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School of Life Sciences

The role of perivascular adipose tissue in vascular function: how hyperglycaemia and adiposity affect vascular control

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Contents

ABSTRACT

Hyperglycaemia associated with diabetes may have detrimental effects on vascular function. Diabetes may be accompanied by obesity which can potentially compound impaired vascular function by altering the physiological state of adipose tissue. Perivascular adipose tissue (PVAT), the exterior covering layer of most blood vessels, is receiving interest as a paracrine modulator of vascular function. Most conventional pharmacological studies dissect off the adherent adipose tissue and so this aspect of vascular control is often neglected. The present study aimed to investigate the effects of hyperglycaemia and PVAT on control of the porcine coronary artery (PCA).

In vitro studies were carried out, using PCAs obtained from the abattoir, in organ-bath set ups. Exposure of PCAs to acute hyperglycaemia (22 mM) caused a significant contractile response, which was similar to that caused by the osmotic control (mannitol) and which was attenuated by superoxide dismutase. Superoxide production was detected in the buffer solution incubated with PCAs during hyperglycaemia. These findings suggest that acute hyperglycaemia increased PCA contractility by inducing oxidative stress, which involved superoxide production. Osmotic stress may possibly have contributed to hyperglycaemia-induced vasoconstriction, which needs to be investigated in future work.

The relaxant responses of PCAs to the NO donor (SNP) in the presence of PVAT showed significant potentiation, compared to the vessels without PVAT. Inhibition of NOS in PCAs (denuded of endothelium) led to a contractile response, which was significantly greater in the presence of PVAT. The Griess reaction detected the presence of nitrite in buffer solutions incubated with PVAT. Moreover, the expression of eNOS was identified in PVAT using Western blotting. These data indicate that the PVAT of PCAs released the relaxant factor NO.

Exposure of cleaned PCAs to PVAT significantly increased the basal tone of the vessels which was significantly attenuated in the presence of a

thromboxane A_2 (TXA_2) receptor antagonist. In addition, PVAT enhanced the contractile responses to 4-AP-induced inhibition of vascular voltage-activated K^+ channels (K_v) channels. This enhancement was attenuated following TXA_2 receptor inhibition. These findings point to the release of TXA_2 from PVAT, which had a contractile effect by augmenting the closure of K_v channels of PCAs.

In addition, isometric tension studies showed that the maximal endothelium-dependent vasorelaxation to cumulative bradykinin was significantly inhibited in the presence of exogenous angiotensin II and PVAT. The later effect was ameliorated by inhibition of the angiotensin II, type 1 (AT1) receptor. ELISA showed the presence of angiotensin II in PVAT. However, Western blotting carried out to detect the expression of ACE1 (which converts angiotensin I to angiotensin II) in PVAT showed non-specific bands and was inconclusive. Angiotensin II may have been released from PVAT which interfered with the endothelium-dependent relaxation responses.

In conclusion, the present study has shown that hyperglycaemia influenced the function of PCAs by causing a contractile response possibly mediated by induction of oxidative stress. Moreover, PVAT impacted on function of the adjacent vascular smooth muscle plausibly via release of the relaxant factor NO and the contractile factor TXA_2. Finally, PVAT-derived angiotensin II may have inhibited the function of endothelium of PCAs in a paracrine manner. Future studies in porcine and human coronary arteries will help to further investigate this area.

ABSTRACTS FOR CONFERENCES

1) **Saleem MS**, Chan S, Randall M. Role of perivascular adipose tissue in endothelial function of porcine coronary artery. Proceedings of the British Pharmacological Society 2015 at: http://www.pa2online.org/abstract/abstract.jsp?abid=32846&cat=29&period=61

2) **Saleem MS**, Chan S, Randall M. Perivascular Adipose Tissue modulates function of Porcine Coronary Artery. School of Life Sciences, University of Nottingham Postgraduate Research Symposium 2016 (best poster presentation).

3) **Saleem MS**, Chan S, Randall M. Perivascular adipose tissue induces relaxation of porcine coronary artery through release of nitric oxide. Proceedings of the British Pharmacological Society 2017 at:

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ABBREVIATIONS

Ab	Antibody
AC	Adenylyl cyclase
ACE	Angiotensin-converting enzyme
ADCF	Adipocyte-derived contractile factor
ADRF	Adipocyte-derived relaxant factor
ADMA	Asymmetrical dimethyl arginine
AGT	Angiotensinogen
AM	Adrenomedullin
AMPK	Adenosine monophosphate-dependent protein kinase
ANOVA	Analysis of variance
4-AP	4-amino pyridine
ATP	Adenosine triphosphate
aPWV	Aortic pulse wave velocity
Arg	Arginine
AT1	Angiotensin II type 1 receptor
BH4	Tetrahydrobiopterin
BK	Bradykinin
BSA	Bovine serum albumin
BK_{Ca}	Large-conductance, calcium-activated potassium channels
eNOS	Endothelial nitric oxide synthase
Ca^{+2}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CAT	Cysteine aminotransferase
CBS	Cystathionine beta synthase
COX	Cyclooxygenase

CSE	Cystathionine gamma lyase
cGMP	Cyclic guanosine monophosphate
$CuSO_4$	Copper sulphate
DAF-2DA	4,5 diamino fluorescein diacetate
DHFR	Dihydro folate reductase
EC_{50}	Agonist concentration that produces 50% of the maximal response
EDRF	Endothelium-derived relaxant factor
EDCF	Endothelium-derived contractile factor
EDH	Endothelium-dependent hyperpolarization
EDHF	Endothelium-dependent hyperpolarization factor
EET	Epoxyeicosatrienoic acid
EGTA	Ethylene glycol tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ET	Endothelin
FAD	Flavin adenine dinucleotide
FFA	Free fatty acids
FMN	Flavin mono nucleotide
gAD	Globular adiponectin
GAPDH	Glyceraldehyde phosphate dehydrogenase
GTP	Guanine triphosphate
HDL	High density lipoprotein
H_2O_2	Hydrogen peroxide
HPLC	High-pressure liquid chromatography
H_2S	Hydrogen Sulphide
IFN	Interferon

IK_{Ca}	Intermediate-conductance, calcium-activated potassium channels
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Prostacyclin receptor
IRS-1	Insulin receptor substrate 1
I-SAP	Iodophenyl sulfonyl aminopinane
K^+	Potassium ion
KCl	Potassium chloride
K_{ATP}	ATP-sensitive potassium channels
K_{Ca}	Calcium-activated K^+ channels
K_{IR}	Inwardly rectifying potassium channels
K_v	Voltage-activated potassium channels
L-364,373	**K_V agonist:** 5-(2-Fluorophenyl)-1,3-dihydro-3-(1H-indol-3-ylmethyl)-1-methyl-2H-1,4-benzodiazepin-2-one
L-NAME	N^G-nitro, L-Arginine, Methyl Ester
LDL	Low density lipoprotein
MLC	Myosin light chain
MLCK	Myosin light chain kinase
mM	Milli molar
MPST	3-mercaptopyruvate sulfurtransferase
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide hydrogen phosphate
NaF	Sodium fluoride
NaHS	Sodium hydrogen sulphide
NBT	Nitroblue tetrazolium
NO	Nitric oxide

NO^-	Nitroxyl
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NOX	NADPH oxidase
NS1619	**BK_{Ca} agonist:** 1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one
O_2	Molecular oxygen
O_2^-	Superoxide anion
$ONOO^-$	Peroxynitrite
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet derived growth factor
pEC_{50}	Negative logarithm (to base 10) of EC_{50}
PCA	Porcine coronary artery
PGI_2	Prostacyclin
PK	Protein kinase
PLC	Phospholipase A_2
PVAT	Perivascular adipose tissue
RAS	Renin angiotensin system
RIA	Radio immunoassay
R_{Max}	Maximal effect of an agonist
ROCK	Rho-activated kinase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum calcium ATPase
sGC	Soluble guanylyl cyclase

SK_{Ca}	Small-conductance, calcium-activated potassium channels
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TBST	Tris buffered saline with Tween 20
TEA	Tetra ethyl ammonium
TNFα	Tumour necrosis factor alpha
TP	Thromboxane A_2 receptor
TXA_2	Thromboxane A_2
U46619	**Thromboxane A_2 agonist:** (5Z)-7-[(1R,4S,5S,6R)-6-[(1E,3S)-3-Hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid
VSMC	Vascular smooth muscle cell

Chapter 1

GENERAL INTRODUCTION

1.1 THE GLOBAL BURDEN OF OBESITY AND DIABETES

Populations worldwide are in the midst of an epidemic of obesity, with about 700 million adults estimated to be currently affected in the developing as well as developed nations (Nguyen *et al.*, 2010). In the UK, about 25% of adults are classed as obese (Wang *et al*., 2011). In the US, in the 1976-1981 period, 44% of adults were categorised as overweight or obese and by 2011-2012, the figure had risen to 69% (Martinez *et al.*, 2017). This epidemic, fuelled by sedentary life style and high caloric intake, is a contributory factor to the rising prevalence of type II diabetes mellitus (Dabelea *et al.*, 2014; Zimmet *et al.*, 2001).

The global prevalence of diabetes in adults (20-79 years of age) was 6.4% in the year 2010 and is projected to increase to 7.7% by 2030, with a significantly greater increase expected in developing (69%) compared to developed (20%) countries over the two decades (Shaw *et al.*, 2010). Internationally, about 552 million individuals are expected to have diabetes by 2030 (International Diabetes Federation Guideline Development, 2014). The underlying mechanism for the development of diabetes includes impaired production of insulin by progressive loss of beta cells in the pancreas (type I diabetes) along with resistance to the peripheral effects of insulin (type II diabetes) (Ndisang *et al.*, 2014).

1.2 OBESITY AND DIABETES ARE LINKED TO VASCULAR DYSFUNCTION

Diabetes is associated with hyperglycaemia and other metabolic abnormalities including increased free fatty acids, modified low-density lipoprotein (LDL)-cholesterol, reduced high-density lipoprotein (HDL)-

cholesterol and raised triglycerides (Rask-Madsen *et al.*, 2013). These changes contribute to formation of glycation end-products, inflammatory cytokines, angiotensin II and increase leucocyte adhesion and protein kinase C (PKC) activation. Simultaneously, the level of protective factors including anti-oxidants, insulin, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and activated protein-C are markedly reduced tilting the balance towards vascular dysfunction (Figure 1.1) (Rask-Madsen *et al.*, 2013).

