-constriction was not different in the presence of L-NAME.

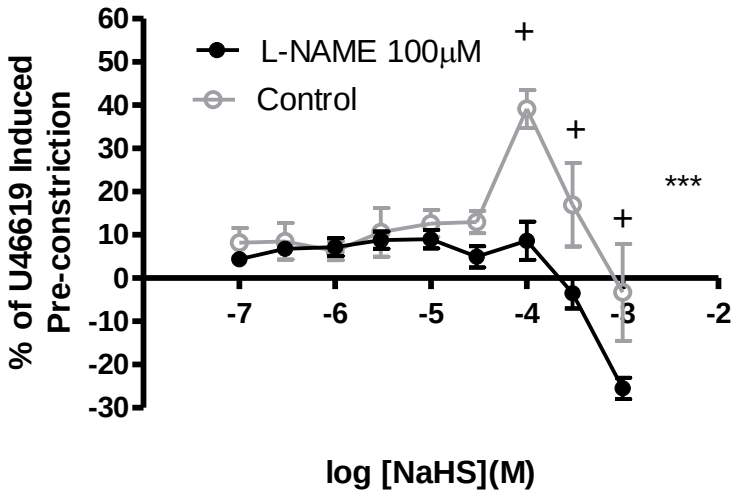

Figure 4.8b Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of L-NAME (100 µM) in porcine splenic arteries pre-constricted with U46619 and gassed with 95% air:5% CO_2. Each point represents the mean ± S.E.M. (n = 7). *** represents a significant difference between the curves ($p<0.001$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

Discussion

In the present study, experiments conducted in 95% O_2:5% CO_2 gassed PSS showed that NaHS caused contractile responses at concentrations above 10 µM. In preparations gassed with 95% air:5% CO_2, NaHS produced concentration-dependent contractions at concentrations up to 100 µM followed by small relaxations at concentrations above 100 µM. Removal of the endothelium, and inhibition of the production of NO with L-NAME, both significantly attenuated NaHS-induced contractions, under either gassing condition, indicating NaHS interacts with endothelium-derived NO, to remove the vasorelaxatory effects of NO and to cause a contractile response. Inhibition of K_{ATP} channels using glibenclamide significantly reduced the small NaHS-induced relaxation responses observed under the 95% air:5% CO_2 gassing condition.

U46619 (up to a concentration of 1 µM) was used to induce pre-constriction in experiments investigating the effects of different O_2 levels used to gas the PSS. Although responses to 60 mM KCl were not significantly different between gassing conditions, U46619 was unable to maintain a stable pre-constricted in splenic arteries using the 95% N_2:5% CO_2 gas mixture. Therefore responses to NaHS were only determined using 95% O_2:5% CO_2 and 95% Air: 5% CO_2. In porcine coronary arteries, changing the gassing mixture from 95% O_2:5% CO_2 to 95% N_2:5% CO_2 produced a hypoxic relaxation of U46619-induced tone in part via activation of K^+ channels (Shimizu *et al.,* 2000). It is possible that under the 95% N_2:5% CO_2 gassing condition that a K^+ channel-dependent hypoxic relaxation

response was inhibiting U46619-induced tone, to such an extent that no receptor-mediated contraction was possible, even though depolarizing responses to KCl were unaffected by these hypoxic conditions.

The effect of removing the endothelium on NaHS responses was investigated. Perhaps surprisingly, vessels that were endothelium-denuded showed no difference in the amount of U46619 required to produce equivalent levels of pre-constriction. Nonetheless, in the absence of endothelium, the contractile response to NaHS was almost completely abolished under either gassing condition. These observations are similar to those made by Kubo *et al.* (2007), in rat aortic rings where removal of the endothelium abolished contractions induced by NaHS and suggests the contractile responses to NaHS are mediated by a factor released from the endothelium.

To investigate if endothelium-dependent NaHS responses were mediated via the generation of cyclooxygenase products, experiments were conducted in the presence of indomethacin. In porcine splenic arteries, indomethacin had no effect on NaHS-induced contractile responses under either gassing condition, suggesting no interaction with cyclooxygenase or its products. In contrast, indomethacin (5 μM) caused a small enhancement of contractile responses to NaHS in rat aorta, suggesting some role for cyclooxygenase in NaHS-induced relaxation responses in other vascular preparations (Koenitzer *et al.,* 2007).

NO has been shown to contribute to contractile responses produced by NaHS and H_2S responses in rat aortic rings (Ali *et al.*, 2006; Kubo *et al.,*

2007; Lim *et al.,* 2008) and human internal mammary artery (Webb *et al.,* 2008). In porcine splenic arteries, L-NAME virtually abolished NaHS-induced contractile responses under both gassing conditions. The interpretation of this data is that NaHS is mopping up NO, to remove its vasorelaxatory action and hence promoting contraction. In support of this, using biochemical techniques, Whiteman *et al.* (2006), showed that NO interacts chemically with H_2S to produce a nitrosothiol compound that is inactive in blood vessels. Similarly, Ali *et al.* (2006), observed that the vasocontraction produced by NaHS in rat aorta was abolished by L-NAME by a similar mechanism. The data in the present study is consistent with the suggestion that NaHS causes vasocontraction by removal of the normal vasorelaxatory function of NO. In another study, Kubo *et al.* (2007), showed that NaHS was directly inhibiting the activity of recombinant bovine eNOS. This alternative mechanism of NaHS inhibition of NO may also be occurring in the current study.

The NaHS-induced vasorelaxation observed when gassing with 95% Air:5% CO_2 was not sensitive to the non-selective K+ channel blocker, TEA (1mM). However, the K_{ATP} channel specific inhibitor, glibenclamide (10 µM) caused a significant reduction in the NaHS-induced relaxation responses. Furthermore, NaHS-induced contractile responses were significantly enhanced by glibenclamide in the 95% O_2:5% CO_2 condition, suggesting the possible inhibition of a relaxation response normally masked by NaHS induced contractions. Using glibenclamide, K_{ATP} channels were shown to be a target of NaHS in the porcine mesenteric arteries under 95% N_2: 5% CO_2. Glibenclamide also attenuated NaHS vasodilator responses in the perfused rat mesenteric arterial bed (Cheng *et al.,* 2004). In addition, in VSMCs

isolated from rat mesenteric arteries, NaHS caused an increase in K_{ATP} channel currents which again was attenuated by glibenclamide (Tang *et al.,* 2005). When gassing with 95% air: 5% CO_2, relaxation response to 1 mM NaHS were small and inconsistent, masked by the competing contractile responses. However, contractile responses obtained when gassing PSS with 95% O_2: 5% CO_2 were enhanced by glibenclamide indicating an underlying relaxation responses to NaHS possibly mediated by K_{ATP} channels. Enhanced NaHS-induced contractile responses in the presence of glibenclamide have also been observed in rat aorta (Lim *et al.,* 2008). It is difficult to understand why TEA had no effect on relaxation responses to NaHS, but it has been reported that it is not a very potent inhibitor of K_{ATP} channels even at a concentration of 1mM (Brayden, 2002).

In the rat urinary bladder, it has been shown that NaHS stimulated capsaicin sensitive transient receptor potential vanilloid receptor 1 (TRPV1), located on sensory nerves, causing the release of neuropeptides and inducing contraction (Patacchini *et al.,* 2005). However, in porcine splenic arteries, capsaicin had no effect on NaHS-induced responses in either gas mixture. This suggests that sensory nerve mediated release of vasoactive neuropeptides is not involved in the porcine splenic arteries response to NaHS, in contrast to rat mesenteric arteries (see chapter 6).

As described in the previous chapter, under 95% O_2:5% CO_2 or 95% air:5% CO_2, the pO_2 was 619 ± 17 mmHg and 140 ± 4 mmHg, respectively. In mammals, the pO_2 is normally around 100mmHg in arterial blood (Erecińska and Silver, 2001; Tsai *et al*., 2003). The majority of studies investigating the effects of H_2S on conduit arteries have been conducted in

preparations gassed with 95% O_2:5% CO_2 (Zhao *et al*., 2001; Ali *et al*., 2006; Lim *et al*., 2008; Webb *et al.*, 2008). The results of these studies may reflect non-physiological conditions. In a study specifically on pigs, Gianotti *et al.* (2010) showed that, under normal conditions they had an arterial pO_2 of 90.65 ± 6.95 mmHg. In this study, gassing with 95% air:5% CO_2 creates an oxygen tension similar to that observed under normal physiological conditions. Thus in the present study, the observed response under these conditions i.e. a contraction (due to an interaction with endothelial-derived NO) at low concentrations and a relaxation (mediated by activation of K_{ATP} channels) may be most relevant.

In summary, NaHS caused contractile responses in porcine splenic arteries when gassing the PSS with a 95% O_2:5% CO_2 gas mixture. These contractile responses were attenuated by removal the endothelium or inhibition of eNOS with L-NAME, suggesting H_2S interacts with endothelium-derived NO or its production from eNOS. When gassing the PSS with 95% air:5% CO_2, NaHS produced concentration dependent contractions in splenic arteries at lower concentrations and relaxations at higher NaHS concentrations. Removal of the endothelium or the presence of L-NAME again abolished the contractile responses. Furthermore, the NaHS-induced relaxation response was significantly attenuated by glibenclamide, suggesting a role of K_{ATP} channels in NaHS-induced vasorelaxatory response.

Chapter 5:

NaHS and NO Interactions in Porcine Splenic Veins

Introduction

In the previous chapters the effects of NaHS was studied in arterial preparations isolated from the pig, namely mesenteric and splenic arteries. It was shown that different oxygen levels in the gassing mixtures changed the nature of the response to NaHS. In the main, contractile responses were observed when gassing with 95% O_2:5% CO_2 and 95% air:5% CO_2 that were attenuated by removing the endothelium and by the eNOS inhibitor L-NAME, suggesting an interaction with NO underlay the contractile response. As oxygen levels were decreased, relaxation responses were generally produced by NaHS. In the main, relaxation responses to NaHS were attenuated by glibenclamide suggesting some involvement of K_{ATP} channels.

Similar observations have been reported in the rat aorta by Koenitzer *et al.* (2007), who observed that, when gassed with a 200 µM O_2 level (equivalent to gassing with 95% Air:5% CO_2), H_2S induced a contractile response at low concentrations. At high concentrations H_2S elicited a vasorelaxant response and the predominant response in preparations gassed with an O_2 level of 40 µM (equivalent to gassing with 95% N_2:5% CO_2) was vasorelaxation. This change in response was suggested to be due to increased H_2S oxidization at 200 µM O_2, leading to a reduced H_2S availability and subsequent loss of vasorelaxation or, to the generation of H_2S-derived oxidation products that had contractile properties. Interestingly, Wills *et al*. (1989) showed that meta(bis)sulphate, an oxidative product of H_2S, caused vasoconstriction in rat aortic rings. These data show that the

oxygen tension is a crucial factor in determining responses to H_2S in mammalian conduit arteries.

As previously shown, PSS gassed with 95% O_2:5% CO_2, 95% air:5% CO_2 and 95% N_2:5% CO_2 have a pO_2 of 619 ± 17 mmHg, 140 ± 4 mmHg and 33 ± 6 mmHg, respectively. In mammals, the pO_2 is normally around 100mmHg in arterial blood and varies considerably in venous blood, between 15 and 50 mmHg (Erecińska and Silver, 2001; Tsai *et al*., 2003). In the majority of *in vitro* studies examining vascular function, preparations are gassed with 95% O_2:5% CO_2. The justification for this is that, while pO_2 is high, it is necessary to ensure the total oxygen content in the PSS can meet the metabolic demands of the tissue. However, the environment created by gassing with 95% O_2:5% CO_2 (619 ± 17 mmHg) is supra-physiological and may lead to oxidative stress and increase the production of reactive oxygen species in tissues (Halliwell, 2007). This is potentially problematic in arterial preparations, but is certainly of concern in venous preparations.

To date, there are no publications reporting the effects of H_2S in veins. Larger veins are exposed to relatively low oxygen concentrations under normal physiological conditions. For example, the pO_2 was 38.2±12.3 mmHg in rat cortical venules (Vovenko, 1999), 26 ± 10 mmHg in the spinotrapezius muscle venules (Shonat and Johnson, 1997), and 36 mmHg in hamster jugular vein (Duling and Berne, 1970). On this basis, the current chapter examined responses to NaHS in the porcine splenic vein, varying the gassing conditions.

Materials and Methods

Set up and General Protocol

Porcine splenic veins were isolated and set up in an isometric tension recording system as described in the methods chapter. Vessels were set up in baths containing 20ml PSS at 37˚C and gassed with either 95% O_2:5% C_{O2}, 95% Air:5% CO_2 or 95% N2:5% CO_2. The PSS was pre-gassed with each mixture for at least 30 minutes to ensure gaseous equilibrium of the PSS. Vessels were pre-tensioned to approximately 8-10 g (this level of pre-tensioning was shown to produce the largest KCl 60 mM response in preliminary experiments) and allowed to relax for 30-40 minutes to achieve a stable baseline tone. Thereafter KCl (60 mM) was added to the bath and vessels were allowed to contract. After the response plateaued, the KCl was washed out with fresh PSS at least 2 times at 5 min intervals. Following the return to a stable baseline and after a further 20 minutes, exposure to KCl, followed by the washout process, was repeated.

After three repeats of responses to KCl, any test compounds used in the experiment were added and allowed at least 30 minutes incubation time. The following test compounds were used: the cyclooxygenase inhibitor, indomethacin (1 µM); the NOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME) (100 µM); to desensize sensory nerves, capsaicin (10 µM); the non-specific K^+ channel blocker, tetraethylammonium (TEA) (1 mM) and the selective K_{ATP} channel inhibitor, glibenclamide (10 µM). After this period, vessels were pre-contracted to a level of 30-70% of the maximum KCl response using the thromboxane A2 receptor agonist, U46619. Once vessel

tension had stabilized, a concentration-response curve to NaHS was obtained by adding it in a cumulative manner, in 3-fold increments, between 100 nM and 3 mM, with a period of 5 minutes between additions.

In all experiments a second segment of the same vessel was set up and used as a control.

Endothelium Denudation

Some experiments were designed to investigate the role of the endothelium in mediating responses to NaHS. This was carried out in a similar manner to that in the previous chapter. Briefly, the endothelium was removed mechanically from vessel segments prior to set up in the organ bath. Substance P (1 μM) was used to assess endothelial function in vessels pre-constricted with noradrenaline. After exposure these compounds were washed out. The criterion was set that only vessels that did not respond to substance P were accepted as successfully denuded, in comparison to a relaxation of at least 15% in their paired control segment.

Results

Experiments investigating response to NaHS in porcine splenic veins were carried out using three different gassing mixtures, 95% O_2:5% CO_2, 95% Air:5% CO_2 and 95% N_2:5% CO_2. The gas mixture had no significant effect on the level of tension induced by the third KCl (60 mM) responses ($p>0.05$; Student's t-test). The average levels of KCl responses were 4.2 ± 1.5 g gassing with 95% O_2:5% CO_2 (n = 24), 4.2 ± 1.6 g gassing with 95% air:5% CO_2 (n = 26) and. 4.0 ± 0.7 g gassing with 95% N_2:5% CO_2 (n = 27).