Apart from diabetes, obesity also leads to vascular dysfunction and hence these twin-factors increase the risk for development of coronary artery disease, stroke and peripheral vascular disease (Gollasch, 2012). A common denominator in the impairment of vascular function in these co-morbidities is the development of endothelial dysfunction (Singhal, 2005). Endothelial dysfunction is characterized by decreased local nitric oxide (NO) bioavailability, increased expression of cytokines and adhesion molecules which interact with leukocytes and platelets, directing local inflammation (Hansson, 2005). Production of reactive oxygen species (ROS) including superoxide (O_2^-) and hydrogen peroxide (H_2O_2) increase, predisposing to decreased endothelial dependent vasodilatation, endothelial cell damage and atherosclerosis (Figure 1.1) (Elmarakby *et al.*, 2010; Furukawa *et al.*, 2004; Rhee, 2006).

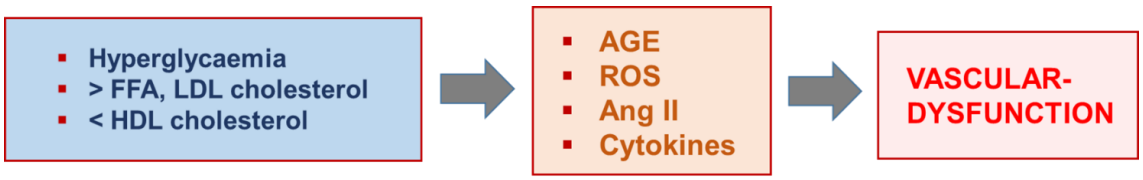

Figure 1.1 Obesity and diabetes predispose to vascular dysfunction. FFA, free fatty acids; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; AGE, advanced glycation end-products; ROS, reactive oxygen species; Ang II, angiotensin II.

Type II diabetes has been reported to be associated with oxidative stress which promotes atherosclerosis and other vascular complications (Emilova *et al.*, 2016). A study conducted on human subjects with type II diabetes who were undergoing coronary-artery bypass grafting demonstrated decreased adiponectin gene expression and diminished circulating levels of adiponectin which were positively correlated with the production of NADPH oxidase (NOX)-derived O_2^- in the arteries. These changes were detected by the overlying PVAT which responded by up-regulating its adiponectin gene expression. The resulting increased release of adiponectin in turn acted in a paracrine manner to inhibit NOX activity in the arterial wall (Antonopoulos *et al.*, 2015).

Another mechanism of vascular dysfunction in obesity and diabetes is the effect of adipose tissue derived biologically active substances, leading to an inflammatory state (Dimitrova *et al*., 2014). These substances including adipokines (leptin, resistin etc.) and pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα), interleukin (IL) 6, and plasminogen activator inhibitor-1 (PAI-1) are produced in increasing amounts (Hajer *et al.*, 2008). They in turn predispose to endothelial dysfunction and subsequent atherosclerosis (Wang *et al*., 2010).

Atherosclerosis, characterized by deposition of lipoproteins and inflammatory cells in the inner arterial layer (intima), causes arterial narrowing and therefore reduced organ perfusion (Glass *et al.*, 2001; Lo *et al.*, 2012). The first step in atherosclerosis is oxidation of low-density lipoproteins (LDL) by free radicals (Hansson *et al.*, 2006). These oxidised LDLs enter the extracellular matrix of the sub-endothelial space of the intima, leading to local inflammation, calcification and plaque formation. With progression of the lesion, the plaque may rupture, leading to thrombosis (Berliner *et al.*, 1995). Obesity and diabetes are associated with a pro-thrombotic state, with increased tendency to platelet aggregation, increased levels of fibrinogen, thrombin and PAI-1 (an

inhibitor of fibrinolysis). These factors then increase the risk of developing thrombosis, including that in the coronary, cerebral and peripheral circulations (Boden, 2008).

Obesity in association with diabetes predispose to development of hypertension (Boden, 2008). There is increased free fatty acid production and insulin resistance, reducing endothelial NO production and increasing production of reactive oxygen species, hence vasodilatation is reduced and development of hypertension is promoted (Inoguchi *et al.*, 2000). Obesity increases the overall blood volume and sympathetic nerve activity, increasing the blood pressure and contributing to hypertension (Fortuño *et al.*, 2003; Hall *et al.*, 2001).

The above evidence highlights that hyperglycaemia in diabetes along with increased adipose tissue found in obesity may perturb vascular function. Excess adipose tissue may accumulate at various sites including in the subcutaneous region, around viscera as well as around blood vessels (Chang *et al.*, 2013) (Grundy, 2015) .

1.3 ROLE OF PERIVASCULAR ADIPOSE TISSUE (PVAT) IN VASCULAR FUNCTION

Blood vessels are surrounded by an outer adipose layer consisting mainly of fat cells, termed perivascular adipose tissue (PVAT) (Figure 1.2), which is present around most large vessels and in some small vascular beds such as the mesentery (Figure 1.3) (Frontini *et al.*, 2010).

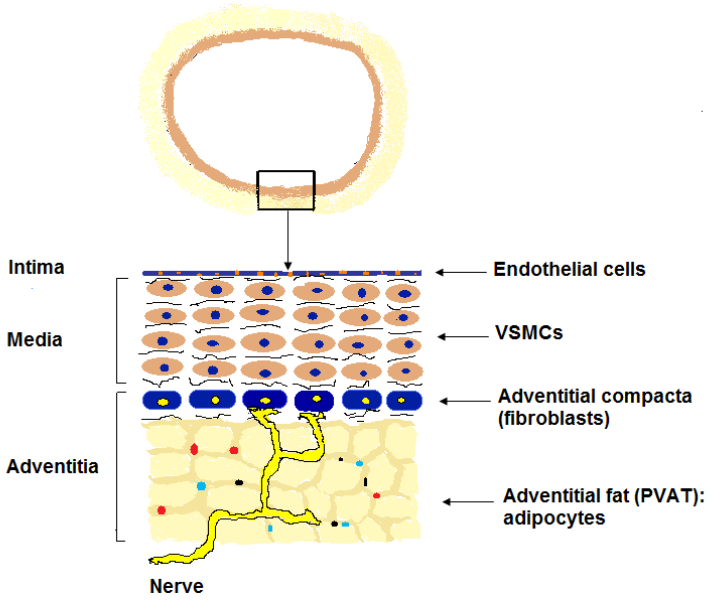

Figure 1.2 Layers of blood vessel wall. Vascular smooth muscle cells (VSMCs) are located in the tunica media. Perivascular adipose tissue (PVAT) contains adipocytes (yellow), vasa vasorum (red) and other cells including macrophages, lymphocytes, adipocyte stem cells and fibroblasts (blue). Adapted from Miao *et al.,* 2012.

PVAT had been thought of as providing only mechanical support for blood vessels and had been routinely removed in experiments (Szasz *et al.*, 2012). So its influence on vascular control had been neglected. Recently, it has received interest as a paracrine structure secreting various active agents which modulate vascular function (Gollasch *et al.*, 2004).

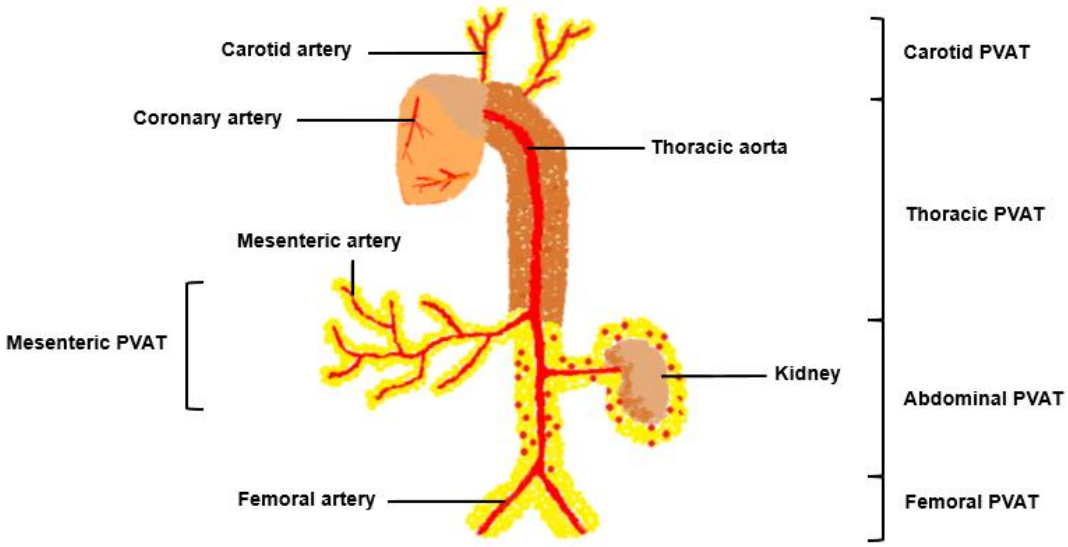

Figure 1.3 Location of perivascular adipose tissue (PVAT) in major vascular beds. Adapted from Brown *et al.,* 2014.

1.3.1 ADIPOCYTE-DERIVED RELAXANT FACTOR (ADRF)

In 2002, Lohn *et al.* and other investigators (Galvez *et al.*, 2006) showed that PVAT released an adipocyte-derived relaxing factor (ADRF), that leads to vasodilatation by opening K^+ channels in vascular smooth muscle (Figure 1.4). Studies (Fésüs *et al.*, 2007) on rat aorta and the mouse mesenteric artery indicated that although adiponectin causes vasodilatation through its action on voltage dependent K^+ (K_v) channels, the ADRF originating from PVAT in these vessels is not adiponectin. However, another study carried out in small human subcutaneous arteries demonstrated complete inhibition of the vasodilator effect of PVAT by blockade of adiponectin receptor with adiponectin receptor 1 blocking peptide (Greenstein *et al.*, 2009).

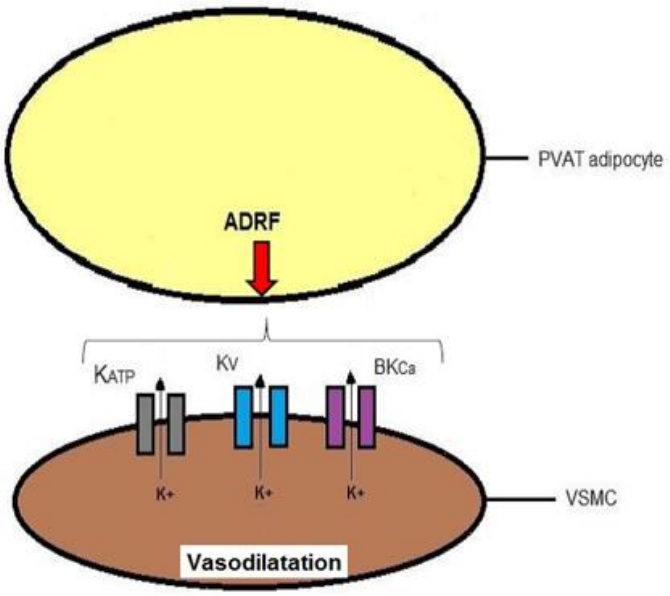

Figure 1.4 Effects of adipocyte derived relaxing factor (ADRF) on potassium (K^+) channels in vascular smooth muscle cells (VSMC). ATP-sensitive potassium channel, K_{ATP}; voltage-dependent potassium channel, K_V; large-conductance, calcium-dependent potassium channel, BK_{Ca}; perivascular adipose tissue, PVAT.

It has been suggested that a gaseous substance, hydrogen sulphide (H_2S), may be a candidate ADRF (Fang *et al.*, 2009). H_2S is produced by action of the enzymes cystathionine-γ- lyase (CSE), cystathionine-ß-synthase (CBS) and by the combined action of cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (MPST) on L-cysteine (Köhn *et al.*, 2012; Szabó, 2007). Investigations have been carried out on the role of CSE-derived H_2S in the relaxant effect of PVAT in rat aorta (Fang *et al.*, 2009) (Schleifenbaum *et al.*, 2010). It was shown that rat aortic rings with PVAT had a significantly reduced contractile response to serotonin, compared to rings without PVAT. Moreover, inhibition of CSE ameliorated the anti-contractile effect of PVAT (Fang *et al.*, 2009; Schleifenbaum *et al.*, 2010).

Other factors which could act as an ADRF include angiotensin 1-7 (Gao *et al.*, 2007) palmitic acid methyl ester (Lee *et al.*, 2011) and prostacyclin

(Chang *et al.*, 2012). The latter is readily detectable in PVAT and it was demonstrated that PVAT caused vasodilatation in carotid artery rings from aged mice, which was inhibited by prostacyclin receptor antagonist (Chang *et al.*, 2012). This suggests that PVAT derived prostacyclin could be an ADRF (Ozen *et al.*, 2013). It is likely that a combination of the above factors is involved in the ADRF response (Szasz *et al.*, 2012). Most of the above findings are from *in-vitro* studies in animals, so their applicability to the *in-vivo* setting as well as to human vessels remains to be determined (Brown *et al.*, 2014).

ADRF is considered to play a major role in the regulation of visceral arteries such as the aorta and mesenteric arteries (Schleifenbaum *et al*., 2010). ADRF is released in response to vasoconstrictors, such as 5-hydroxytryptamine, and produces its effect by multiple mechanisms: endothelium-independent vasorelaxation by opening voltage-dependent K^+ (K_v) channels in the plasma membrane of VSMCs (Fésüs *et al*., 2007) and by H_2O_2 release from PVAT, with subsequent activation of guanylyl cyclase (Gao *et al.*, 2007). However, another study in the rat aorta indicated that ATP-sensitive potassium channels (K_{ATP}) channel activation is involved in the ADRF response and the response is regulated by tyrosine kinase and protein kinase A (PKA) - dependent mechanisms (Lohn *et al*., 2002). The variations seen in the proposed type of K^+ channel involved in the ADRF response could be due to difference in the species and vascular bed studied as well as the methodology employed in different studies (Girouard *et al*., 2010).

1.3.1.1 NITRIC OXIDE (NO) AND THE ADRF RESPONSE

It has been suggested that endothelium-dependent mechanisms involving NO release and calcium-dependent K^+ channels (K_{Ca}) activation are also linked to the ADRF effect (Gao *et al*., 2007). NO is a soluble gas, continuously produced by endothelial cells through the interaction of the amino acid arginine with molecular oxygen under the

influence of the enzyme NO synthase and plays a significant role in maintenance of vascular tone, prevention of inflammation and oxidative stress (Tousoulis *et al*., 2012). NO has also been reported to be produced by PVAT (Lynch *et al.*, 2013; Zaborska *et al.*, 2016).

HISTORY OF RESEARCH ON NITRIC OXIDE (NO)

NO was discovered in 1772 by the British chemist Joseph Priestly (Smith, 1972). For the ensuing period of more than two centuries, this colourless and odourless gas was considered to be of no benefit and even detrimental towards human health. Its importance in human physiology and pathology only began to be recognised in late 20th century (Rapoport *et al.*, 1983). In the 1970s/1980s, Robert Furchgott, Louis Ignaro and Ferid Murad discovered that the endothelium produces a factor which relaxes vascular smooth muscle cells leading to vasodilatation (Furchgott *et al.*, 1980). In 1987, Palmer *et al* postulated that the endothelium-derived relaxing factor (EDRF) discovered by Furchgott *et al* is NO (Palmer *et al.*, 1987). Subsequently it was reported that most cells in the body are able to produce NO which plays a key role in defence (Moncada *et al.*, 1991a).

NO has been found to play an essential role in the cardiovascular, renal, endocrine and reproductive systems as an intercellular signalling molecule (Malinski, 2005) (Ambrosino *et al.*, 2003). NO produced in the central and peripheral nervous systems acts a potential neurotransmitter and may cause neuronal damage (Steinert *et al.*, 2010). It has been implicated in the development of hypertension, stroke, myocardial infarction, metabolic syndrome and even cancer (Clemons *et al.*, 2010; Siervo *et al.*, 2011). Excessive levels of NO have been seen to be associated with sepsis, hypotension, rheumatoid and osteoarthritis (Abramson, 2008; Leiper *et al.*, 2007; Li *et al.*, 2013). Increased NO production induced by TNF-α, IL1 and IFN-γ in macrophages, smooth muscle and other cells plays an important role in the pathogenesis of inflammation and circulatory shock (Leiper *et al.*, 2007).

PRODUCTION AND EFFECTS OF NITRIC OXIDE (NO)

NO is produced by endothelial, neuronal and inflammatory cells through oxidation of the amino acid arginine under the influence of the calcium-calmodulin dependent enzyme NO synthase (NOS) in the presence of the cofactors tetrahydrobiopterin (BH_4), nicotinamide adenine dinucleotide hydrogen phosphate (NADPH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Cannon, 1998) (Figure 1.5). Cells with the capacity to produce NO have their own specific NOS isoforms differing in structure termed endothelial (eNOS), neuronal (nNOS) (both constitutively expressed) and inducible (iNOS) (manufactured during the inflammatory response) forms respectively (Tousoulis *et al.*, 2012).

NO production in the vascular endothelium is brought about by the constitutive enzyme eNOS, whose activity is stimulated by agonists such as bradykinin, acetyl-choline and histamine as well as by shear-stress caused by blood flow along the vessel (Zhao *et al.*, 2015). NO being a lipophilic gaseous mediator, diffuses rapidly to the adjacent vascular smooth muscle cells (VSMCs), where it activates soluble guanylyl cyclase (sGC) resulting in conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP).

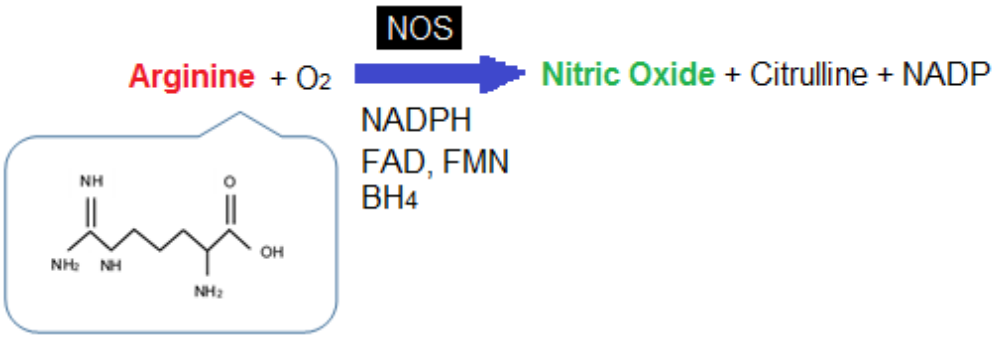

Figure 1.5 Nitric oxide production from arginine. Oxygen molecule, O_2; nitric oxide- synthase, NOS; nicotinamide adenine dinucleotide hydrogen phosphate, NADPH; nicotinamide adenine dinucleotide phosphate, NADP.

The cGMP interacts with its dependent protein kinases (PKGs) to cause phosphorylation of myosin light chain kinase (MLCK) inhibiting it and so reducing phosphorylation of myosin light chains (MLC) and hence leading to a decrease in interaction of myosin heads with actin filaments (Zhao *et al.*, 2015). In parallel, cGMP causes phosphorylation of sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), increasing its activity and so resulting in sequestration of cytoplasmic Ca^{2+} into the endoplasmic reticulum (ER) leading to a drop in intracellular Ca^{2+} concentration (Van Hove *et al.*, 2009). The above combination of events precipitates VSMC relaxation and hence vasodilatation (Moncada *et al.*, 1991a; O'Rourke, 2002).

Apart from maintenance of vascular tone, NO plays a significant role in controlling tissue blood flow, oxygen delivery, sodium balance and blood pressure (Panza *et al.*, 1993). NO also inhibits platelet aggregation, inflammation and oxidative stress (Tousoulis *et al.*, 2012). A reduced anionic nitroxyl form of NO (NO^- or HNO) has also been identified which contributes to the physiological effects of the uncharged NO molecule, including vasorelaxation, cardiac protection and neurotransmission (Triggle *et al.*, 2012). The significance of HNO in maintaining vascular function may be enhanced in the setting of deficiency of BH_4, an essential co-factor required for normal functioning of eNOS (Fukuto *et al.*, 1992).

NITRIC OXIDE PRODUCTION BY PVAT

NO has also been reported to be produced by PVAT (Lynch *et al.*, 2013). Work on male Sprague-Dawley rat mesenteric arteries showed that PVAT exhibited an anti-contractile effect, which was lost following inhibition of NOS (Zaborska *et al.*, 2016).

In experiments on rat aorta, transfer of organ bath solution incubated with aortic rings with intact PVAT, to a bath solution incubated with aortic rings without PVAT, caused relaxation in the later rings (Gao *et al.*, 2007),

suggesting the release of a relaxant factor from PVAT. This relaxation response was attenuated by removal of the endothelium, NOS inhibition and blockage of calcium-dependent K^+ channels (K_{Ca}). It was also demonstrated that this relaxant factor did not originate from the endothelium as organ bath solution incubated with PVAT negative, endothelium positive aortic rings did not induce vasorelaxation in another PVAT and endothelium negative ring (Gao *et al.*, 2007; Triggle *et al.*, 2012).