In addition, there was no significant difference in the concentration of U46619 required to induce similar levels of tone under each gassing condition ($p>0.05$; Student's t-test). The average level of pre-constriction of the 95% O_2: 5% CO_2 data was 48 ± 8% using an average U46619 concentration of $3 \times 10^{-9} \pm 1 \times 10^{-9}$ M (n = 24), in the 95% air: 5% CO_2 data was 47 ± 9% using an average U46619 concentration of $3 \times 10^{-9} \pm 2 \times 10^{-9}$ M (n = 26) and in the 95% N_2: 5% CO_2 data was 52 ± 9% using an average U46619 concentration of $3 \times 10^{-9} \pm 1 \times 10^{-9}$ M (n = 27). Representative traces of the responses to NaHS under these conditions are shown in Figure 5.1 and summarised in Figure 5.2. When gassing with 95% O_2:5% CO_2 or 95% air:5% CO_2 mixtures, NaHS concentration response curves were similar, consisting of contractions at concentrations below 300 µM followed by relaxations at higher concentrations. When gassing with 95% N_2:5% CO_2, NaHS generally produced only a vasorelaxation response, although these responses were not very well maintained until the NaHS concentration exceeded 100 µM.

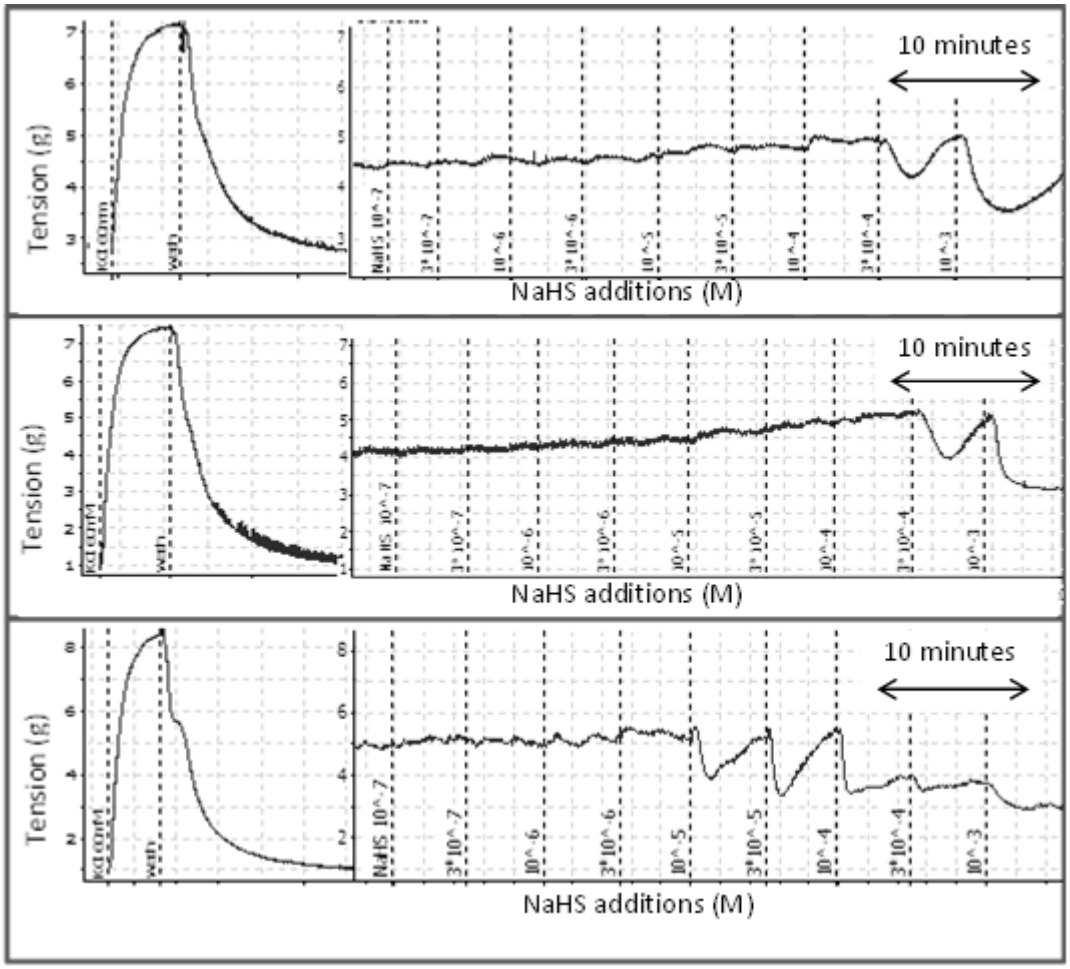

Figure 5.1 Representative traces showing responses to NaHS in porcine splenic veins under different gassing conditions. Responses to 60 mM KCl were obtained (left panels) then U46619 was used to pre-constrict vessels by 30-70% of the 60 mM KCl response, after which concentration response curves to NaHS were obtained. Example a) shows responses to NaHS when gassing with 95% O_2:5% CO_2, illustrating small contractile responses followed by transient relaxations at concentration exceeding 300 µM. Example (b) shows a similar pattern of responses to NaHS in vessels gassed with 95% air:5% CO_2. Example (c) shows that NaHS caused a transient relaxation starting at concentrations of 10 µM, which became more sustained at concentration exceeding 100 µM, when vessels were gassed with 95% N_2:5% CO_2.

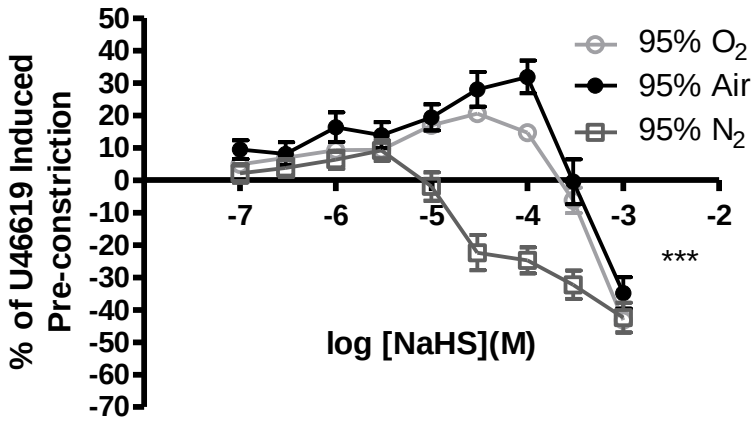

Figure 5.2 Concentration response curves to NaHS gassed with 95% O_2:5% CO_2 (open circles), 95% air:5% CO_2 (closed circles) or 95% N_2:5% CO_2 (open squares) in porcine splenic veins pre-constricted with U46619. Each point represents the mean ± S.E.M. (n = 24 for 95% O_2:5% CO_2, n = 26 for 95% air:5% CO_2 and n = 27 for 95% N_2:5% CO_2). *** represents a significant difference between the curves ($p<0.001$; 2 way ANOVA).

Under all three gassing conditions there was a relaxation response produced by higher concentrations of NaHS. To investigate the involvement of K_{ATP} channel in this relaxation, the specific K_{ATP} channel inhibitor, glibenclamide (10 µM) was used. In 95% O_2:5% CO_2 gassed PSS, NaHS responses were not attenuated by glibenclamide (Figure 5.3a). In the presence of glibenclamide a higher concentration of U46619 was required to achieve the same level of pre-constriction as in its absence ($4 \times 10^{-8} \pm 1 \times 10^{-8}$ M compared to $3 \times 10^{-9} \pm 5 \times 10^{-10}$ M; n = 5).

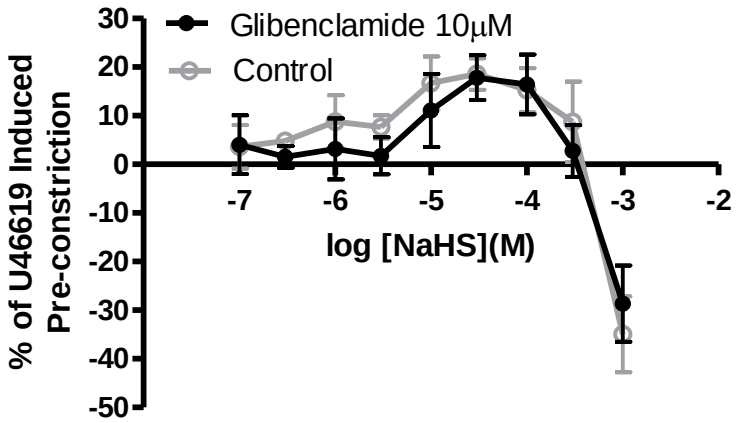

Figure 5.3a Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of glibenclamide (10 µM) in porcine splenic veins pre-constricted with U46619 and gassed with 95% O_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 5). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

In the 95% air:5% CO_2 mixture glibenclamide had no significant effect on responses to NaHS (Figure 5.3b), although there was very little relaxation observed at the higher concentration of NaHS in this series of experiments. Again, glibenclamide increased the concentration of U46619 required to induce tone in its presence ($9 \times 10^{-8} \pm 2 \times 10^{-8}$ M compared with $2 \times 10^{-9} \pm 1 \times 10^{-9}$ M; n = 7)

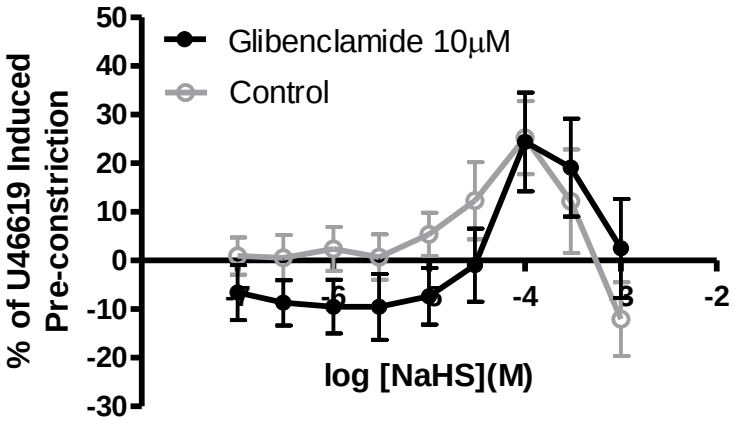

Figure 5.3b Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of glibenclamide (10 μM) in porcine splenic veins pre-constricted with U46619 and gassed with 95% air:5% CO_2. Each point represents the mean ± S.E.M. (n = 7). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

In 95% N_2:5% CO_2, glibenclamide showed no effect on NaHS responses, that were rather inconsistent in this series (Figure 5.3c). In the 95% N_2:5% CO_2 vessels were pre-constricted to similar levels using an average U46619 concentration of $4 \times 10^{-8} \pm 3 \times 10^{-8}$ M in the presence and $3 \times 10^{-9} \pm 2 \times 10^{-9}$ M in the absence of glibenclamide (n = 6).

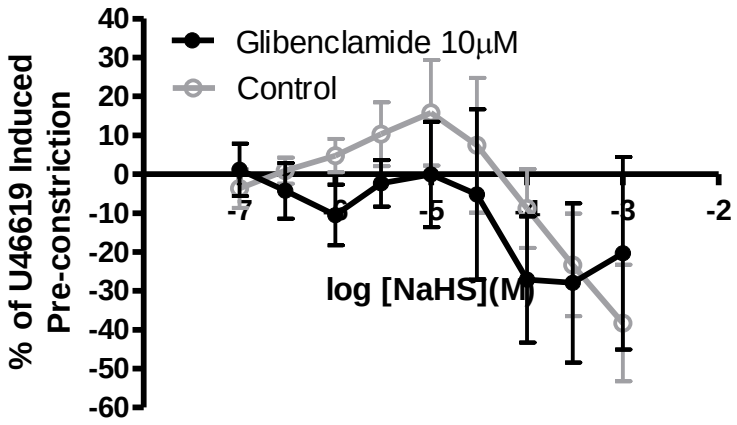

Figure 5.3c Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of glibenclamide (10 μM) in porcine splenic veins pre-constricted with U46619 and gassed with 95% N_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 6). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

As there was no evidence for the involvement of K_{ATP} in mediating NaHS-induced relaxations, the possible role of other potassium channels, was assessed in the presence and absence of TEA (1 mM), the non-selective K^+ channel blocker. In 95% O_2:5% CO_2, TEA had no significant effect on NaHS-induced relaxation responses (Figure 5.4a). TEA had no effect on the concentration of U46619 required to pre-constrict the splenic veins.

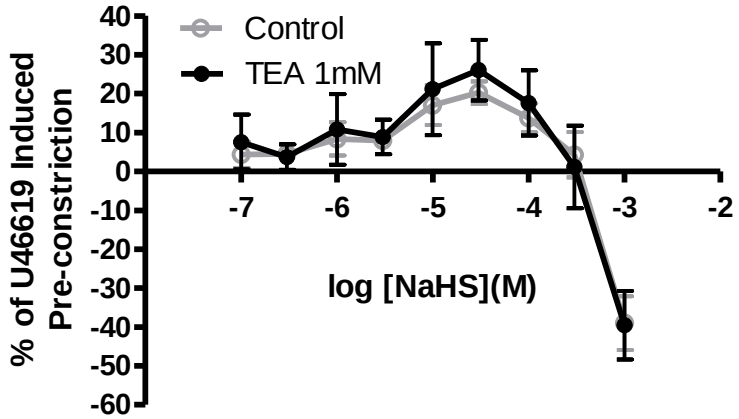

Figure 5.4a Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of TEA (1 mM) in porcine splenic veins pre-constricted with U46619 and gassed with 95% O_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 8). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

In the 95% air:5% CO_2 mixture, TEA showed no significant effect on the NaHS concentration responses curve. However, there was a modest reduction in the relaxation response at 1 mM NaHS (Figure 5.4b). In 95% air:5% CO_2, vessels were pre-constricted using an average U46619 concentration of $3 \times 10^{-9} \pm 1 \times 10^{-9}$ M in the control, compared to $8 \times 10^{-10} \pm 6 \times 10^{-10}$ M in the presence of TEA to induced the same pre-constriction level (n = 6).

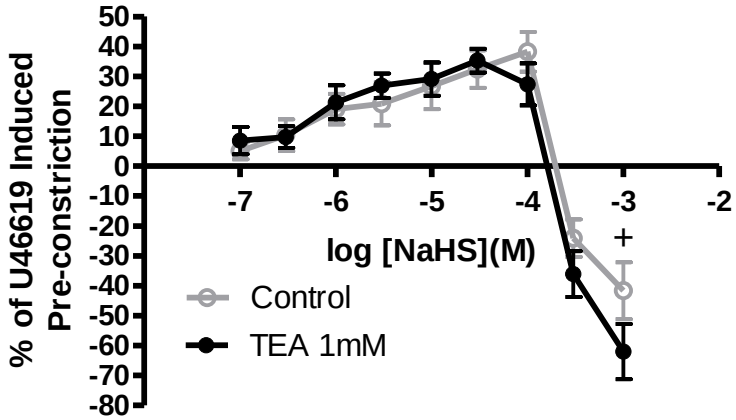

Figure 5.4b Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of TEA (1 mM) in porcine splenic veins pre-constricted with U46619 and gassed with 95% air:5% CO_2. Each point represents the mean ± S.E.M. (n = 6). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

In 95% N_2:5% CO_2, TEA had no significant effect on NaHS responses (Figure 5.4c). TEA did not affect the concentration of U46619 required to produce pre-constriction.

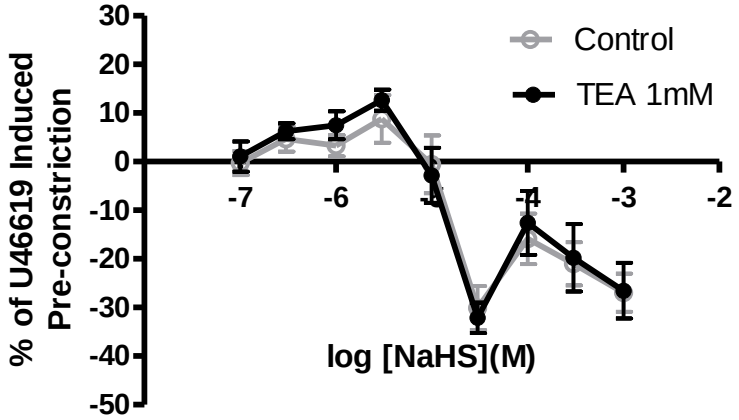

Figure 5.4c Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of TEA (1 mM) in porcine splenic veins pre-constricted with U46619 and gassed with 95% N_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 6). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

To investigate the potential involvement of sensory nerves in mediating NaHS responses, experiments were carried out after exposure to capsaicin (10 μM) or methanol. Under all gassing conditions capsaicin had no significant effect on responses to NaHS (Figure 5.5). There was no difference in concentration of U46619 or pre-constriction level between capsaicin and the controls.