Work on isolated rat mesenteric arteries using wire-myography showed that the contractile response in endothelium-intact vessels to cumulative concentrations of noradrenaline was significantly inhibited in the presence of PVAT. This effect was reversed following NOS inhibition, pointing to NO release from PVAT causing its anti-contractile effect. Moreover, it was demonstrated that *in-vitro* beta-adrenergic receptor stimulation of isolated mesenteric PVAT led to NO production, which may contribute to its anti-contractile effect (Bussey *et al.*, 2013).

In vitro investigations by Melrose *et al* on mesenteric arteries (with PVAT) obtained from male Wistar rats demonstrated inhibition of vessel contractility to the TXA_2 agonist U46619 and the α-adrenoceptor agonist phenylephrine in young animals, which was reversed by pre-incubation of vessel segments with a NOS inhibitor. However, this effect was absent in older animals, suggesting that NO contributed to the anti-contractile effect of PVAT in young rats. Moreover, it was found that the ratio of phosphorylated eNOS to total eNOS was significantly decreased in older animals, indicating that the reduced NO effect in these animals may be due to impaired eNOS phosphorylation (Melrose *et al.*, 2015).

Studies by Xia *et al* on endothelium-denuded aortic rings of lean mice showed a significantly increased relaxant response of pre-contracted vessels to acetyl-choline in the segments with intact PVAT compared to the control vessels without PVAT. Also, this PVAT-dependent vasodilatation was absent when NOS was inhibited with L-NAME. Fluorescence-imaging of aortic sections using 4, 5 diaminofluoroscein

diacetate (DAF-2 DA) showed basal NO production in PVAT which was enhanced in the presence of acetylcholine and reduced after pre-incubation with L-NAME. Furthermore, immunohistochemistry detected positive staining of PVAT with anti-eNOS antibody. These findings indicate that NO released from PVAT contributes to vasorelaxation induced by acetylcholine (Xia *et al.*, 2016a).

FACTORS WHICH MODULATE THE EFFECTS OF NITRIC OXIDE ON VASCULAR FUNCTION

HYDROGEN SULPHIDE

Endogenous hydrogen sulphide (H_2S) by forming a novel nitrosothiol compound (Whiteman *et al.*, 2006) may down-regulate the vascular effect of NO. *In vitro* experiments showed that the relaxant effect of NO donor compounds on isolated rat aortic rings was significantly inhibited in the presence of the H_2S donor sodium hydrosulphide (NaHS) in physiological concentrations indicating that H_2S may be inactivating NO. Also, low concentrations of NaHS (50 and 100 µM), corresponding to physiological H_2S plasma concentrations caused a contractile response in the endothelium intact aortic rings which was significantly inhibited in the presence of copper sulphate ($CuSO_4$) (which converts nitrosothiol to nitrite and nitrate), indicating that H_2S donated by NaHS caused the contractile response by inactivating endogenous NO. Moreover, evidence from an *in vivo* study in anaesthetized rats demonstrated that the mean arterial pressure lowering effect of a bolus injection of the NO-donor sodium nitroprusside (SNP) was lost when SNP was co-administered with NaHS. So, it was suggested that local vascular concentrations of NO may be regulated physiologically by H_2S (Ali *et al.*, 2006; Lo Faro *et al.*, 2014; Nagpure *et al.*, 2016).

INSULIN

Circulating insulin has been shown to potentiates endothelial NO synthesis in healthy individuals (Yu *et al.*, 2011). In an *in vivo* study in humans, intra-brachial infusion of insulin lead to progressive increase in forearm blood flow in healthy controls, which was absent in patients with diabetes. This response was accompanied by a significant increase in the flux of L-hydroxy-L-arginine (precursor of NO) and clearance of radio-labelled L-arginine from the forearm circulation in controls but not in the diabetic group, suggesting that insulin facilitates L-arginine transport to facilitate endothelial NO production leading to vasodilatation, which is impaired in diabetes (Rajapakse *et al.*, 2013).

Research work on human subjects found impaired endothelium-dependent vasodilatation in response to L-arginine infusion in patients with hypertension when compared to healthy controls. This was accompanied by decreased plasma cyclic guanosine mono-phosphate (cGMP) level along with insulin resistance, pointing to an association between insulin-resistance and diminished endothelial NO-cGMP release and the involvement of insulin in NO production in healthy subjects (Higashi *et al.*, 1997).

The underlying signalling process begins by the binding of insulin to its receptor on the endothelial membrane leading to activation of the receptor tyrosine kinase that phosphorylates insulin receptor substrate-1 (IRS-1). This then activates phosphatidylinositol 3’ kinase-protein kinase B-eNOS pathway which results in local NO production, mediating vasorelaxation, and hence post-prandial increase in blood flow (Yu *et al.*, 2011). Interruption of the above signalling occurs in insulin resistance (a hall mark of diabetes, obesity and hypertension) in association with impairment of the NO generation pathway leading to cardiovascular complications, which are a feature of diabetes and obesity (Huang, 2009). This effect of insulin may also be impaired in obesity due to release of adipokines and inflammatory factors from PVAT, including TNF-α and lL-6, exacerbating insulin resistance (Yudkin *et al.*, 2005).

LEPTIN

The peptide leptin is produced by white adipose tissue and its levels increase in obesity (Considine *et al.*, 1996). Intravenous infusion of leptin leads to increase in sympathetic nervous system activity resulting in vasoconstriction and an increase in blood pressure. However, other evidence points to a direct vasodilator action of this adipokine (Morioka *et al.*, 2014), which may counteract its pressor effect. This latter effect becomes evident following experimental chemical sympathectomy (Lembo *et al.*, 2000).

An *in vivo* investigation in male Wistar rats with normal blood pressure showed a dose-dependent increase of serum nitrate/nitrite concentration in response to bolus leptin injections. This effect required functional leptin receptors as the leptin-induced increase in serum nitrate/nitrite concentration was absent in obese Zucker rats (with a mutated leptin receptor gene). When the Wistar rats were administered L-NAME to inhibit NOS and were then given a bolus leptin injection (in physiological dose), there was a significant increase in mean-arterial pressure. These findings imply that leptin modulates vascular tone partly by causing release of NO leading to a vasorelaxant response (Fruhbeck, 1999).

Evidence from work in sympathectomised rats showed an acute dose-dependent drop in blood pressure following intra-venous administration of leptin in contrast to the control group. Furthermore, isolated rings of aorta from these animals showed a dose-dependent relaxation to leptin, which was abrogated by removal of the endothelium and by inhibition of NOS. This indicates that leptin induced vasorelaxation is endothelial in origin and involves vascular release of NO (Kimura *et al.*, 2000; Lembo *et al.*, 2000). NO-dependent vasodilatation caused by leptin has also been reported to occur in rat and canine coronary arterioles (Lembo *et al.*, 2000).

A study using DAF-2DA fluorescent probe showed direct NO release from cultured human aortic endothelial cells and rat aorta, under the influence of leptin, which was attenuated by NOS inhibition and abolished

by a tyrosine-kinase inhibitor (erbstatin A) (Vecchione *et al.*, 2002). Moreover, immunoblotting demonstrated that leptin stimulated phosphorylation of protein kinase B (Akt) in both human aortic endothelial cells and rat aorta in a time-dependent manner (maximum at 10 minutes), which was sensitive to inhibition of tyrosine-kinase by erbstatin A. Also, treatment of vessels with leptin lead to a significant increase in phosphorylation of eNOS at the Serine1177 site. These data indicate that leptin causes vascular NO production by intracellular phosphorylation of Akt and tyrosine, culminating in eNOS phosphorylation (Vecchione *et al.*, 2002). It has been shown that Akt plays an essential role in regulation of cellular metabolism and also controls endothelial NO production by phosphorylating eNOS at the Serine1177 location (Dimmeler *et al.*, 1999). The evidence presented above adds Akt as an essential link in the pathway involved in the NO-inducing effect of leptin in the vascular endothelium (Vecchione *et al.*, 2002).

An *in vivo* study in humans reported in 2013 looked at the effect of endothelin type A (ET_A) receptor antagonism and NOS inhibition before and after exogenous leptin administration on forearm blood flow (using plethysmography) in lean-controls and subjects with metabolic syndrome (MS). The results demonstrated that in lean-controls, after leptin infusion, ET_A antagonism caused a greater vasodilator response while NOS inhibition lead to higher vasoconstriction, compared to the responses before leptin infusion. Interestingly, these responses to hyperleptinaemia were absent in subjects with MS. This implies that under physiological conditions, hyperleptinaemia enhances ET-dependent vasoconstriction and NO-induced vasodilatation in a balanced manner, which is not the case in MS. It follows that hyperleptinaemia in MS may indicate underlying vascular dysfunction (Schinzari *et al.*, 2013).

ADIPONECTIN

Adiponectin is a 30 kDa protein abundantly found in the circulation and adipose tissue. It has a full length form acting through the R2 adiponectin

receptor and a smaller globular form which acts at the R1 adiponectin receptor (Aghamohammadzadeh *et al.*, 2012). Adiponectin facilitates endothelial NO production by phosphorylation of eNOS mediated by phosphorylated adenosine monophosphate-activated protein kinase (AMPK) (Wang *et al.*, 2008). This was directly demonstrated in experiments on isolated aortae of obese male Sprague-Dawley rats fed a high fat diet. The results showed markedly reduced endothelium-dependent vasorelaxation in these animals which improved significantly after incubation of the vessels with globular adiponectin (gAD) whereas inhibition of NO synthase led to significant reversal of the afore-mentioned beneficial effect of gAD on endothelial cells. Moreover, total NO production by aortic segments was significantly reduced in obese animals compared to controls and administration of gAD increased vascular NO production which was reversed after NO synthase inhibition (Deng *et al.*, 2010).

Further work by the same group in cultured human umblical-vein endothelial-cells showed significantly increased eNOS phosphorylation at the Serine 1177 site in the presence of gAD compared to the control group which was absent in the presence of an AMPK inhibitor (compound C), supporting the aforementioned concept that adiponectin potentiates NO production by activation of eNOS through stimulation of AMPK (Deng *et al.*, 2010). Other experiments showed significantly increased phosphorylation of AMPK at the threonine 172 site in the presence of gAD and the AMPK activator metformin. The latter is used for diabetes control and is known to have protective effects on the vasculature. Accumulating evidence indicates that AMPK, a key regulator of cell metabolism may play a protective role in regulation of vascular function (Davis *et al.*, 2006). These data point to a salutary effect of adiponectin in association with AMPK during vascular dysfunction in obesity, mediated by increased endothelial NO production (Deng *et al.*, 2010).