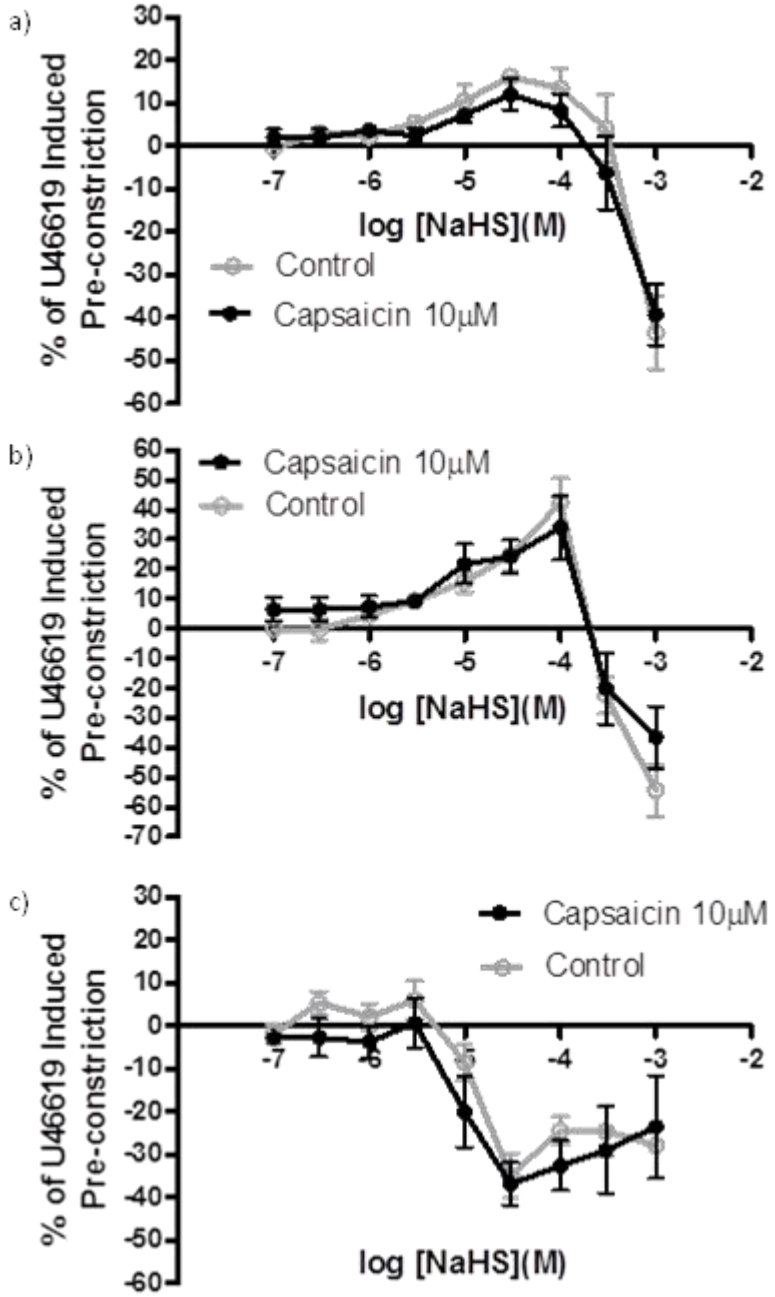

Figure 5.5 Concentration response curves to NaHS in non-treated (closed circles) or capsaicin- treated (1 μM) (open circles) porcine splenic veins pre-constricted with U46619 and gassed with; a) 95% O_2:5% CO_2 (n = 6), b) 95% air:5% CO_2 (n = 6), and c) 95% N_2:5% CO_2 (n = 4). Each point represents the mean ± S.E.M. There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

Responses to NaHS were assessed after removal of the endothelium. Vessels that did not response to substance P (0 ± 0%) were considered successfully denuded of their endothelium. In 95% O_2:5% CO_2, substance P caused a transient relaxation of 23 ± 7% of the noradrenaline pre-constriction in unrubbed control segments of the splenic vein, compared with 0 ± 0% in rubbed segments. Average KCl responses were 4.1 ± 1.4 g in endothelium-intact vessels and 3.8 ± 0.9 g in endothelium-denuded vessels (n = 8). There was no significant difference between responses to KCl between the two groups ($p>0.05$; Student's t-test). The removal of the endothelium attenuated responses to NaHS, preventing both the contraction and relaxation responses (Figure 5.6a). There was a significant difference between both the peak contraction and peak relaxation of endothelium intact and endothelium denuded vessels ($p<0.05$; Mann Whitney test). The level of pre-constriction was not significantly different in endothelium intact or endothelium denuded vessels, using similar concentrations of U46619 to induce tone. The same average concentration of U46619 ($3 \times 10^{-9} \pm 1 \times 10^{-9}$ M) was used. There was no significant difference in the level of pre-constriction induced, averaging 49 ± 7% in the endothelium-intact vessels and 52 ± 3% in endothelium-denuded vessels ($p>0.05$; Student's t-test; n = 8).

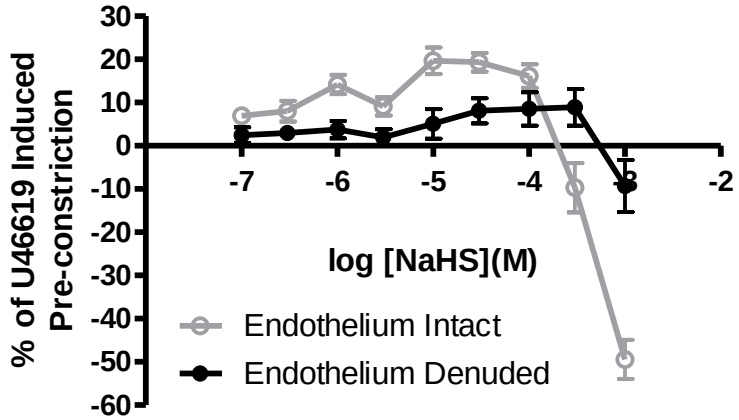

Figure 5.6a Concentration response curves to NaHS in endothelium intact (open circles) or endothelium denuded (closed circles) porcine splenic veins, pre-constricted with U46619 and gassed with 95% O_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 8). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

In 95% air:5% CO_2, removal of the endothelium effectively abolished responses to NaHS, attenuating the majority of both the contractile and the relaxation response to NaHS (Figure 5.6b). In vessels with the endothelium intact, substance P caused a transient relaxation by 45 ± 16% of noradrenaline pre-constriction compared with 0 ± 0% in those that were endothelium denuded. Average KCl responses were 3.9 ± 1.0 g in endothelium-intact vessels and 3.7 ± 1.2 g in endothelium-denuded vessels (n = 6). There was no significant difference between responses to KCl between the two groups ($p>0.05$; Student's t-test). The concentration of U46619 required to induce tone was not affected by removal of the endothelium. The same average concentration of U46619 ($1 \times 10^{-9} \pm 7 \times 10^{-10}$ M) was used. There was no significant difference in the level of pre-constriction induced, averaging 40 ± 6% in the endothelium-intact vessels and 42 ± 6% in endothelium-denuded vessels ($p>0.05$; Student's t-test; n = 6).

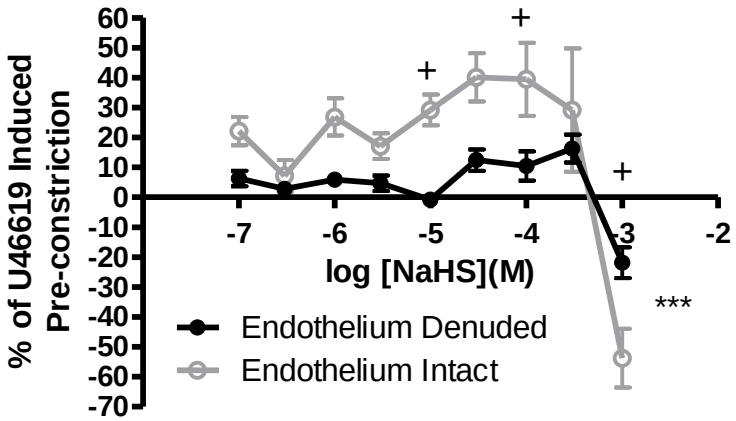

Figure 5.6b Concentration response curves to NaHS in endothelium intact (open circles) or endothelium denuded (closed circles) porcine splenic veins pre-constricted with U46619 and gassed with 95% air:5% CO_2. Each point represents the mean ± S.E.M. (n = 6). *** represents a significant difference between the curves ($p<0.001$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

In 95% N_2:5% CO_2, removal of the endothelium significantly attenuated responses to NaHS, reducing the extent of the relaxation response (Figure 5.6c). substance P caused a transient relaxation by 23 ± 6% of noradrenaline pre-constriction compared with 0 ± 0% in endothelium denuded vessels. Average KCl responses were 4.0 ± 0.6 g in endothelium-intact vessels and 3.9 ± 1.1 g in endothelium-denuded vessels (n = 8). There was no significant difference between responses to KCl between the two groups ($p>0.05$; Student's t-test). The same average concentration of U46619 ($2 \times 10^{-9} \pm 1 \times 10^{-9}$ M) was used. There was no significant difference in the level of pre-constriction induced, averaging 48 ± 11% in the endothelium-intact vessels and 46 ± 10% in endothelium-denuded vessels ($p>0.05$; Student's t-test; n = 8).

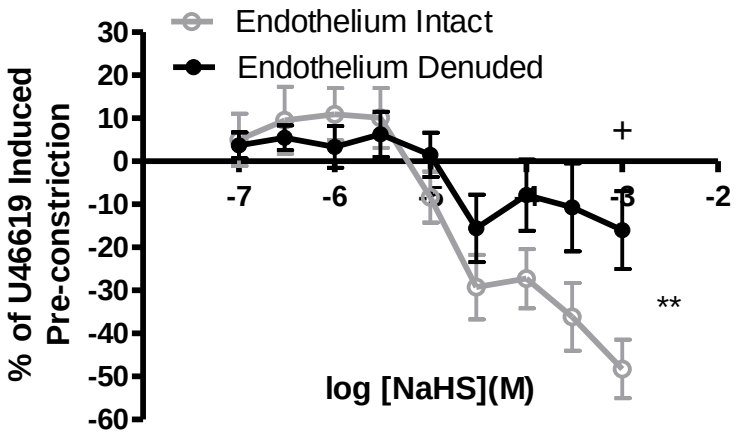

Figure 5.6c Concentration response curves to NaHS in endothelium intact (open circles) or endothelium-denuded (closed circles) segments of porcine splenic veins pre-constricted with U46619 and gassed with 95%N_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 8). ** represents a significant difference between the curves ($p<0.01$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

In preparations gassed with 95% O_2:5% CO_2, L-NAME had no significant effect on the NaHS response curve but modestly reduced the small amount of contractile response observed at 30 μM (Figure 5.7a). In addition, there was a significant difference between the peak contraction of endothelium intact and endothelium denuded vessels ($p<0.05$; Mann Whitney test). A similar level of pre-constriction was achieved in the presence of L-NAME, using a lower average U46619 concentration ($5 \times 10^{-10} \pm 3 \times 10^{-10}$ M) compared to the controls ($3 \times 10^{-9} \pm 2 \times 10^{-9}$ M). There was a significant difference between the concentrations of U46619 used in the two groups ($p<0.05$; Student's t-test; n = 8).

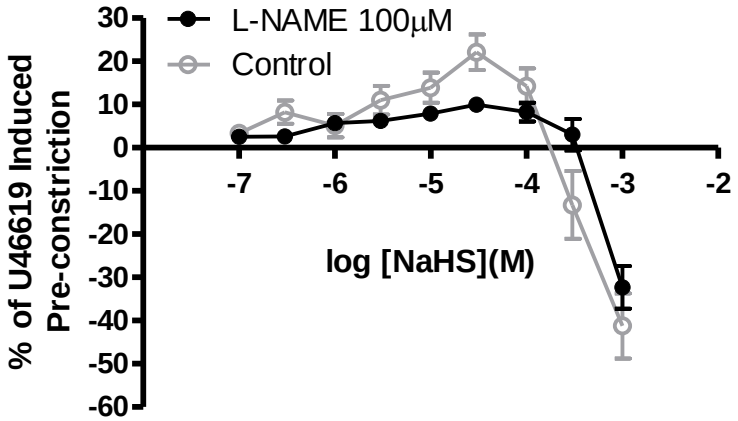

Figure 5.7a Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of L-NAME (100 μM) in porcine splenic veins pre-constricted with U46619 and gassed with 95% O_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 8). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA)

In 95% air:5% CO_2, L-NAME attenuated NaHS-induced contractile responses (Figure 5.7b). The level of pre-constriction was 57 ± 8% using an average U46619 concentration of 2 x 10^{-9} ± 1 x 10^{-9} M in the presence of L-NAME and 46 ± 10% using an average U46619 concentration of 8 x 10^{-9} ± 2 x 10^{-9} M in its absence. There was a significant difference between the concentrations of U46619 used in the two groups ($p<0.05$; Student's t-test; n = 7).

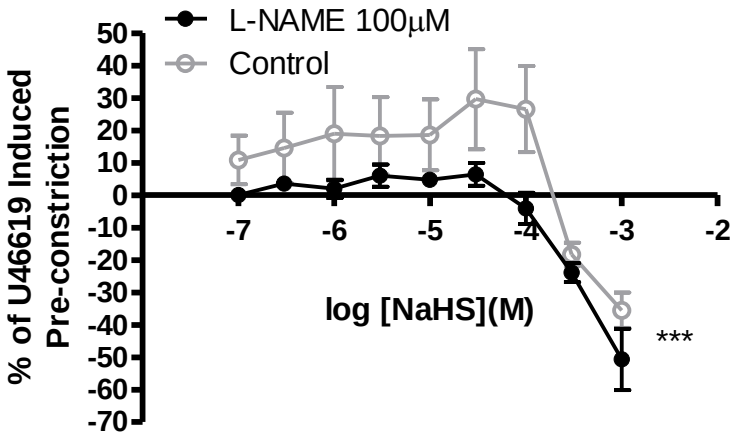

Figure 5.7b Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of L-NAME (100 μM) in porcine splenic veins pre-constricted with U46619 and gassed with 95% air:5% CO_2. Each point represents the mean ± S.E.M. (n = 7). *** represents a significant difference between the curves ($p<0.001$; 2 way ANOVA).

In 95% N_2:5% CO_2, L-NAME significantly reduced the relaxation response to NaHS (Figure 5.7c). In this series of experiments, there was no difference in the concentration of U46619 required to produce a similar level of pre-constriction in the presence or absence of L-NAME.