In vitro experiments were performed, using myography, on small gluteal arteries obtained from patients with obesity associated with insulin

resistance and compared to arteries of healthy controls. It was observed that healthy PVAT of the vessels of controls had an anti-contractile effect accompanied by increased endothelial NO bioavailability, which was lost following incubation with adiponectin blocking peptide. Whereas, the PVAT of arteries sourced from obese subjects lacked the anti-contractile effect seen with healthy vessels (Greenstein *et al.*, 2009). Furthermore, in isolated mesenteric arteries of healthy Wistar rats, vessels with PVAT exhibited an anti-contractile effect which was abrogated after exposure to the adiponectin blocking peptide and exogenous adiponectin added to the organ bath solution led to prompt vasodilatation of pre-constricted vessels. These findings indicate that healthy PVAT may produce an anti-contractile effect through release of adiponectin which involves endothelial NO production (Greenstein *et al.*, 2009).

ADRENOMEDULLIN

Adrenomedullin (AM), a novel peptide first extracted from human phaeochromocytoma, was also found to be synthesized by blood vessels, white adipose tissue, heart, lungs, kidneys and adrenal gland (Fukai *et al.*, 2005; Patel *et al.*, 2017; Sakata *et al.*, 1994). It is composed of 52 amino acid residues held together by a di-sulphide bond (Wong *et al.*, 2014a). Porcine AM is similar to the human form except for an asparagine substituted with glycine at position 40 (Kitamura *et al.*, 1994). The AM receptor consists of seven trans-membrane domains and is included in the G protein coupled-receptor super-family. This receptor has been isolated from endothelial cells, VSMCs, cardiac myocytes, pulmonary cells and astrocytes (Wong *et al.*, 2012).

The production of AM is increased by oxidative stress, angiotensin II, aldosterone, hypoxia, inflammation, hyperglycaemia as well as by natriuretic peptide (Krzeminski, 2016). AM leads to vasodilatation, hypotension, angiogenesis, altered endothelial permeability, myocyte protein synthesis and fibroblast proliferation as well as inhibition of

proliferation and migration of VSMCs (Schonauer *et al.*, 2017). AM may have a role in control of blood volume through its natriuretic and diuretic effect and by its central modulation of thirst and salt appetite (Nikitenko *et al.*, 2006).

The vasodilatation induced by AM may result from endothelium-dependent increase in NO production brought about by increased eNOS activity resulting from increased intracellular calcium concentration (Nakamura *et al.*, 1997; Patel *et al.*, 2017). AM may also cause vasodilatation through an endothelium-independent increase in adenylyl-cyclase activity in VSMCs which in turn enhances cAMP production, stimulation of protein kinase A (PKA), opening of K^+ channels, sequestration of intracellular Ca^{2+} culminating in hyperpolarization of VSMCs and vasorelaxation (Nuki *et al.*, 1993).

The vasodilator effect of AM on rat mesenteric vessels was first reported in 1993 (Nuki *et al.*, 1993). AM in aerosol form delivered into the trachea was found to be beneficial in patients with idiopathic pulmonary hypertension (Nagaya *et al.*, 2004). Inhalation of AM lead to a significant decrease in mean pulmonary arterial pressure and vascular resistance in these patients, along with significantly increased oxygen consumption during exercise (Nagaya *et al.*, 2004). AM has also been shown to induce vasodilatation in human coronary and skeletal arteries through an NO-mediated mechanism (Terata *et al.*, 2000) and may be of benefit in vascular dysfunction associated with obesity and aging by decreasing reactive oxygen species production in VSMCs (Shimosawa *et al.*, 2003; Yoshimoto *et al.*, 2005).

THROMBOXANE A_2

Thromboxane A_2 (TXA_2) is produced in platelets, monocytes/macrophages and blood vessels from arachidonic acid through the action of the constitutively expressed enzyme cyclooxygenase (COX)-1. In turn, TXA_2 acts on its target tissues including

blood vessels (VSMCs, endothelial cells) and platelets via its receptor (TP) facilitating vasoconstriction, platelet aggregation, endothelial adhesion molecule expression as well as smooth muscle cell migration and proliferation (Smyth, 2010).

A study on isolated rat middle cerebral arteries demonstrated that blocking NOS with N^G-nitro-L-arginine induced a contractile response which was significantly attenuated by inhibition of TXA_2 synthase as well as by antagonism of the TP receptor. This phenomenon suggests that NO regulates vascular tone by suppressing TXA_2 and therefore removal of endogenous NO facilitates vascular TXA_2 production (Benyo' *et al.*, 1998).

Exposure of cultured endothelial cells derived from bovine aorta to TXA_2 mimetics for 24 hours led to significantly increased production of the free radicals superoxide (O_2^-) and peroxynitrite ($ONOO^-$) accompanied by membrane translocation of a NADPH oxidase (NOX) subunit as well as a marked decrease in eNOS activity, NO release and NO bioactivity. Inhibition of NOX or TP pharmacologically or genetically abrogated the TXA_2 mimetic-induced O_2^- production and improved eNOS activity, along with NO release and activity. This work indicates that activation of NOX by TXA_2 uncouples NOS from NO production towards manufacture of reactive nitrogen species and other free radicals which are detrimental towards vascular function, predisposing to cardiovascular disease (Zhang *et al.*, 2011).

PATHOPHYSIOLOGY OF NITRIC OXIDE

Impaired vascular NO production may lead to pathological vasoconstriction, tissue ischaemia with organ dysfunction and development of hypertension. In obesity and aging, dysfunction of PVAT occurs which is associated with impairment of its anti-contractile effect and decreased NO production (Britton *et al.*, 2011).

The NOS enzyme may be uncoupled from its physiological role of producing NO towards manufacture of superoxide anion (O_2^-) (Roe *et al.*, 2012). eNOS has multiple sites of phosphorylation and investigations showed that phosphorylation at its serine 1177 location can stimulate O_2^- production in a Ca^{+2} independent manner (Chen *et al.*, 2008). Decreased availability of the NOS cofactor BH_4 due to oxidation or decreased expression of its recycling enzyme dihydrofolate reductase (DHFR) is another possible reason for NOS uncoupling, which is associated with endothelial dysfunction predisposing to cardiovascular disease. Interestingly, ascorbic acid, folic acid and other anti-oxidants increase the bioavailability of BH_4, improving endothelial function (Shi *et al.*, 2004).

Work by Xia *et al* on aortae from male mice with diet induced obesity showed significant inhibition of the relaxant response to acetylcholine compared to lean controls in vessels with intact PVAT. The acetylcholine-induced vasorelaxation was dependent on NO release as the response was completely inhibited following pre-incubation of the vessels with L-NAME. When the experiments were repeated in the aortic segments after removal of PVAT, the relaxant response to acetyl-choline was not inhibited compared to vessel segments from lean controls. These data suggest that the PVAT of obese animals may have impaired NO production. This was confirmed by fluorescence imaging using DAF-2 DA. To identify the mechanisms involved, the group looked at the phosphorylation of eNOS in PVAT by immunohistochemistry, which was found to be reduced in animals fed a high fat diet. Moreover, O_2^- production was found to be increased in PVAT of obese mice which was ameliorated in the presence of L-NAME, suggesting the involvement of NOS in O_2^- production and pointing to its uncoupling. The eNOS uncoupling was seen to be due to reduced L-arginine content and induction of arginase expression in PVAT of animals fed a high fat diet (Xia *et al.*, 2016a).

Increased NO production due to induction of iNOS by TNF-α, IL1β and IFN-γ by macrophages, smooth muscle and other cells play an important

role in pathogenesis of inflammation and circulatory shock (Leiper *et al.*, 2007). Experimental shock was produced in anaesthetised rats by clamping the celiac and superior mesenteric splanchnic arteries for 45 minutes after which the occlusion was removed (Squadrito *et al.*, 1996). Observations during the reperfusion period showed marked drop in blood pressure and white blood cell count along with significantly reduced contractile and relaxant responses of the isolated aortae of the animals to phenylephrine (with and without endothelium) and acetyl choline respectively. *In vitro* treatment of the aortic rings (without endothelium) with a NOS inhibitor restored the contractile responses of the vessels to phenylephrine and treatment of vessels (in the presence of endothelium) with an NO donor restored their responsiveness to acetyl choline. Moreover, *in vivo* treatment of the animals with a combination of a NOS inhibitor and NO donor prevented mortality while significantly improving the blood pressure, white cell count and impaired aortic responsiveness to phenylephrine and acetyl choline (Squadrito *et al.*, 1996). These data are consistent with the observation that shock due to splanchnic artery occlusion is associated with a decreased endothelial and enhanced vascular smooth muscle NO production, the later due to increased activity of iNOS (Klemm *et al.*, 1995; Squadrito *et al.*, 1996).

1.3.1.2 IMPAIRMENT OF THE ADRF RESPONSE

In metabolic syndrome and obesity, the capacity of PVAT to cause vasorelaxation is greatly reduced predisposing to vasoconstriction, increased vascular resistance and development of hypertension (Marchesi *et al.*, 2009; Meijer *et al.*, 2011). This is likely to be due to altered production of paracrine modulators from PVAT (Yudkin *et al.*, 2005). Specifically, decreased release of adiponectin and therefore diminished endothelial NO production coupled with excessive ROS and inflammatory cytokine production along with infiltration of macrophages

in PVAT may contribute to this phenomenon (Chatterjee *et al.*, 2009; Eringa *et al.*, 2012).

1.3.2 ADIPOCYTE-DERIVED CONTRACTILE FACTOR (ADCF)

Apart from the vasodilator effect, evidence indicates that PVAT causes contraction of blood vessels. The initial data from 1991 by Soltis and Cassil showed that the contractions of rat aortae with intact PVAT were significantly increased in response to the sympathomimetic tyramine and to electrical field stimulation, compared to vessels devoid of PVAT. The increased responses of the aortae to electrical field stimulation were absent without PVAT and in the presence of antagonists of angiotensin II and the alpha-adrenergic receptor (Soltis *et al.*, 1991). Subsequently, another study in the rat superior mesenteric artery suggested a contractile role for PVAT mediated by O_2^- production in perivascular adipocytes (Gao *et al*., 2006). This effect was ameliorated by an angiotensin-converting enzyme (ACE) inhibitor and angiotensin II type I (AT1) receptor blocker (Lu *et al.*, 2010).

Most components of the renin-angiotensin system (RAS) including angiotensinogen, angiotensin I, angiotensin II, angiotensin-converting enzyme (ACE 1) and aldosterone have been shown to be expressed in PVAT (Galvez-Prieto *et al.*, 2008; Gu *et al.*, 2013) (Figure 1.6). Angiotensin-II originating from PVAT was shown to be involved in vessel contraction to electrical stimulation (Lu *et al.*, 2010). It has been demonstrated that PVAT-derived angiotensin II leads to local production of adipokines and aldosterone, which may induce vasoconstriction, inflammation and oxidative stress in the adjacent smooth muscle cells in a paracrine manner (Nguyen Dinh Cat *et al.*, 2011b; Nguyen Dinh Cat *et al.*, 2011c).