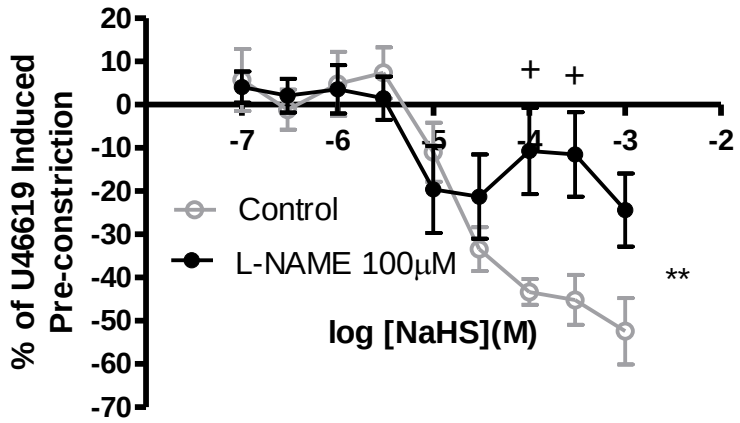

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Figure 5.7c Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of L-NAME (100 µM) in porcine splenic veins pre-constricted with U46619 and gassed with 95% N_2:5% CO_2. Each point represents the mean ± S.E.M. (n = 7). ** represents a significant difference between the curves ($p<0.01$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

To investigate the potential role of cyclooxygenase and its products in NaHS-induced relaxation responses, the cyclooxygenase inhibitor, indomethacin (1 μM) was used. The presence of indomethacin had no significantly effect on NaHS responses under any gassing conditions (Figure 5.8a). In the presence of indomethacin, there was no difference in concentration of U46619 was required to achieve a similar level of pre-constriction.

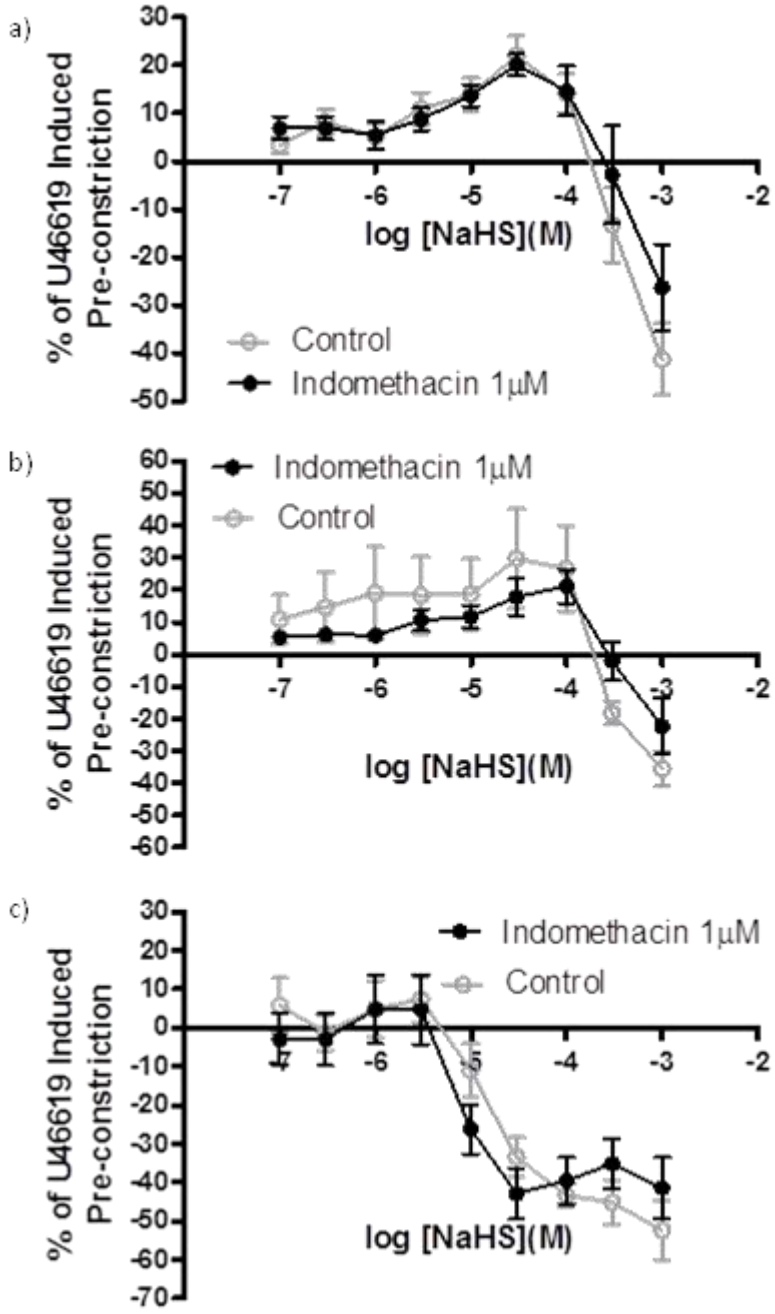

Figure 5.8 Concentration response curves to NaHS in the presence (closed circles) or absence (open circles) of indomethacin (1 μM) in porcine splenic veins pre-constricted with U46619 gassed with; a) 95% O_2:5% CO_2 (n = 8), b) 95% air:5% CO_2 (n = 7), and c) 95% N_2:5% CO_2 (n = 7). Each point represents the mean ± S.E.M. There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

Discussion

In the present study, under conditions where splenic veins were gassed with 95% O_2:5% CO_2, or 95% air:5% CO_2, NaHS caused contractions at concentrations below 100 µM followed by relaxation at higher concentrations. In experiments in which preparations were gassed with 95% N_2:5% CO_2, NaHS only produced vasorelaxation. Both the removal of the endothelium and inhibition of NOS with L-NAME, abolished contractile responses to NaHS when preparations were gassed with either 95% O_2:5% CO_2, or 95% air:5% CO_2. Thus the contractile responses to NaHS are via interactions with NO produced from eNOS in a similar manner to that described in mesenteric and splenic arteries (chapters 3 & 4). In preparations gassed with 95% N_2:5% CO_2, removal of the endothelium or L-NAME both attenuated the relaxation response. This suggests that NaHS is causing the release of NO from the endothelium.

Unlike porcine splenic arteries, there was no significant difference in the U46619 concentration required to induce the same level of pre-constriction in porcine splenic veins under any of the gassing conditions, including 95% N_2:5% CO_2. This suggests that in porcine splenic veins, U46619-induced pre-constriction is more stable when gassing with 95% N_2:5% CO_2 than in the equivalent arteries. This is possibly due to the oxygen tension presented by 95% N_2:5% CO_2 (33 ± 6 mmHg) being similar to values reported in venous blood (15-50 mmHg) and thus not hypoxic in the way this would be for arterial preparations (Erecińska and Silver, 2001; Tsai *et al*., 2003).

In porcine splenic veins, removal of the endothelium abolished contractile responses and significantly attenuated relaxatory responses to NaHS, regardless of the gassing condition. Thus responses to NaHS are mediated via a substance released from the endothelium in porcine splenic veins. This contrasts with porcine splenic arteries, where removal of the endothelium abolished contractile response to NaHS, but enhanced the relaxatory response. This suggests that, in splenic veins, the release of EDRFs mediates the relaxatory response to NaHS.

In tissue gassed with either 95% O_2:5% CO_2 or 95% air:5% CO_2, NaHS relaxation responses were not blocked by L-NAME. In contrast, L-NAME attenuated relaxation responses to NaHS produced when using the 95% N_2:5% CO_2 gas mixture. This reduction in the response was similar to the effect of removing the endothelium on NaHS-induced relaxation. This suggests that under N_2:5% CO_2 conditions, NO is being released from the endothelium. Both endothelium-dependent and L-NAME-sensitive relaxations induced by NaHS have also been observed in rat aorta using a 95% O_2:5% CO_2 gas mixture (Zhao *et al.*, 2001). However, the endothelium-dependent NaHS-induced relaxation responses in tissue gassed with either 95% O_2:5% CO_2 or 95% air:5% CO_2 were not L-NAME- sensitive, suggesting a different EDRF is being released under these conditions.

To assess the role of cyclooxygenase and its products as an EDRF released by NaHS, experiments were carried out in the presence and absence of indomethacin. In porcine splenic veins, indomethacin (1 µM) had no effect on NaHS responses under any gassing condition. In contrast, Koenitzer *et al.* (2007), found the presence of indomethacin (5 µM) caused

an enhancement of contractile responses to NaHS in rat aorta at higher oxygen concentration (equivalent to gassing PSS with 95% air:5% CO_2) but not at a lower oxygen concentration. They suggested that this may indicate that H_2S was potential causing the release of vasorelaxant prostaglandins at higher oxygen concentrations reducing contractile effects. Despite observing slightly smaller contractile responses when gassing with 95% O_2:5% CO_2 compared with 95% air:5% CO_2, there was no evidence of a similar interaction of NaHS with the cyclooxygenase enzyme in the porcine splenic veins. An alternative hypothesis is that NaHS is causing the release of an EDHF (Cheng *et al.,* 2004).

Relaxation responses in both porcine splenic and mesenteric arteries were in part mediated via the activation of K_{ATP} channels. However, glibenclamide did not affect NaHS relaxation responses in porcine splenic veins under any gassing condition. Furthermore, TEA had no effect on NaHS responses. This suggests that in porcine splenic veins, the mechanism of NaHS relaxation is not mediated via activation of K^+ channels. Alternatively, it is possible that NaHS is acting via TEA insensitive K^+ channels. For example, di Villa Bianca *et al.* (2011), showed NaHS vasodilator responses in rat mesenteric artery beds were significantly attenuated by inhibition of K_{Ca2+} channels. Small and intermediate conductance K_{Ca2+} channels are not inhibited by TEA (Alexander *et al.,* 2009). Lee *et al*. (2007) showed in rat aorta and A7r5 cells (derived from rat BD1X embryonic rat aortic smooth muscle), NaHS induced vasodilatation via activation Cl^-/HCO_3^- exchangers and an alteration of pH_i. It is possible one of these alternative mechanisms may be involved in mediating the L-NAME-resistant response to NaHS,

although this will need to involve an endothelium-derived vasorelaxant such as EDHF.

In the previous chapter, NO produced from NOS was shown to be involved in mediating the NaHS-induced contractile response observed in porcine splenic arteries when gassed with either 95% O_2:5% CO_2 or 95% air:5% CO_2. In porcine splenic veins, NaHS contractile responses were significantly attenuated by L-NAME in preparations gassed with either 95% O_2:5% CO_2 or 95% air:5% CO_2, to a similar extent as removal of the endothelium. As described in the previous chapter, Whiteman *et al*. (2006), showed evidence for the formation of a nitrosothiol compound from NO and H_2S under normal experimental conditions. Thus the data in porcine splenic veins is again consistent with an interaction between NO and H_2S (perhaps creating a non-vasorelaxatory nitrosothiol) mediating contraction when the ambient oxygen concentration is high. The study conducted by Whiteman *et al*. (2006), used 95% O_2:5% CO_2 for gassing the tissue and this may have influenced their observations. Under these conditions, the higher oxygen tension increases the level of free radical oxygen and reactive oxygen species (Kashiba *et al*., 2002). NO readily reacts with super oxide radicals, forming amongst others things, peroxynitrite ($ONOO^-$) (Henry *et al.*, 1991). Whiteman *et al*. (2006), showed synthetic $ONOO^-$ also reacts with H_2S in *vitro,* to form nitrosothiol. It is thus feasible that the contraction occurs due to the generation of oxygen-derived free radicals that mop up and remove the vasorelaxatory effects of both NO and H_2S. Since fewer of these oxygen-derived free radicals would be produced when gassing with 95% N_2:5% CO_2,

then the vasorelaxatory response will predominate under these experimental conditions.

Since the reported range of oxygen tension in mammalian venous blood is between 15 and 50 mmHg (Erecińska and Silver, 2001; Tsai *et al*., 2003), this suggests that responses obtained under 95% N_2: 5% CO_2 are the most physiologically relevant, and inducing relaxation may be the predominant effect of NaHS.

In summary, porcine splenic veins gassed with either 95% O_2:5% CO_2 or 95% air:5% CO_2 contracted in response to lower concentrations of NaHS but relaxed at higher concentrations. When gassing with 95% N_2:5% CO_2, NaHS caused only relaxation responses. Removing the endothelium or, inhibition of eNOS, abolished the majority of the contractile response, suggesting they are mediated via interactions between NO and NaHS. Removal of the endothelium attenuated the relaxation responses under all gassing conditions. The specific endothelium derived relaxing factor was not identified under 95% O_2:5% CO_2 or 95% air:5% CO_2. However, when gassing with 95% N_2:5% CO_2, inhibition of eNOS attenuated NaHS-induced vasorelaxation. This suggests under physiological conditions, NaHS induces vasorelaxation, mediated by NO derived from endothelium.

Chapter 6:

NaHS Causes Sensory Nerve Mediated Vasodilatation in 2nd Order Mesenteric Arteries Isolated from Rats

Introduction

In the previous chapters, responses to NaHS were investigated using porcine conduit arteries and veins. In both porcine splenic and mesenteric arteries, NaHS caused contractile responses when gassed with 95% O_2: 5% CO_2 or 95% Air: 5% CO_2. In general, the NaHS-induced contractile responses involved removal of the vasorelaxing effects of NO, in a similar manner to that described by Ali *et al.* (2006). In porcine arteries, under lower oxygen conditions, NaHS induced vasorelaxation responses. Generally, these responses were mediated via the activation of K_{ATP} channels.

In other vascular preparations, NaHS-induced vasorelaxation has been observed that is not mediated via activation of K_{ATP} channels, including NaHS-induced relaxation responses in porcine splenic veins which were not attenuated by glibenclamide. This relaxation response was partially attenuated by removal of the endothelium and the presence of the NOS inhibitor, L-NAME. Similarly, in rat aorta, L-NAME also attenuated H_2S-induced relaxation (Zhao *et al.,* 2001). In contrast, another study in rat aorta showed NaHS-induced relaxation responses were attenuated by the non-specific Cl^- channel blocker, 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS), but not glibenclamide or, the non-selective NOS inhibitor, N^G-methyl-L-arginine (Kiss *et al.,* 2008). This suggests that depending on the preparation or experiment conditions, H_2S-induced responses are mediated via different mechanisms.

The majority of studies examining the role of H_2S in mammalian vasculature have been conducted using large conduit arteries. In vivo, Zhao

et al. (2001), showed intravenous bolus injection of H_2S transiently decreased blood pressure in anaesthetized rats, suggesting an effect on the smaller arteries that control vascular resistance and hence blood pressure. More importantly, it has been shown that mice with the CSE gene deleted, are hypertensive (Yang *et al*., 2008). Cheng *et al.* (2004), showed that H_2S produced glibenclamide sensitive vasorelaxation in perfused mesenteric resistance arterial beds. In addition, NaHS has been shown to activate K_{ATP} channels, in vascular smooth muscle cells isolated from rat small resistance mesenteric arteries (Cheng *et al.*, 2004; Tang *et al.*, 2005). This supports the argument that H_2S may have a more significant vasodilatory role in small resistance arteries.

To date, no studies have been conducted investigating the effects of H_2S on isolated small arteries. To investigate the role of H_2S in small arteries the present study was conducted on 2^{nd} order rat mesenteric arteries. The effects of NaHS on 2^{nd} order rat mesenteric arteries were investigated using pressure myography. Investigation into the underlying mechanisms of NaHS responses was carried out, as was investigation into the potential endogenous production of H_2S from the substrate L-cysteine.

Materials and Methods

Set up and General Protocol

2^{nd} Order mesenteric arteries were isolated and set up in a pressure myograph using the method described in the general materials and methods chapter. Vessels were pressurized to 90 mmHg and checked to ensure there were no leaks. Once pressurized the vessel was allowed to equilibrate for 20-30 minutes with circulating PSS at a rate of approximately 50 ml/min. This was maintained at 37˚C and gassed with 95% air: 5% CO_2 mixture unless otherwise stated.