Recent evidence has shown that in PVAT, a homologue of ACE 1, ACE 2 converts angiotensin II to Ang 1-7 which then induces endothelial NO production and hence vasorelaxation (Gu *et al*., 2013). The RAS based

in PVAT may contribute to vascular dysfunction in metabolic syndrome, obesity, hypertension and hyperaldosteronism (Nguyen Dinh Cat *et al.*, 2011c).

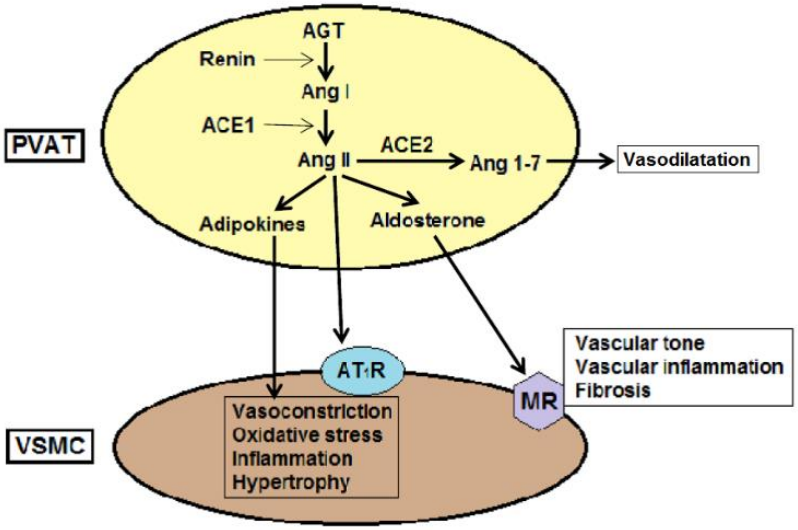

Figure 1.6 The renin-angiotensin system in perivascular adipose tissue (PVAT) influences vascular function. PVAT-derived angiotensin II (Ang II), angiotensin 1-7 (Ang 1-7) and aldosterone affect function of vascular smooth muscle cell (VSMC) via specific receptors. Adipokines produced in response to locally produced Ang II may also impact on vascular function. AGT, angiotensinogen; AT_1R, Ang II type I receptor; MR, mineralocorticoid receptor (Nguyen Dinh Cat *et al.*, 2011c).

Catecholamines including dopamine, noradrenaline and adrenaline were shown to be present in PVAT (Ayala-Lopez *et al.*, 2014). A study on PVAT in rats showed release of functional catechol amines, which may have a role in control of vascular tone by PVAT. In this study, tyramine (an indirect sympathomimetic) caused a concentration-dependent contraction in thoracic aorta and superior mesenteric artery which required the presence of PVAT. Moreover, tyramine stimulated release of noradrenaline, dopamine and 5-hydroxytryptamine from PVAT of both vessels (Ayala-Lopez *et al.*, 2014).

Investigations in aortae from obese male mice showed that PVAT enhanced the contractile effects of 5-hydroxytryptamine and phenylephrine, which was prevented by administration of a cyclooxygenase (COX) inhibition. Also, this study quantified the COX derived vasoconstrictor TXA_2 in PVAT, whose levels were found to be increased. Therefore, TXA_2 may represent a putative ADCF (Meyer *et al.*, 2013).

The potential ADCFs are heterogeneous and their effects depend on the location of the vessel and the species (Brown *et al*., 2014). PVAT of murine aorta is similar to brown fat and has a reduced tendency to development of an inflammatory state (Fitzgibbons *et al*., 2011). Studies have shown that with onset of obesity, the contractile effects of PVAT are enhanced in small human gluteal arteries (Greenstein *et al.*, 2009), murine mesenteric arteries (Fésüs *et al*., 2007), but not in the aortae from obese rats (Ma *et al*., 2010). Other studies in rat aorta and mesenteric arteries demonstrated stronger enhancement of the contractile effects of PVAT in obesity (Lohn *et al*., 2002; Ma *et al*., 2010) compared to a study in aorta of obese mice (Meyer *et al*., 2013).

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1.3.3 ADIPOKINES AND CYTOKINES

PVAT also produces a number of biologically active adipokines, some of which are pro-inflammatory (leptin, resistin and visfatin), while others oppose inflammation (adiponectin and adrenomedullin) (Rajsheker *et al.*, 2010). A study on human coronary PVAT showed decreased expression

of adiponectin and an increased inflammatory state (Chatterjee *et al.*, 2009). Another study on murine periaortic adipose tissue demonstrated markedly decreased expression of adiponectin in PVAT following two weeks of a high fat diet (Chatterjee *et al.*, 2009). Adiponectin also stimulates NO production by the endothelium, leading to an anti-contractile effect (Wang *et al.*, 2008).

Leptin production correlates with the amount of adipose tissue and causes vasodilatation by inducing endothelial release of NO (Galvez-Prieto *et al.*, 2012). A study in aortae of spontaneously hypertensive rats showed that leptin expression in PVAT was significantly lower and its endothelium-dependent relaxation was significantly reduced, compared to controls (Galvez-Prieto *et al.*, 2012).

PVAT has been shown to produce a number of cytokines involved in the inflammatory response during injury to the blood vessel including interleukin 1, interleukin 6 and tumour necrosis factor-alpha (Tilg *et al.*, 2006). They are released by infiltrating macrophages and T lymphocytes in PVAT (Szasz *et al.*, 2012). Inflammatory cell infiltration is markedly increased in PVAT surrounding human aorta with atherosclerosis (Henrichot *et al.*, 2005).

Evidence from studies in small human subcutaneous arteries from obese subjects indicates that the adipocytokines secreted by PVAT during the inflammatory response combined with oxidative stress in PVAT cause a contractile effect on the vessel (Greenstein *et al.*, 2009).

1.3.4 PVAT DYSFUNCTION

In lean individuals, healthy PVAT helps to keep the vessels dilated and inhibits the effect of contractile agents (Van de Voorde *et al.*, 2014). The total mass of PVAT and size of adipocytes is increased in obesity (Marchesi *et al.*, 2009; Van de Voorde *et al.*, 2014). Macrophage infiltration and inflammation starts in the PVAT, causing alteration in the

profile of paracrine mediators released from it, leading to decreased vasorelaxation and hence development of hypertension (Van de Voorde *et al.*, 2014). PVAT dysfunction in obesity also results in increased smooth muscle proliferation and development of atherosclerosis (Ma *et al.*, 2010).

In a study carried out in New Zealand obese (NZO) mice with metabolic syndrome, macrophage infiltration in PVAT along with increased production of reactive oxygen species (ROS) was noted, leading to PVAT dysfunction (Marchesi *et al.*, 2009). The NZO mice showed insulin resistance, hypertension and increased visceral fat, compared to control mice. Mesenteric arteries from the NZO mice showed hypertrophy of the media and decreased endothelial dependent vasodilation, which was not affected by inhibition of endothelial NO synthase, but was improved by an antioxidant. This suggested decreased NO bioavailability and increased oxidant stress. Mesenteric and aortic PVAT of the NZO mice showed increased superoxide production, inflammation and hypertrophy of the adipocytes (Marchesi *et al.*, 2009).

The mass of PVAT correlates negatively with insulin sensitivity in the skeletal muscle, liver, blood vessels and increase in blood flow following ischaemia (Rittig *et al.*, 2008). The decreased insulin sensitivity in adiposity may be linked to altered adipokine release, inflammation and oxidative stress (Zhang *et al.*, 2010). The release of leptin has been found to increase (Payne *et al.*, 2010) and that of adiponectin decrease in obese mice and humans (in coronary perivascular, subcutaneous and perirenal adipocytes), contributing to impaired insulin sensitivity and vascular dysfunction (Chatterjee *et al.*, 2009).

1.4 ENDOTHELIUM AND ITS FUNCTIONS

1.4.1 ENDOTHELIUM AND VASCULAR TONE

The endothelial layer plays an important role in the regulation of blood pressure (Silverthorn, 2013) and maintenance of vascular homeostasis by producing a number of relaxant and contractile factors (Deanfield *et al.*, 2007). Arterial tone is maintained by endothelial NO, which also prevents inflammation, cellular proliferation and thrombosis (Feletou *et al.*, 2008). Production of NO by the endothelium may be enhanced by estrogens, dietary components and exercise, while downregulation of endothelial NO release can be brought about by the effect of oxidative stress, oxidised LDL, ageing, diabetes and hypertension (Vanhoutte *et al.*, 2017).

NO works in concert with locally produced prostacyclin and endothelium-dependent hyperpolarization (EDH) factors in the control of vascular tone (Feletou *et al.*, 2006). Their action is antagonised by a number of contractile factors also synthesized in the endothelium including ET-1, contractile prostanoids and angiotensin II, whose effects are balanced out by the relaxant factors in the physiological milieu (Deanfield *et al.*, 2007).

Endothelial vasorelaxation occurs under the influence of shear-stress created by flow of blood along the vessel as well as by agonists including acetyl-choline, substance P and bradykinin (BK) (Félétou, 2009). BK can cause arterial relaxation through endothelial release of NO, prostacyclin as well as via endothelium-dependent hyperpolarization (EDH) by acting on its specific receptors on endothelial cells (Figure 1.7) which has been demonstrated in animal and human studies (Nakashima *et al.*, 1993). Other neuro-humoral mediators can also attach to their specific endothelial receptors to prompt the release of endothelium-derived relaxant factors (EDRF). These factors include NO, prostacyclin and the mediators involved in the EDH response. The EDRFs in turn diffuse

across to the adjacent vascular smooth muscle cells to cause their relaxation (Figure 1.7) (Triggle *et al.*, 2012; Vanhoutte *et al.*, 2017).

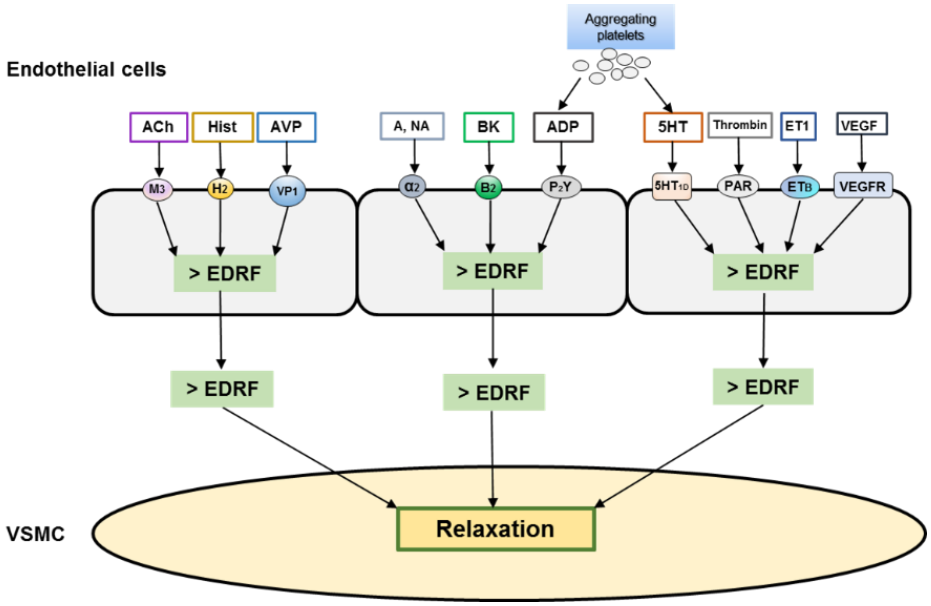


Figure 1.7 Endothelium-dependent vasorelaxation stimulated by neurohumoral factors acting through their specific receptors on endothelial cells. A, adrenaline; ACh, acetyl choline; ADP, adenosine diphosphate; α, adrenergic receptor; AVP, arginine vasopressin; B, kinin receptor; BK, bradykinin; EDRF, endothelium-derived relaxant factors; ET, endothelin; H, histaminergic receptor; Hist, histamine; 5HT, 5-hydroxytryptamine; M, muscarinic receptor; NA, noradrenaline; P, purinergic receptor; PAR, protease activated receptor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VSMC, vascular smooth muscle cell. Adapted from Vanhoutte *et al*., 2017.

Healthy endothelial cells protect against the damaging effects of thrombin and platelet aggregation by upregulating the activity of eNOS, resulting in an increase of endothelial NO production (Figure 1.7) (Motley *et al.*, 2007; Touyz, 2007). In the physiological state, thrombin and platelet-derived products such as 5-hydroxytryptamine and adenosine diphosphate act via their specific endothelial receptors (PAR, $5HT_{1D}$ and P2Y respectively) to enhance phosphorylation of eNOS and thus its

activity, leading to increase in endothelial NO production. The augmented NO release causes vasodilatation, feedback inhibition of platelet aggregation and blood coagulation (Radomski *et al.*, 1987). Whereas, during vascular damage associated with disruption of the endothelium, the aggregating platelets can directly access the vascular smooth muscle cells, inducing their contraction by releasing products such as 5-hydroxy tryptamine and TXA_2 (Flavahan *et al.*, 1995).

1.4.2 ENDOTHELIUM-DEPENDENT HYPERPOLARIZATION

In endothelial dysfunction, there is a compensatory increase in other vasodilators which are involved in endothelium-dependent hyperpolarization (EDH) (McCulloch *et al.*, 1997). The EDH response is thought to be mediated by calcium activated potassium channels (K_{Ca}) (Félétou, 2009), of which there are three types: small (SK_{Ca}), intermediate (IK_{Ca}) and large conductance (BK_{Ca}) (Bolton *et al.*, 1984; Taylor *et al.*, 2003; Weston *et al.*, 2005).

The SK_{Ca} and IK_{Ca} are located in the endothelium, while BK_{Ca} are found in the vascular smooth muscle. Activation of SK_{Ca} and IK_{Ca} leads to hyperpolarization, which is transmitted to VSMC of the underlying media through myo-endothelial gap junctions or by endothelium-derived potassium (K^+) ions (Bellien *et al.*, 2008; Chaytor *et al.*, 2005). Alternatively, endothelium-derived products, such as epoxyeicosatrienoic acid (Weston *et al.*, 2005) and hydrogen peroxide (H_2O_2) (Hatoum *et al.*, 2005) stimulate BK_{Ca} which hyperpolarizes and relaxes the VSMCs (Figure 1.8). Evidence indicates that H_2O_2 may play a dominant role in the EDH response (Freed *et al.*, 2014). EDHFs may be ‘site- and species-specific’ and their contribution to vasodilatation has been noted to increase as the vessel size decreases (conduit vessels versus resistance vessels) and in states of decreased NO availability (Clark *et al.*, 2000).

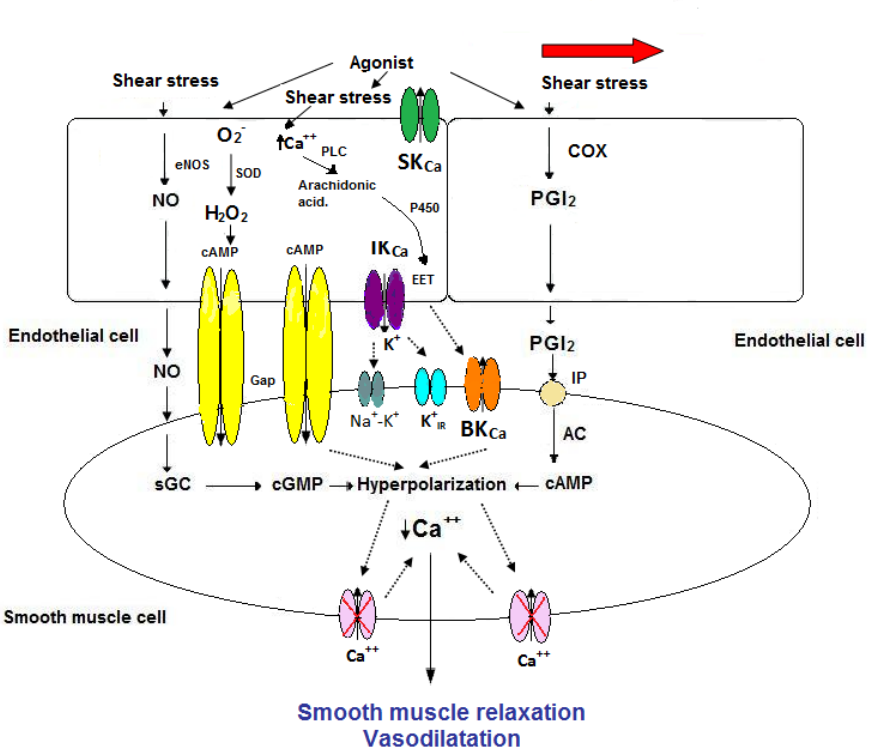


Figure 1.8 Mechanisms of endothelial-dependent vasorelaxation. Agonist (bradykinin/acetyl-choline/substance P) or shear stress lead to nitric oxide (NO) or prostacyclin (PGI_2)-mediated vasodilatation. Multiple potential endothelial dependent hyperpolarization factor/s (EDHF) pathways are also involved. Increases in intracellular calcium activates phospholipase A_2 (PLC), which produces arachidonic acid. This is then converted to epoxyeicosatrienoic acids (EETs), which activate calcium dependent potassium (K_{Ca}) channels in endothelial and smooth muscle cells. The resulting hyperpolarization leads to efflux of K^+ ions into the sub-endothelial space. This in turn may activate large conductance calcium-activated potassium channels (BK_{Ca}), inwardly rectifying K^+ channels (K_{IR}) or the Na^+-K^+ pump on the smooth muscle cells, causing their hyperpolarization and subsequent closure of voltage gated Ca^{+2} channels. This reduces Ca^{+2} concentration in the smooth muscle cells and hence vasodilatation ensues. Endothelial nitric oxide-synthase (eNOS) and oxidases act on molecular oxygen to produce the reactive oxygen species superoxide (O_2^-). Superoxide dismutase (SOD) converts

the later to hydrogen peroxide (H_2O_2) which may cause hyperpolarization by stimulating endothelial or smooth muscle K_{Ca} channels or through the myo-endothelial gap junctions (Gap). Adenylyl cyclase: AC; cyclic adenosine monophosphate: cAMP; cyclic guanosine monophosphate: cGMP; cyclooxygenase: COX; sGC: soluble guanylyl cyclase; SK_{Ca}: small-conductance, calcium-activated, potassium-channels; IK_{Ca}: intermediate-conductance, calcium- activated, potassium-channels; IP: prostacyclin receptor; P450: cytochrome P450. Adapted from Feletou 2009; Ozkor *et al*., 2011.

Recent evidence indicates that H_2O_2 may play a dominant role in the EDH response (Freed *et al.*, 2014). EDHFs may be 'site- and species-specific' and their contribution to vasodilatation has been noted to increase as the vessel size decreases (conduit vessels versus resistance vessels) and in states of decreased NO availability (Clark *et al.*, 2000).

EDH-mediated responses are impaired in the states of decreased NO availability (endothelial dysfunction) which play an important role in atherosclerosis, hypertension and diabetes mellitus (De Vriese *et al.*, 2000; Gollasch, 2012). The contribution of prostacyclin to endothelium-dependent vasodilatation is reported to increase as a compensation in diabetes associated with diminished NO bioavailability and EDH responses (Mokhtar *et al.*, 2015).

1.4.3 ENDOTHELIUM-DEPENDENT VASOCONSTRICTION

The endothelium has been reported to secrete a number of contractile factors, termed endothelium-derived contractile factors (EDCFs), which modulate local vascular tone, participating in the maintenance of homeostasis, in tandem with the EDRFs, in the physiological environment (Gilbert *et al.*, 2001). Stimuli such as sudden stretch, ET1, adenosine diphosphate and 5-hydroxytryptamine prompt the manufacture of various EDCFs (Figure 1.9). Candidate EDCFs include

ROS, angiotensin II, ET-1 and contractile prostanoids such as TXA_2 (Castellon *et al.*, 2016; Chu *et al.*, 2016; Cooper *et al.*, 2002; Virdis *et al.*, 2015).

Experiments (*in vivo* and *ex vivo*) were performed on swine models to investigate the effect of mechanical stimuli (pressure or volume overload) on coronary artery function. The right coronary artery was exposed to pressure overload by banding the aorta or volume overload by banding of the pulmonary artery. These mechanical stimuli resulted in increased vascular expression and activation of the AT1 receptor, enhanced ROS production along with endothelial dysfunction. The later partially recovered by inhibition of AT1 receptor and NOX. These data suggest that local mechanical stimulation of coronary artery activated the AT1 receptor, which may have caused ROS production accompanied by endothelial dysfunction, supporting the role of ROS and angiotensin II (ligand for AT1 receptor) as candidate EDCFs (Lu *et al.*, 2013).