After tone had stabilized, vessels were pre-constricted by 40-60% of the initial diameter using either, U46619, methoxamine or raised extracellular KCl. For the majority of experiments the thromboxane A2 receptor agonist U46619 was used to pre-constrict the vessels. In most cases, in the absence of inhibitors/agonists, concentrations of 3×10^{-8} - 1×10^{-7} M of U46619 and 1×10^{-6} to 3×10^{-6} M of methoxamine, were required to achieve an appropriate level of pre-constriction. High K^+ PSS was made by substituting NaCl with KCl.

Once vessel diameter had stabilized at the appropriate level, a concentration response curve to NaHS was obtained by adding it to the circulating PSS in a cumulative manner. NaHS was added to the perfusate in 3-fold increments from 100 nM to 300 µM, with a period of at least 5 minutes between further additions, to ensure equilibrium responses were achieved with each concentration.

Reproducibility

In some experiments the reproducibility of NaHS responses was assessed. After completion of the 1st concentration response curve, NaHS was washed out of the system with fresh PSS and the vessel was left for at least 30 minutes, to re-equilibrate, before further pre-constriction with U46619 and the generation of a 2nd concentration response curve.

NaHS responses were found not to be reproducible and therefore, in subsequent experiments, only one concentration responses curve to NaHS was carried out on each vessel. A second vessel from the same tissue was set up and used as a control

Gassing

In some experiments the effects of altering the O_2 concentration on responses to NaHS was assessed using either 95% O_2:5% CO_2, 95% Air:5% CO_2 or 95% N_2: 5% CO_2. The PSS was pre-gassed with each mixture for at least 30 minutes before set up of the vessel to give the PSS time to ensure gaseous equilibration. After set up, the vessel was again allowed 30 minutes to equilibrate before pre-constriction with U46619 and the generation of the concentration response curve to NaHS.

Putative Inhibitors of NaHS Responses

Inhibitors were added before pre-constriction of the vessels with U46619 and were added to the PSS a minimum of 30 minutes before the generation of the concentration response curve to NaHS.

To investigate the potential interactions of NaHS with NO, the NOS inhibitor nitro-L-arginine methyl ester (L-NAME) (100 µM; Zhao *et al.*, 2001) was used. When investigating the role of K^+ channels; the non-specific K^+ channel inhibitor, tetraethylammonium (TEA) (1 mM; White and Hiley, 1997), the K_{ATP} channel inhibitor, glibenclamide (10 µM; Zhao *et al.*, 2001) or raised extracellular K^+ PSS were used. When investigating the possible role of Cl^- channels the non-specific Cl^- channels inhibitors; 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) (100 µM; Al-Magableh and Hart 2011), 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) (10 µM; Jentsch *et al.,* 2002) and anthracere-9-carboxylic acid (A9C) (100 µM; Jentsch *et al.,* 2002) were used. To investigate the potential role of CGRP released from sensory nerves, the CGRP antagonist, BIBN 4096 (1 µM; Gupta *et al.,* 2007) was used.

Desensitization of Sensory Nerves

Some experiments were designed to desensitize the sensory nerves. In these experiments vessels were pre-constricted with U46619 by 40-60% as previously described. Vessels were then exposed to either capsaicin (1 µM; Dunn *et al.*, 2003) or methanol, the vehicle control. Vessels were left for

10-20 minutes during which time capsaicin caused a transient vasodilator response before a second exposure to capsaicin (1 µM) or control. After confirmation of sensory nerve desensitization, capsaicin or methanol was washed out of the system with fresh PSS. The vessel was left at least 30 minutes to re-equilibrate before pre-constriction with U46619 and the commencement of the NaHS concentration response curve.

Endogenous Production of H_2S

In experiments investigating the potential endogenous production of H_2S, U46619 was used to pre-constrict the vessel diameter by 40-60% of the initial diameter. Thereafter, responses to L-cysteine, an H_2S substrate, were obtained by adding it in 3-fold increments over the concentration range, 10 µM to 30 mM, with a period of at least 5 minutes between additions. Once the first concentration response curve was completed, L-cysteine was washed out of the system and the vessel was left for at least 40 minutes to re-equilibrate before pre-constriction with U46619 and the generation of a 2nd concentration response curve. During this time some vessels were exposed to dl-proparagylglycine (PPG) (10 µM; Cheng *et al.*, 2004) an inhibitor of CSE and/or aminooxyacetic acid (AOAA) (10 µM; Abe *et al.*, 1996) an inhibitor of CBS. Control experiments in the absence of enzyme inhibitors tested the reproducibility of L-cysteine responses.

Results

In the majority of experiments where concentration responses to NaHS were obtained, NaHS caused a vasodilatation with a threshold concentration of between 10 and 30 μM. This vasodilatation was concentration-dependent and, in most cases, fully dilated the artery as the concentration of NaHS was increased. In some experiments however, the vasodilatation was not maintained and diameter returned towards baseline. In all experiments vasodilatation was produced by the highest concentration of NaHS assessed (i.e. 300 μM). These patterns of responses are shown with representative traces in Figure 6.1.

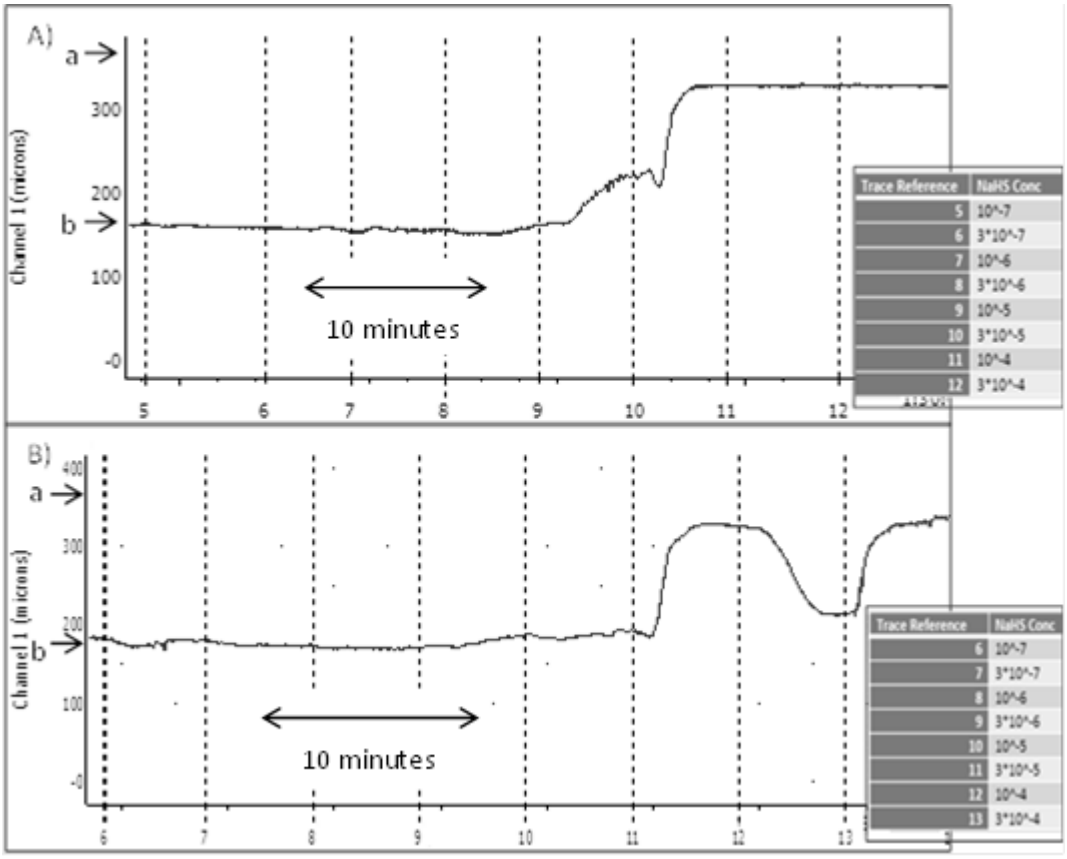

Figure 6.1 Two representative traces showing responses to NaHS in rat 2nd order mesenteric arteries pressurized to 90 mmHg. U46619 was used to pre-constrict vessels by 40-60% of original vessel diameter and then a concentration response curve was generated. The arrows indicate the maximum vessel diameter (a) and the U46619 induced tone (b). A is an example of a response showing maximal vasodilatation at 30 µM NaHS that was maintained. B is an example of a 2nd type of response showing maximal vasodilatation at 30 µM NaHS that was not maintained, returning towards baseline before a further vasodilatation was observed with NaHS at a concentration of 300 µM (B).

Responses to NaHS were investigated to see if they were reproducible. NaHS caused a concentration-dependent vasodilatation at 10 µM but this was not observed on repeat exposure to NaHS (Figure 6.2a). Vasodilatation was still apparent with the repeat exposure but required a 10-fold increase in concentration of NaHS (100 µM) to induce this. In this set of experiments, there were no significant differences in either the U46619 concentration used to induce tone or the level of pre-constriction induced.

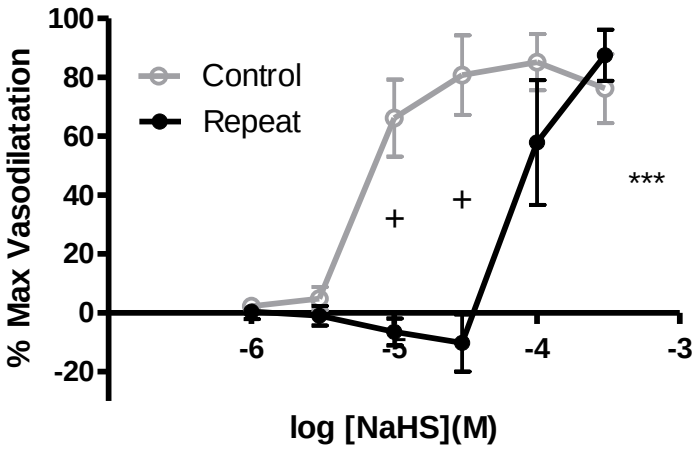

Figure 6.2a Reproducibility of concentration response curves to NaHS in rat 2nd order mesenteric arteries held at 90 mmHg, pre-constricted with U46619. Each point represents the mean ± S.E.M. (n=6). *** represents a significant difference between the curves ($p<0.001$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

To test whether the lack of reproducibility of responses to NaHS was due to exposure to an excessive concentration of NaHS, the highest concentration used in the first curve was reduced from 300 µM to 30 µM. In these experiments, NaHS again caused a concentration-dependent vasodilatation at a concentration of 10 µM (Figure 6.2b). However, even under these conditions responses to NaHS were not reproducible. As a consequence, all further experiments were carried out by obtaining only a single concentration response curve to NaHS per vessel using a paired segment from the same animal for a comparison of the effects of inhibitors/agonists.

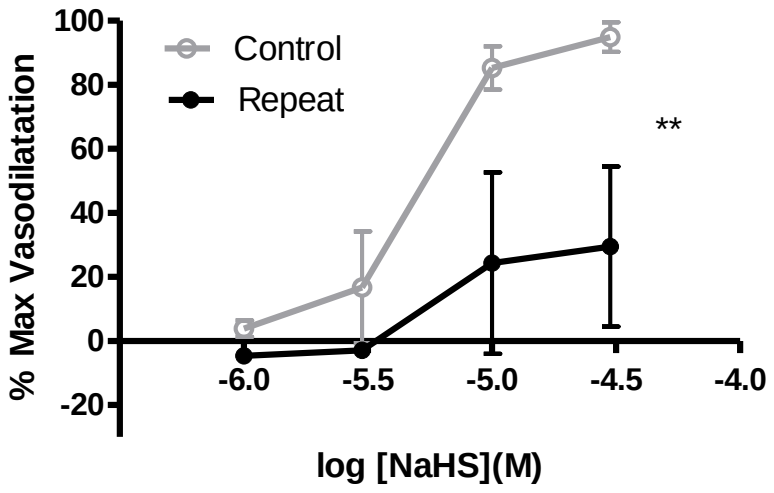

Figure 6.2b Reproducibility of concentration response curves to NaHS when the concentration was restricted to 30 µM in the 1st concentration response curve, in rat 2nd order mesenteric arteries held at 90 mmHg. Each point represents the mean ± S.E.M. (n=3). ** represents a significant difference between the curves ($p<0.01$; 2 way ANOVA).

Responses to NaHS were also obtained after pre-constriction was induced using a different vasoconstrictor i.e. methoxamine, an α1-adrenoreceptor agonist (Figure 6.3). With a similar level of pre-constriction induced in comparison to U46619, NaHS also produced a similar pattern of vasodilator responses

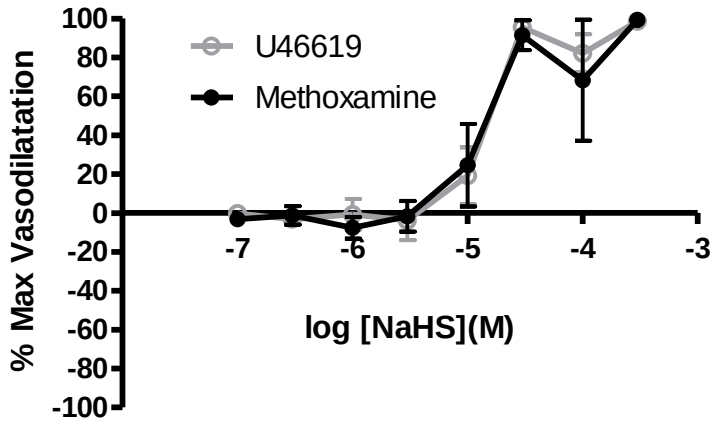

Figure 6.3 Concentration response curves to NaHS in rat 2nd order mesenteric arteries held at 90 mmHg, pre-constricted with either U46619 (open circles) or methoxamine (closed circles). Each point represents the mean ± S.E.M. (n=4). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

To investigate the influence of altering the O_2 level used to gas the PSS on responses to NaHS, responses were compared after gassing with 95% O_2:5% CO_2, 95% air:5% CO_2 or 95% N_2:5% CO_2. There was no significant difference in responses to NaHS when gassed with the different gassing mixtures (Figure 6.4). In addition, changing the gas mixtures did not alter the amount of U46619 required to reduce the diameter by 40-60%.

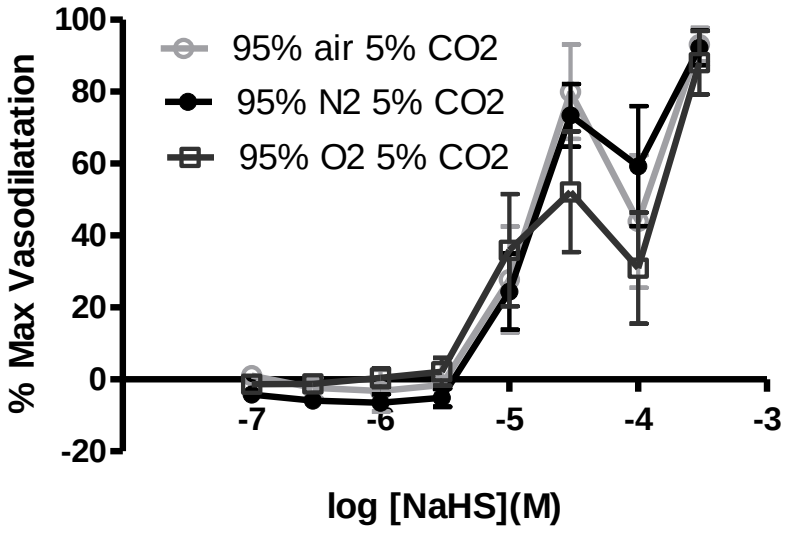

Figure 6.4 Concentration response curves to NaHS under different gassing conditions in U46619 pre-constricted rat 2nd order mesenteric arteries held at 90 mmHg. Each point represents the mean ± S.E.M. (n=6). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

To investigate the potential involvement of NO in mediating vasodilator responses to NaHS, concentration response curves were obtained in the presence and absence of L-NAME (100 µM). Vessels were pre-constricted with lower concentrations of U46619 in the presence of L-NAME (between 3 X 10^{-8} and 2 X 10^{-7} M), than in its absence (between 1 X 10^{-7} and 2 X 10^{-6} M). However, L-NAME did not attenuate the vasodilator responses to NaHS (Figure 6.5).

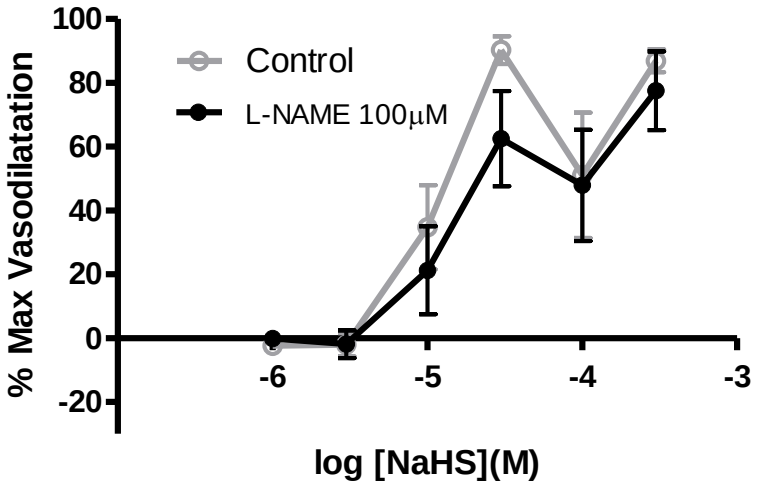

Figure 6.5 Concentration response curves to NaHS in the presence (closed circles) and absence (open circles) of L-NAME (100 µM) in rat 2nd order mesenteric arteries held at 90 mmHg, pre-constricted with U46619. Each point represents the mean ± S.E.M. (n=7). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

To investigate the potential involvement of a hyperpolarization mechanism in mediating NaHS responses, vasoconstriction was induced using raised extracellular K^+. The extracellular concentration of K^+ was raised by an average concentration of 31 ± 4 mM (n=6) to pre-constrict the vessel to 45 ± 4% of the initial diameter. In control experiments, vessels were pre-constricted by 52 ± 4% with U46619. In the presence of raised extracellular K^+, the vasodilator response to NaHS was abolished at concentrations below 100 µM uncovering a modest vasoconstriction. At 300 µM NaHS a vasodilatation was evident (Figure 6.6a).

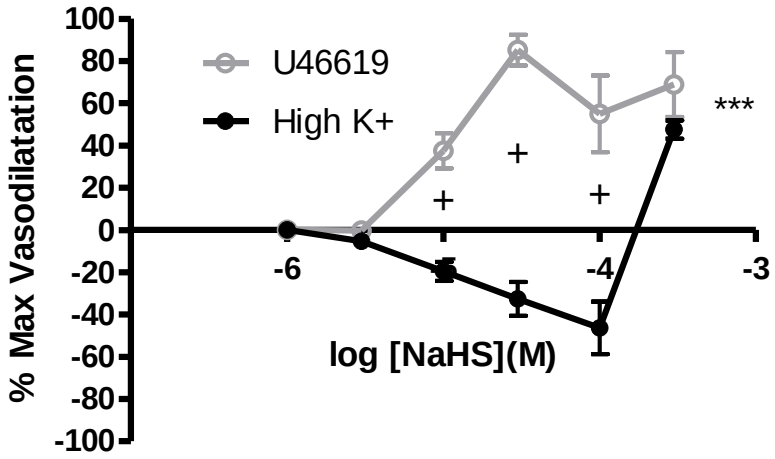

Figure 6.6a Concentration response curves to NaHS in rat 2nd order mesenteric arteries held at 90 mmHg, pre-constricted with either U46619 (open circles) or raised extracellular K+ PSS (closed circles). Each point represents the mean ± S.E.M. (n=6). *** represents a significant difference between the curves ($p<0.001$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

Since raising extracellular K^+ attenuated NaHS responses, the potential role of K_{ATP} channels in mediating NaHS-induced vasodilator responses was investigated by carrying out experiments in the presence and absence of the K_{ATP} channel inhibitor, glibenclamide (10 μM). For these experiments, vessels were pre-constricted by 46 ± 8% in the presence of glibenclamide and 48 ± 6% in its absence. It was noted that the range of U46619 concentration required to pre-constrict vessels in the presence of glibenclamide (1 X 10^{-7} to 3 X 10^{-7} M) was significantly higher than the control group (1 X 10^{-8} to 8 X 10^{-8} M). Despite this, glibenclamide had no significant effect on NaHS responses (Figure 6.6b).

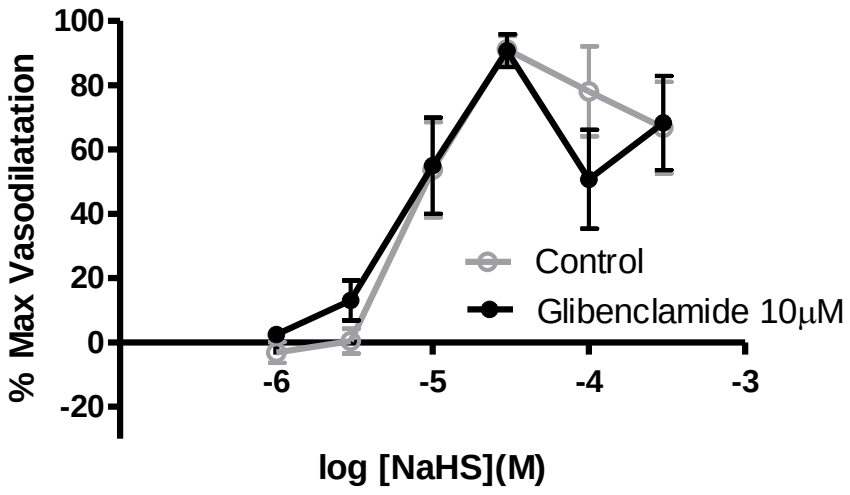

Figure 6.6b Concentration response curves to NaHS in the presence (closed circles) and absence (open circles) of glibenclamide (10 μM) in rat 2nd order mesenteric arteries held at 90 mmHg, pre-constricted with U46619. Each point represents the mean ± S.E.M. (n=6). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

NaHS responses were subsequently assessed in the presence and absence of the non-selective K^+ channel blocker TEA (1 mM). In these experiments, vessels were pre-constricted with U46619 to 49 ± 3% in the absence of TEA and 47 ± 2% in its presence. The amount of U46619 required to induce vasoconstriction was not altered by TEA. TEA did not attenuate NaHS responses (Figure 6.6c).

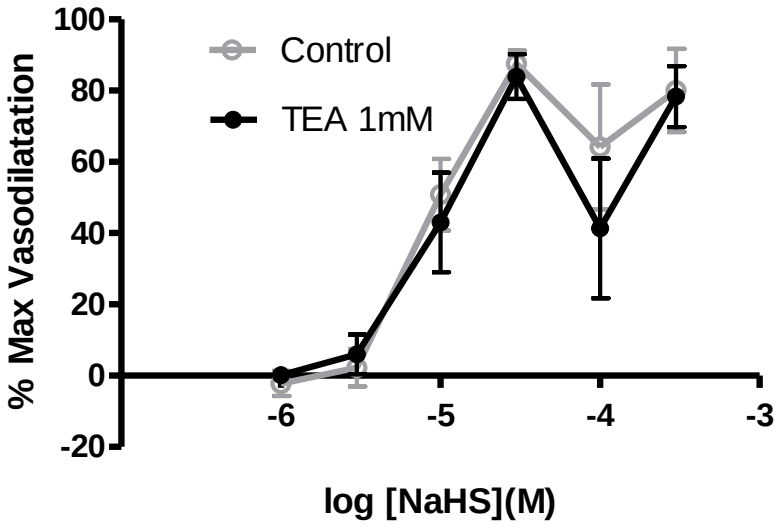

Figure 6.6c Concentration response curves to NaHS in the presence (closed circles) and absence (open circles) of TEA (1 mM) in U46619-induced pre-constricted rat 2nd order mesenteric arteries held at 90 mmHg. Each point represents the mean ± S.E.M. (n=6). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

To investigate the potential involvement of Cl^- Channels in mediating NaHS-induced vasodilator responses, concentration response curves were carried out in the presence and absence of DIDS (100 µM), a non-specific inhibitor of Cl^- Channels. In the presence of DIDS, NaHS vasodilator responses were abolished (Figure 6.7a). Vessels were pre-constricted to a similar level however, the concentration of U46619 required to induce vasoconstriction was significantly higher in the presence of DIDS (range 2 X 10^{-7} to 2 X 10^{-6} M) than in the control group (range 1 X 10^{-8} to 2 X 10^{-7} M).

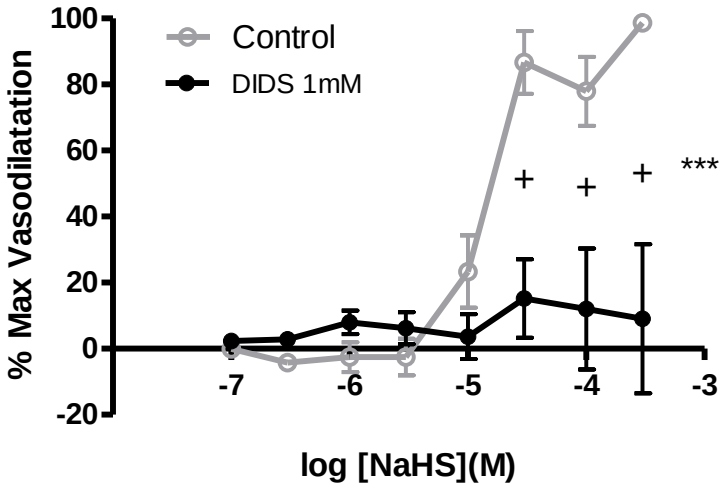

Figure 6.7a Concentration response curves to NaHS in the presence (closed circles) and absence (open circles) of DIDS (100 µM) in rat 2nd order mesenteric arteries held at 90 mmHg, pre-constricted with U46619. Each point represents the mean ± S.E.M. (n=6). *** represents a significant difference between the curves ($p<0.001$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

Experiments were also carried out in the presence of the non-specific Cl^- channel inhibitor, NPPB (10 μM), although maintaining stable tone within the 40-60% range with U46619 upon incubation with NPPB was not possible. Therefore, vessels were pre-constricted with the α1-adrenergic receptor agonist, methoxamine, by 48 ± 1% in the presence of NPPB and 49 ± 2% in its absence. There was no impact of NPPB on the concentration of methoxamine required to induce this level of vasoconstriction. NPPB did not affect NaHS responses (Figure 6.7b).

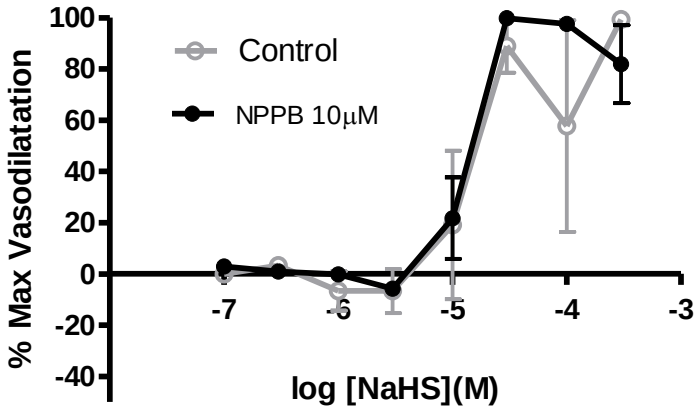

Figure 6.7b Concentration response curves to NaHS in the presence (closed circles) and absence (open circles) of NPPB (10 μM) in rat 2nd order mesenteric arteries held at 90 mmHg pre-constricted with methoxamine. Each point represents the mean ± S.E.M. (n=3). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

Additional experiments were conducted in the presence and absence of another non-specific Cl^- channel inhibitor, A9C (100 µM). Methoxamine was again required to induce tone in these experiments. A9C showed no significant attenuation of the NaHS-induced vasodilator responses (Figure 6.7c).

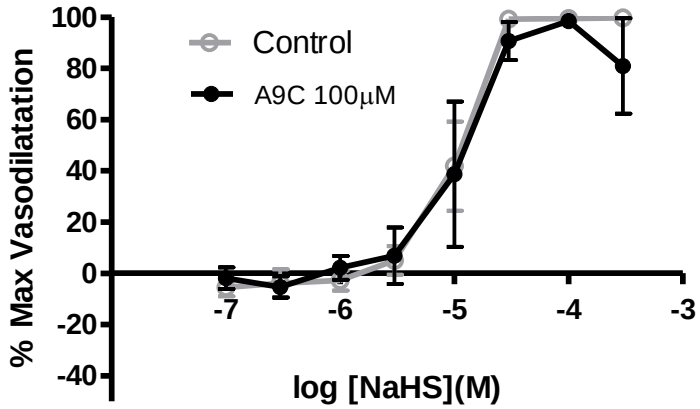

Figure 6.7c Concentration response curves to NaHS in the presence (closed circles) and absence (open circles) of A9C (10 µM) in rat 2nd order mesenteric arteries held at 90 mmHg pre-constricted with methoxamine. Each point represents the mean ± S.E.M. (n=3). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

In guinea-pig and human colon (Kruger *et al.,* 2010) and rat urinary bladder (Patacchini *et al.,* 2005), NaHS responses were shown to be mediated by activation of TRPV1 receptors located on sensory nerves. To investigate the possible role of sensory nerves in mediating NaHS-induced vasodilatation, vessels were exposed to capsaicin (2 x 1 µM). Vessels were pre-constricted and then exposed to two additions of 1 µM capsaicin that were separated by between 10 and 20 minutes. The first exposure caused a transient vasodilator response, which peaked at 74 ± 25% of U46619-induced pre-constriction and gradually returned to baseline. The second exposure to capsaicin produced no response, indicating depletion of vasodilator neurotransmitters from sensory nerves. These response patterns are shown with a representative trace (Figure 6.8a).

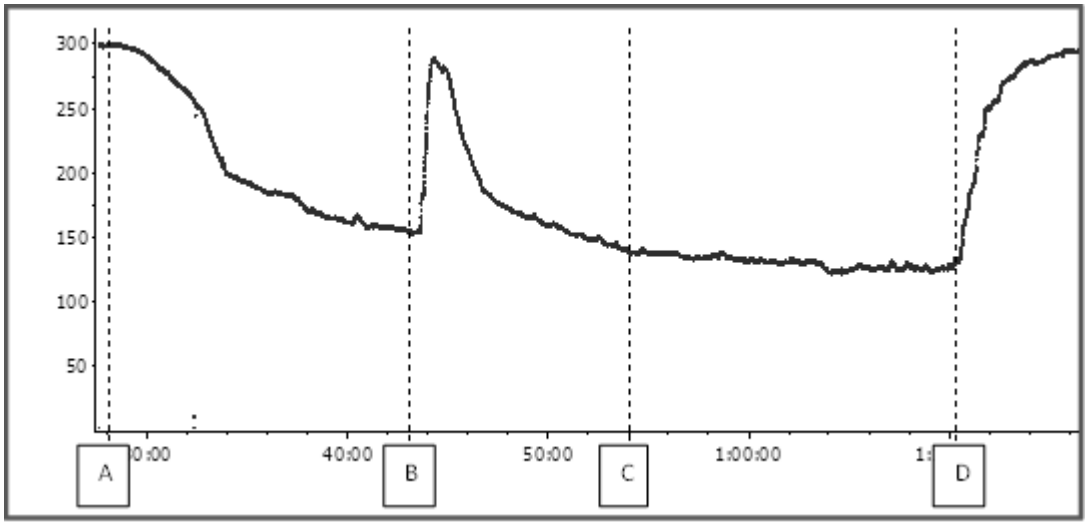

Figure 6.8a representative traces showing desensitization of sensory nerves with capsaicin in a rat pressurized 2nd order mesenteric artery. A) U46619 was used to pre-constrict vessels. B) Addition of 1 µM capsaicin causes a transient vasodilatation. C) Second addition of 1 µM capsaicin caused no further vasodilator responses suggesting desensitization of sensory nerves. D) Vessel washed with fresh PSS.

After washout of capsaicin or methanol (the vehicle control) vessels were pre-constricted with similar concentrations of U46619 by 49 ± 3% after desensitization of sensory nerves and 49 ± 3% in the methanol control group. Concentration response curves to NaHS were then obtained. In vessels, exposed to capsaicin, NaHS vasodilatation was significantly attenuated, uncovering a small vasoconstrictor response at concentrations up to 100 µM (Figure 6.8b). Vasodilator responses were evident at 300 µM NaHS, mirroring the effect of repeat exposure to NaHS (Figure 6.2a).

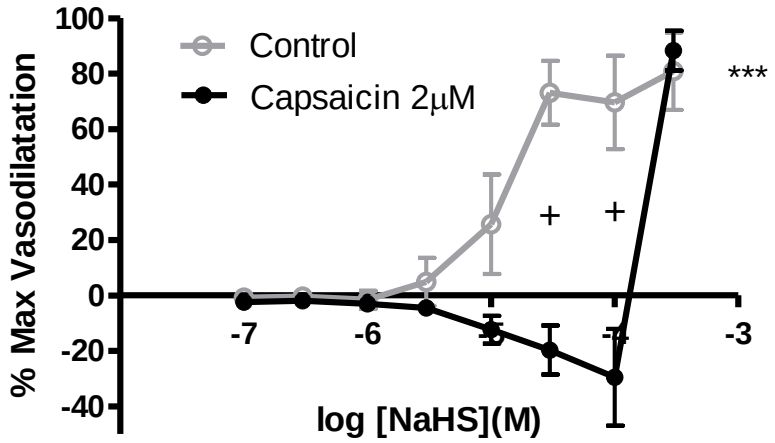

Figure 6.8b Concentration response curves to NaHS with (closed circles) and without (open circles) prior exposure to capsaicin in rat 2nd order mesenteric arteries held at 90 mmHg. Each point represents the mean ± S.E.M. (n=8). *** represents a significant difference between the curves ($p<0.001$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test)

Subsequent experiments were carried out in the presence and absence of the CGRP antagonist BIBN 4096 (1 μM). In these experiments, vessels were pre-constriction with U46619 by 49 ± 1% the presence of BIBN 4096 and 52 ± 3% in its absence using similar concentrations. BIBN 4096, significantly attenuated NaHS vasodilatation at lower concentrations (up to and including 100 μM) uncovering a small vasoconstrictor response (Figure 6.8c). A large vasodilator response was still apparent with 300 μM NaHS in a similar manner to that seen after capsaicin treatment.

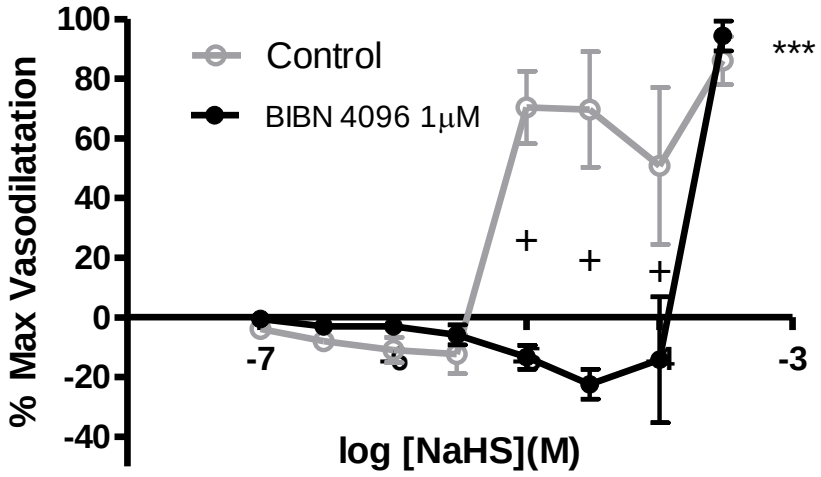

Figure 6.8c Concentration response curves to NaHS in the presence (closed circles) and absence (open circles) of BIBN 4096 (1 μM) in rat 2nd order mesenteric arteries held at 90 mmHg pre-constricted with U46619. Each point represents the mean ± S.E.M. (n=6). *** represents a significant difference between the curves ($p<0.001$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

To test the possibility that H_2S is produced endogenously in rat mesenteric arteries responses to L-cysteine, a substrate for H_2S production were investigated. L-cysteine had little effect on vessel tone until very high concentrations were used (10 and 30 mM) where it caused vasodilatation. Responses to L-cysteine were reproducible (Figure 6.9a).

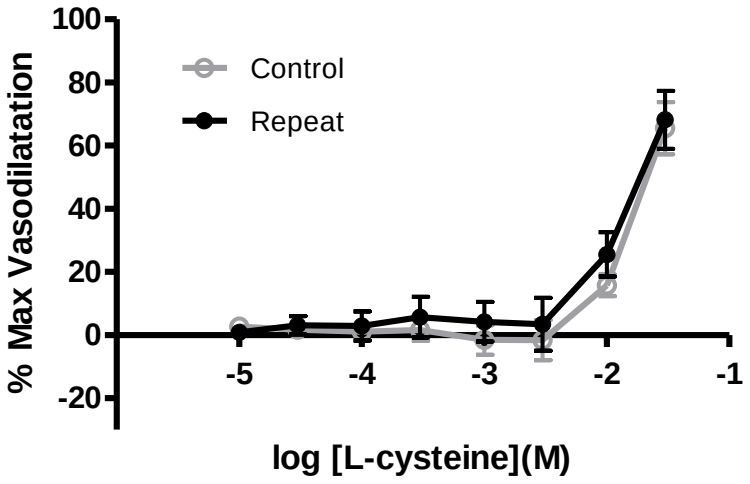

Figure 6.9a Reproducibility of concentration response curves to L-cysteine in U46619-induced pre-constricted rat 2nd order mesenteric arteries held at 90 mmHg. Each point represents the mean ± S.E.M. (n=6). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

To investigate if L-cysteine-induced responses were due to endogenous production of H_2S, NaHS concentration response curves were obtained in the presence and absence of the CSE inhibitor, PPG (10 µM) or the CBS inhibitor, AOAA (10 µM). PPG produced a small but statistically significant reduction of L-cysteine-induced vasodilator responses (Figure 6.9b).

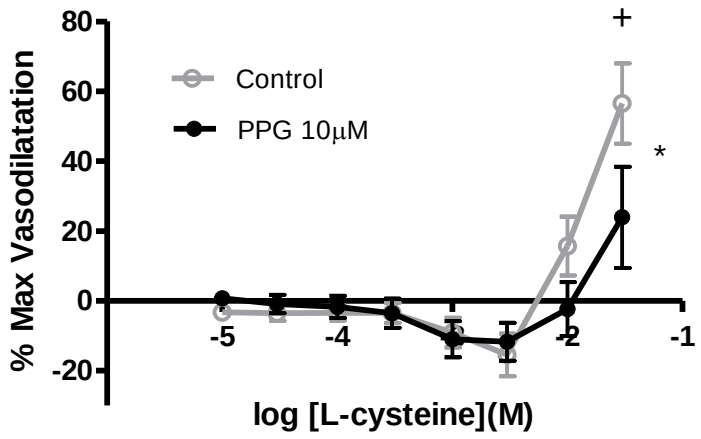

Figure 6.9b Concentration response curves to L-cysteine in the presence (closed circles) and absence (open circles) of PPG (10 µM) in rat 2nd order mesenteric arteries held at 90 mmHg. Each point represents the mean ± S.E.M. (n=6). * represents a significant difference between the curves ($p<0.05$; 2 way ANOVA) and + represents a significant difference between individual points (Bonferroni post-hoc test).

The CBS inhibitor, AOAA had no significant effect on L-cysteine responses (Figure 6.9c).

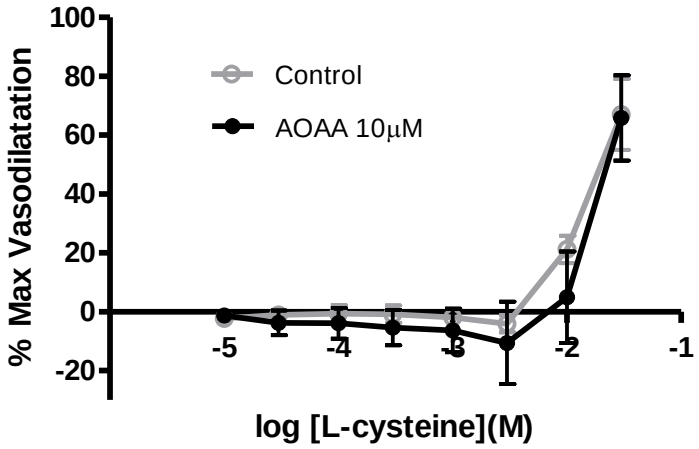

Figure 6.9c Concentration response curves to L-cysteine in the presence (closed circles) and absence (open circles) of AOAA (10 μM) in rat 2nd order mesenteric arteries held at 90 mmHg, pre-constricted with U46619. Each point represents the mean ± S.E.M. (n=5). There was no significant difference between the curves ($p>0.05$; 2 way ANOVA).

The combination of both PPG and AOAA caused a significant decrease in responses to l-cysteine, although they were not abolished (Figure 6.9d).

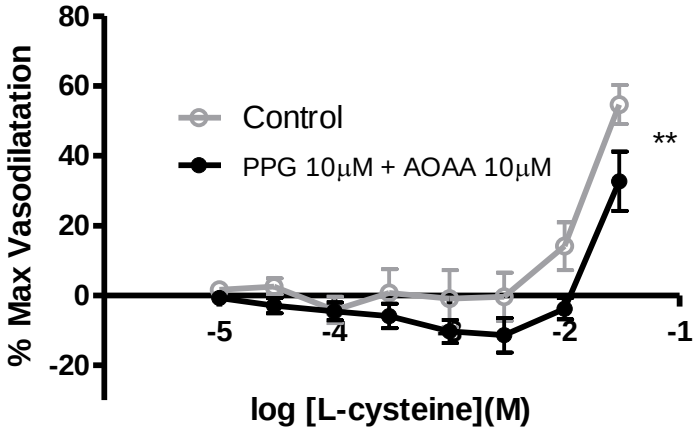

Figure 6.9d Concentration response curves to L-cysteine in the presence (closed circles) and absence (open circles) of AOAA (10 μM) + PPG (10 μM) in rat2nd order mesenteric arteries held at 90 mmHg. Each point represents the mean ± S.E.M. (n=4). ** represents a significant difference between the curves ($p<0.01$; 2 way ANOVA).

Discussion

In the present study it has been shown that NaHS, the H_2S, donor, produced vasodilator responses in rat pressurized 2nd order mesenteric arteries. The NaHS vasodilator responses were not reproducible and desensitized, NaHS-induced vasodilator responses were sensitive to pre-treatment with capsaicin and the presence of the CRGP antagonist, BIBN 4096. This indicates the involvement of CGRP release from sensory nerves in mediating NaHS-induced vasodilator responses observed between 10 and 100 µM NaHS.

In the current study, vasodilator responses were not reproducible. NaHS caused a concentration-dependent vasodilatation at 10 µM but this was not observed on repeat exposure to NaHS until concentrations above 100 µM. Therefore, a single concentration curve to NaHS was carried out per vessel segment set up. Changing the O_2 concentration in the gassing mixture had no effect on the vasodilatator response to NaHS. Evidence for oxygen dependency in H_2S responses has been shown in larger rat vessels. Koenitzer *et al.* (2007), found that at normal O_2 levels (200 µM ~ air equilibrated buffer), H_2S produced contractile responses while at lower O_2 levels (40 µM), H_2S produced relaxation responses. The present data suggests that oxygen sensitivity of H_2S responses is a feature of conduit arteries rather than resistance arteries. In addition, there was no substantial constrictor response in resistance arteries. This also contrasts with the data in conduit arteries and veins from the previous chapters, indicating vasodilatation is predominant in small arteries. This fits with the in vivo

depressor effect and the hypertension observed in the CSE knockout mouse (Yang *et al*., 2008)

In a previous study, Zhao *et al*., (2001) showed NaHS vasodilator responses in rat aorta were significantly reduced in the presence of the NOS inhibitor, L-NAME. In the present study, while L-NAME reduced the concentration of U46619 required to produce a similar level of pre-constriction to the control, suggesting that basal NO production was present, L-NAME has no effect on NaHS concentration responses curves. Thus the NaHS-induced vasodilatation was not due to the release of NO.

When vessels were pre-constricted with raised extracellular K^+, NaHS vasodilator responses were significantly attenuated except at the highest concentration of NaHS used (300 µM). This suggests that vasodilator responses observed at concentrations between 10 and 100 µM NaHS required a hyperpolarization mechanism. Previous studies have shown K_{ATP} channels are a target for NaHS (Zhao *et al.*, 2001; Cheng *et al.*, 2004; Tang *et al.,* 2005). For example, in perfused rat mesenteric arterial beds, Cheng *et al.* (2004), demonstrated that glibenclamide attenuated NaHS vasodilator responses. More specifically, in VSMCs isolated from 2nd order rat mesenteric arteries, it has been has been directly shown that NaHS caused an increase in K_{ATP} channel currents which was attenuated by glibenclamide (Tang *et al.,* 2005). Although this activation occurs in isolated cells, di Villa Bianca *et al.* (2011), showed NaHS vasodilator responses were not attenuated by glibenclamide in the rat mesenteric arterial bed. In the current study, glibenclamide also had no effect on NaHS-induced vasodilatation of 2nd order rat mesenteric arteries. Furthermore, TEA also failed to attenuate

responses to NaHS, suggesting K^+ channels were not involved in mediating the vasodilator response. It is possible that the efflux of K^+ ions occurs via TEA insensitive K^+ channels, such as small and intermediate conductance K_{Ca2+} channels which are less sensitive to TEA (Alexander *et al.,* 2009). In another study where glibenclamide failed to attenuate NaHS response, di Villa Bianca *et al.* (2011), showed NaHS vasodilator responses in the rat mesenteric arterial bed were significantly attenuated by inhibition of K_{Ca2+} channels with the combination of charybdotoxin (100 nM) and apamin (5 µM)and future experiments could be directed to examine this.

In the rat aorta, Kiss *et al.* (2008) showed that inhibition of K_{ATP} channels had no effect on relaxation responses to NaHS; however DIDS, the non-selective Cl^- channel inhibitor, completely abolished responses, suggesting a possible role for Cl^- channels in mediating this response. Furthermore, in the mouse aorta, NaHS-mediated vasodilatation (that was sensitive to glibenclamide), was also sensitive to DIDS (Al-Magableh and Hart, 2011). In the present study, DIDS abolished responses to NaHS in rat 2^{nd} order mesenteric arteries inclusive of the highest concentration of NaHS used (300 µM) potentially indicating the involvement of Cl^- channels. To test this possibility in rat mesenteric arteries, two non-specific Cl^- channel blockers were used. However, neither, NPPB nor, A9C had any effect on NaHS responses. Overall, NPPB and A9C inhibit the majority of the Cl^- channels that are inhibited by DIDS, thus some other mechanism must explain the action of DIDS (Jentsch *et al.,* 2002; Takahashi *et al*., 2005). Lee *et al.* (2007), using rat aortic SMCs, found the NaHS caused a DIDS-sensitive decrease in intracellular pH (pH_i) of vascular smooth muscle cells

and suggested the involvement of a DIDS-sensitive Cl^-/HCO_3^- exchanger. Lu *et al.*, (2010) also showed that DIDS inhibited NaHS-induced regulation of intracellular pH in rat primary cultured glia cells by a similar mechanism. This could suggest that DIDS was affecting the DIDS-sensitive Cl^-/HCO_3^- exchanger and inhibiting NaHS-induced regulation of pH_i to cause vasodilatation in rat mesenteric arteries.

Vasodilator responses to NaHS were frequently not maintained returning back to baseline after exposure to 100 µM NaHS. Furthermore, NaHS responses were not reproducible. These observations suggested NaHS responses were being caused by a mechanism that was desensitizing or involved the depletion of a vasodilator mediator. In the rat urinary bladder, it has been shown that NaHS stimulated a capsaicin sensitive, transient receptor potential vanilloid receptor 1 (TRPV1) on sensory nerves causing the release of neuropeptides and a contractile responses (Patacchini *et al.,* 2005). Furthermore, in the guinea-pig and human colon, NaHS has been shown to activate TRPV1 receptors located on afferent nerves to cause the release of neuropeptides, subsequently evoking mucosal secretions (Kruger *et al.,* 2010). In the present study, investigation into desensitization of sensory nerves was achieved using capsaicin. The first exposure to capsaicin caused a transient vasodilatation. Subsequent exposure to capsaicin did not cause vasodilatation, indicating desensitization of the sensory nerves. After capsaicin treatment, NaHS vasodilatation was significantly attenuated at concentrations up to 300 µM. This suggests that NaHS-induced vasodilatation was mediated by sensory nerves, subsequent to via activation of TRPV1 and release of sensory neuropeptides. Calcitonin

gene-related peptide (CGRP) is an important neurotransmitter in capsaicin-sensitive sensory nerves in the rat mesenteric arterial bed (Kawasaki *et al.,* 1988). In the present study, the CGRP antagonist BIBN4096 (Gupta *et al.,* 2007), significantly attenuated NaHS-induced vasodilatation in a similar manner to capsaicin. Thus this data is consistent with NaHS inducing the release of CGRP from sensory nerves in rat 2nd order mesenteric arteries to cause vasodilatation.

Since the NaHS vasodilator response was sensitive to raised extracellular K^+, this indicates a hyperpolarization mechanism. Furthermore, by implication, the NaHS-induced hyperpolarization must have been inhibited by the CGRP antagonist, BIBN4096. This suggests that NaHS responses are mediated via release of CRGP from sensory nerves possibly via activation K_{Ca2+} Channels. In previous studies using mesenteric arteries isolated from female rats, CGRP released from primary afferent axons hyperpolarized the vascular smooth muscle by activating glibenclamide-sensitive K_{ATP} channels in non-pressurized vessels (Dunn *et al.*, 2003). By contrast the NaHS (sensory-nerve-mediated) vasodilatation was not sensitive to glibenclamide. Thus, another potassium channel must be involved in the action of NaHS/CGRP, perhaps changing from K_{ATP} channels due to the sex difference of the rats or, due to alterations in vessel function under pressure. Interestingly, mesenteric arterial relaxation to CGRP in pregnant rats was caused by activation of large and intermediate conductance K_{Ca2+} channels (Gangula et al., 2004). As mentioned previously intermediate and small K_{Ca2+} channels are not inhibited by TEA, thus CGRP-induced responses to NaHS in the present study could be mediated via these channels.

In the current study, both DIDS and capsaicin treatment attenuated vasodilator responses in rat mesenteric arteries. In a study by Lee *et al.* (2007), NaHS-induced relaxation of rat aortic SMCs was mediated via a DIDS sensitive decrease in pH_i. Decreased pH has been shown to significantly enhance responses of TRPV1 to capsaicin activation (Caterina *et al.*, 1997). Therefore, it is plausible that NaHS is causing activation of the DIDS-sensitive Cl^-/HCO_3^- exchanger in sensory nerves of 2nd order rat mesenteric arteries and without this effect the NaHS-induced activation of TRPV1 may require a higher concentration of NaHS than was used to cause the subsequent release of CGRP. The NaHS-induced vasodilatation at higher concentrations of NaHS, that was not capsaicin sensitive, is potentially a direct effect of pH_i change within the VSMCs. The fact that DIDS abolished the vasodilator response at the highest concentration of NaHS (which was not blocked by capsaicin or raised extracellular potassium) is consistent with this hypothesis.

In this chapter attempts were made to assess the potential endogenous production of H_2S from L-cysteine. L-cysteine produced a vasodilatation response at high concentrations (≥10 mM). This effect was modestly attenuated by the CSE inhibitor, PPG but unaffected by AOAA, suggesting a very modest amount of endogenous H_2S production. In a previous study, Cheng *et al.* (2004), found 1 mM l-cysteine caused a relaxation response in rat mesenteric arterial beds and that this response was completely abolished using the same concentration of PPG (10 µM) used in the present study. Thus, the effect of PPG in rat mesenteric arteries was disappointing. In another study, Al-Magableh and Hart (2011), showed,

using the mouse aorta, that PPG effects on responses to l-cysteine were modest, even using PPG at a concentration of 1 mM. They suggested that in conduit vessels l-cysteine-induced vasorelaxation was of minor importance and suggested that the CSE pathway of H_2S production may be more important in resistance than conduit vessels. However, the current study does not support this. Furthermore, responses to l-cysteine were reproducible when, in comparison, responses to NaHS were not. Thus L-cysteine is likely to be producing vasodilatation largely via a non H_2S-dependent mechanism.

In conclusion, NaHS caused vasodilatation of pressurized 2nd order rat mesenteric arteries. The desensitization of sensory nerves with capsaicin and the CGRP antagonist BIBN4096 attenuated these responses. This suggests that NaHS is activating TRPV1 on sensory nerves to cause the release of CGRP and induce vasodilatation.

Chapter 7:

General Discussion

The aim of this thesis was to examine the relationship between H_2S and oxygen in determining vascular responsiveness. Studies were conducted using porcine splenic and mesenteric arteries and extended to include porcine splenic veins. In addition, since there is little information on the effects of H_2S in smaller arteries, and these vessels are more important in determining vascular resistance and consequently blood pressure, this study also examined small arteries isolated from the rat mesentery.

To investigate the relationship between oxygen level and responses to NaHS in blood vessels, gas mixtures containing different oxygen levels were used; 95% O_2:5% CO_2, 95% Air:5% CO_2 and 95% N_2:5% CO_2. In mammals, the arterial pO_2 is normally around 100 mmHg and venous pO_2 is between 15-50 mmHg (Erecińska and Silver, 2001; Tsai *et al*., 2003). In sample studies, the partial pressure of O_2 of PSS gassed with these gas mixtures was measured and was 619 ± 17 mmHg (n = 3) when gassing with 95% O_2:5% CO_2, 140 ± 4 mmHg (n = 3) when gassing with 95% Air:5% CO_2 and 33 ± 6 mmHg O_2 (n = 3) when gassing with 95% N_2:5% CO_2. This suggests that the most physiologically relevant gassing conditions are created by gassing with 95% Air:5% CO_2 in arteries and 95% N_2:5% CO_2 in veins. However, most studies on the effects of H_2S on blood vessels have been traditionally conducted gassing with 95% O_2:5% CO_2 and this may have produced misleading outcomes (Zhao and Wang, 2002, Cheng *at el*., 2004; Ali *et al*., 2006).

In the present study, responses to NaHS in both porcine arteries and veins were greatly affected by the oxygen level of the gassing mixture. Generally, in both porcine splenic and mesenteric arteries as well as splenic

veins, gassing with a higher oxygen levels (95% O_2:5% CO_2 or 95% Air:5% CO_2), was associated predominantly with NaHS causing a contractile response. However, in both porcine mesenteric arteries and splenic veins, gassing with a lower oxygen level (95% N_2:5% CO_2), promoted predominantly vasorelaxatory responses to NaHS. Olson (2008) proposed that H_2S was an endogenous oxygen sensor whereby an increase in endogenous levels of H_2S under hypoxia induced relaxation of blood vessels and thus increasing the supply of oxygenated blood to the tissue. Although the present study does not confirm the endogenous production of H_2S, it does show that at low oxygen levels the predominant response of exogenously applied NaHS is to relax blood vessels.

Removal of the endothelium from both porcine splenic arteries and veins abolished NaHS-induced vasocontractile responses. It is unlikely that the rubbing processes used to remove the endothelium biased responses to NaHS by causing damage to the smooth muscle layer as responses to KCl and U46619 were not significantly different. This suggests that the vasocontractile responses to NaHS in both porcine splenic arteries and veins are mediated via a factor released from the endothelium and not a direct effect on the contractile machinery of vascular smooth muscle. In addition to the removal of the endothelium, L-NAME abolished NaHS-induced contractile responses at higher oxygen levels in porcine splenic arteries and veins and significantly attenuated them in porcine mesenteric arteries. It has been shown that NO and H_2S interact chemically to produce a non-vasoactive nitrosothiol, an effect that can remove the vasodilator effects of NO (Whiteman *et al*., 2006). The data presented in this thesis suggests that

the removal of NO, derived from the endothelium, by the exogenous addition of NaHS is the predominant factor mediating the NaHS-induced vasoconstriction in both arteries and veins under higher oxygen levels.

NaHS-induced vasorelaxation was observed in both porcine mesenteric arteries under 95% N_2:5% CO_2 gassing conditions and at high concentrations of NaHS (above 100 µM) under 95% Air:5% CO_2 gassing conditions in porcine splenic arteries. . These vasorelaxatory responses were abolished by the specific K_{ATP} channel inhibitor, glibenclamide, showing that NaHS is causing relaxation via activation of K_{ATP} channels in porcine arteries. In isolated VSMCs from rat arteries, H_2S has been shown to directly stimulate K_{ATP} channels, independently of membrane receptors (Zhao et al., 2001; Tang et al., 2005). The fact that this vasorelaxation is most evident at lower oxygen concentrations or, with higher concentrations of NaHS, may reflect increased oxidation of H_2S at higher oxygen levels, decreasing the level of active H_2S (Kajimura *et al.*, 2010) or the interaction between NO and H_2S may be reducing the levels of H_2S available to activate K_{ATP} channels.

In porcine splenic veins, as well as abolishing the vasocontractile reponses of NaHS, removal of the endothelium also attenuated the vasorelaxatory responses produced by NaHS. However, unlike porcine arteries, inhibition of K_{ATP} channels with glibenclamide had no effect on NaHS-induced vasorelaxation. Furthermore, using the non-specific K^+ channel blocker, TEA, also had no effect on NaHS responses. This suggests that unlike arteries, the vasorelaxation induced by NaHS is not mediated via K^+ channels. When gassing with 95% N_2:5% CO_2, the NaHS-induced

vasorelaxation was significantly attenuated by L-NAME. This shows that NaHS is activating eNOS to generate NO and cause vasorelaxation.

In porcine splenic veins, under higher oxygen levels (95% Air:5% CO_2 or 95% O_2:5% CO_2) the vasorelaxation response to NaHS were not sensitive to L-NAME, but were significantly attenuated by the removal of the endothelium. Therefore, under these conditions, it is plausible that NaHS is causing the release of another EDRF from the endothelium in addition to NO, although its identity was not defined.

In contrast to porcine conduit arteries and veins, the oxygen level in the PSS had no effect on response to NaHS in rat resistance mesenteric arteries. Interestingly, there was little evidence for a large vasoconstriction produced by NaHS in these vessels. Thus, the influence of removing NO seems to be of less importance in smaller resistance arteries. Woodman *et al*. (2000) showed that ACh-induced dilatation of the rat aorta was dependent NO, whereas hindquarters resistance arteries were more dependent on an EDHF. They suggested that EDHF may be more important than NO in the resistance vasculature.

In rat mesenteric resistance arteries, NaHS caused transient vasodilator responses that were not reproducible upon repeat exposure to NaHS. This vasodilatation was abolished by desensitization of sensory nerves with capsaicin and by the presence of CGRP antagonist, BIBN4096. This indicated that the release of vasoactive neuropeptides from sensory nerves, most probably after activation of TRPV1 and subsequent release of CGRP, mediates the NaHS-induced vasodilatation in rat resistance mesenteric arteries. The mechanism of CGRP-induced vasodilatation was

shown to be via a hyperpolarization. However, the specific target of CGRP was not identified.

NaHS-induced vasodilatation was also inhibited by the non-specific Cl^- channel inhibitor, DIDS. Further studies using other Cl^- inhibitors suggested the most likely target of NaHS was the Cl^-/HCO_3^- exchanger. Therefore, it is plausible NaHS-induced vasodilatation is mediated via a decrease in $[pH]_i$ as well as the release of vasoactive neuropeptides from sensory nerves. In another study, NaHS has been shown to cause a decrease in $[pH]_I$ via activation of the Cl^-/HCO_3^- exchanger in A7r5 cells (derived from rat embryonic rat aortic smooth muscle) (Lee *et al*., 2007). In addition, it has been shown that a decrease in $[pH]_I$ can activate TRPV1 causing the release of neuropeptides (Caterina *et al.*, 1997). Therefore, it is plausible that NaHS may also cause the activation of the Cl^-/HCO_3^- exchanger in sensory nerves, subsequently decreasing $[pH]_I$, which may activate TRPV1 and cause the release of CGRP. In addition to the transient vasodilatation, at higher concentrations of NaHS (300 µM) a secondary vasodilatation was produced. This NaHS-induced vasodilatation was also abolished in the presence of DIDS and therefore it is plausible that activation of the Cl^-/HCO_3^- exchanger in the VSMCs is responsible for vasodilatation induced by higher concentrations of NaHS.

This study also investigated the endogenous production of H_2S from exogenously added L-Cysteine in both rat small mesenteric arteries and porcine large mesenteric arteries. Although L-cysteine caused similar responses to NaHS (vasodilatation in rat resistance mesenteric arteries and contraction in porcine large mesenteric arteries), the presence of inhibitors of

CSE (PPG) and CBS (AOAA) had little effect on responses. However, the concentration of PPG used was 10 µM, which, although shown in some studies to block CSE (Cheng *et al.,* 2004), may require a higher concentration (Al-Magableh and Hart, 2011). Therefore, there may be benefit in using higher concentrations of PPG in future studies.

The aim of this study was to investigate the effects of oxygen levels on responses to NaHS in different blood vessel. This study has shown that responses to NaHS are oxygen-sensitive in both porcine conduit arteries and veins, with vasocontractile responses at higher oxygen levels and vasorelaxatory responses at lower oxygen levels. Therefore, studies should take the oxygen-sensitive nature of NaHS responses into consideration particularly when using 95% O_2:5% CO_2 (as is the experimental norm) where the data may hold little relevance to normal physiological conditions. In addition, more work needs to be done investigating the role and production of H_2S in veins. This study showed that under a physiologically relevant gassing condition, low concentrations of NaHS induce relaxation responses which may increase venous capacitance, thus reducing venous return and decreasing cardiac output. Therefore, if endogenous production of H_2S is occurring to any significant level in veins this could be import in mediating a depressor response. Furthermore, in rat resistance arteries, NaHS caused vasodilator responses that would promote a role for H_2S in maintaining blood pressure at a low level. Any change in the production of H_2S could promote hypertension as has been reported in the CSE knockout mouse (Yang *et al.,* 2008).

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