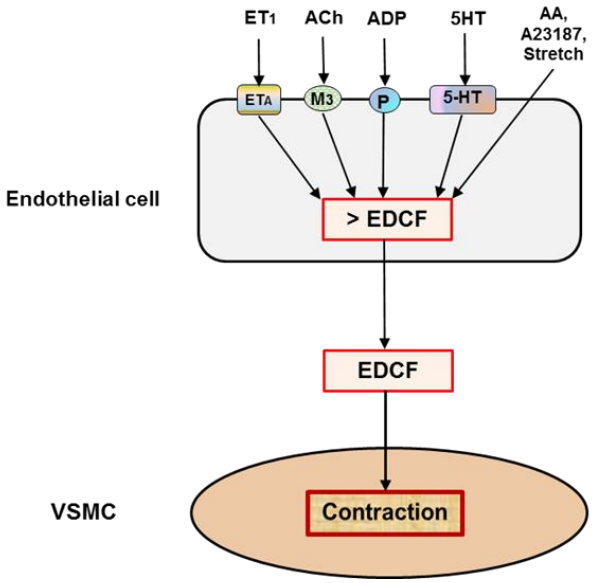


Figure 1.9 In some circumstances, when exposed to sudden-stretch, the calcium ionophore A23187 or neuro humoral factors (acting via their specific receptors), the endothelial cells release vasoconstricting factors termed endothelium-derived contractile factors (EDCF), which disperse to the adjacent vascular smooth muscle cells (VSMC) to stimulate their contraction. AA, arachidonic acid; ACh, acetyl choline; ADP, adenosine

diphosphate; EDCF, endothelium-derived contractile factors; ET, endothelin; 5HT, 5 hydroxytryptamine; M, muscarinic receptor; P, purinergic receptor; VSMC, vascular smooth muscle cell. Adapted from Vanhoutte *et al*., 2017.

Accordingly, a study in isolated aortic segments of rats with chronic arthritis showed the presence of endothelial dysfunction in association with increased NOX activity and O_2^- production. These changes were accompanied by an initial compensatory increase in activity of eNOS, which was not sustained in later stages of arthritis (Totoson *et al.*, 2016).

The peptide ET-1 is produced by the endothelium and exerts a contractile effect by binding to its receptors on VSMCs (ET_A and ET_B). These receptors are also located on endothelial cells, where the binding of ET-1 with ET_A leads to release of EDCFs and hence vasoconstriction (Figure 1.9). On the other hand, ET-1 stimulation of ET_B receptor on endothelial cells increases eNOS activity and NO production, causing vasorelaxation, which aids in counterbalancing the contractile effects of EDCFs (Virdis *et al.*, 2015). Experiments on porcine coronary arteries, rat pulmonary artery and human placental vessels suggested that ET-1 may act on endothelial cells to activate the COX enzyme leading to production of the contractile prostanoid TXA_2, adding to the contractile response (Park *et al.*, 1999).

If the endothelium is exposed to cardiac risk factors (such as smoking, obesity, hypertension and dyslipidaemia) imbalance occurs in its production of vasoactive substances (Ozkor *et al.*, 2011; Singhal, 2007). This includes decreased NO generation, increase in vasoconstrictors like O_2^- anions and ET-1, leading to a state of endothelial dysfunction (Malmsjo *et al.*, 1999).

1.4.4 EFFECTS OF PVAT ON THE ENDOTHELIUM

Factors released from PVAT may directly influence function of the adjacent endothelium through paracrine signalling to produce vasorelaxation in the physiological state (Brown *et al.*, 2014). PVAT can release adiponectin, leptin and angiotensin 1-7 which may diffuse to the endothelium to increase local NO production. Moreover, leptin also stimulates the EDH response in a direct manner to cause vasorelaxation (Gu *et al.*, 2013). In pathological conditions such as hypertension and obesity, PVAT release of contractile factors may be enhanced, which act in an endothelium-dependent manner (Gu *et al.*, 2013). One such factor is the glycoprotein lipocalin-2, which has the capacity to uncouple endothelial NOS and increase COX expression in the endothelium (Liu *et al.*, 2012). Also, angiotensin II and aldosterone manufactured in the local RAS system in PVAT may cause vasoconstriction by binding to their specific receptors on the endothelium, whereas angiotensin II may also increase ROS and inflammation in the endothelium (Daugherty *et al.*, 2000; Nakashima *et al.*, 2006; Nguyen Dinh Cat *et al.*, 2011a).

The above evidence indicates that PVAT has the capacity to affect the function of blood vessels through the release of relaxant and contractile factors, in physiological and pathological states. In obesity, the effect of the contractile factors may dominate, which, in combination with ROS and inflammatory cytokines, may lead to vascular dysfunction (Chatterjee *et al.*, 2009). Moreover, hyperglycaemia associated with diabetes, which may accompany obesity, can potentially impair vascular function by a number of mechanisms, which culminate in increase of vascular contractility and proliferation as well as dysfunction of the endothelium, associated with decreased NO bioavailability (sections 1.2, 2.1.1 and Figure 1.10) (Reho *et al.*, 2017).

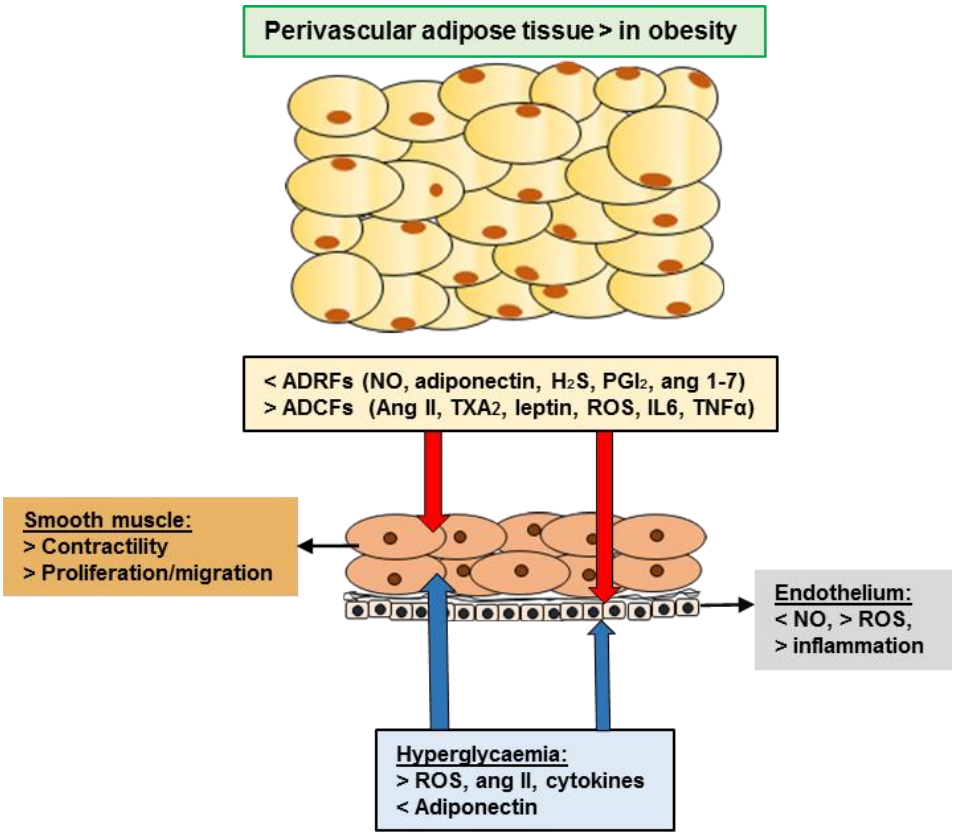


Figure 1.10 The mass of perivascular adipose tissue increases and its function is impaired in obesity, leading to its augmented release of contractile factors, inflammatory cytokines and reactive oxygen species. Crucially, the production of protective relaxant factors may be diminished in parallel. Hyperglycaemia associated with diabetes may accompany obesity, which may also impair vascular function, potentially leading to increased smooth muscle contractility and endothelial dysfunction. Ang, angiotensin; H_2S, hydrogen sulphide; IL6, interleukin 6; NO, nitric oxide; PGI_2, prostaglandin I_2 (prostacyclin); ROS, reactive oxygen species; TNFα, tumor necrosis factor alpha; TXA_2, thromboxane A_2. Adapted from Reho *et al*., 2017.

AIMS

Hyperglycaemia associated with diabetes as well as excess and dysfunctional adipose tissue may have detrimental effects on vascular function. Adipose tissue forms the external covering of most blood vessels (perivascular adipose tissue or PVAT), which is being increasingly recognised as a paracrine modulator of vascular function (Guzik *et al.*, 2007; Oriowo, 2015). Therefore, this study aimed to investigate:

- The influence of PVAT on basal tone and porcine coronary artery (PCA) contraction during exposure to high extra-cellular potassium (KCl) and cumulative U46619 (thromboxane A_2 receptor agonist), postulating that PVAT may have an anti or pro-contractile effect under basal and stimulated conditions as observed by other researchers (Gao *et al.*, 2007). The study also aimed to determine the role of acute hyperglycaemia (simulated by exposing the tissue to high glucose concentrations in the organ-baths) in vasomotor activity of PCAs, to elucidate the potential mechanisms of vascular dysfunction linked with pathologies such as poorly controlled diabetes, building on the findings of previous experiments (Antonopoulos *et al.*, 2015; Inoguchi *et al.*, 2000).

- The potential protective effect of PVAT on PCAs may be mediated by the relaxant factor NO, as reported by other investigators (Bussey *et al.*, 2013). Therefore this part of the work aimed to determine the role of perivascular adipose tissue (PVAT) in vascular reactivity of cleaned PCAs (with intact or disrupted endothelium) to an exogenous NO donor (SNP) and NO-synthase inhibition (by L-NAME). The study also aimed to measure levels of nitrite (a stable and non-volatile product of NO) in buffer solution incubated with PCAs (with intact endothelium) and PVAT by the Griess reaction and expression of endothelial-NOS (eNOS) in PVAT by Western blotting.

℘ Factors released from PVAT, including components of the renin-angiotensin system may interfere with function of the underlying endothelium (Agabiti-Rosei *et al.*, 2018) and so this study aimed to investigate the role of PVAT and exogenous angiotensin II in endothelium-dependent relaxation of porcine coronary arteries induced by bradykinin. In addition, the study aimed to determine the presence in PVAT of angiotensin II by ELISA and type-1 angiotensin-converting enzyme (ACE 1) (converts angiotensin I to angiotensin II) using Western blotting.

℘ It has been reported that the vasoactive factors originating from PVAT may impact on arterial function by stimulating or inhibiting the vascular potassium channels (Lohn *et al.*, 2002) (Girouard *et al.*, 2010). Therefore, this part of the project aimed to investigate the influence of PVAT on arterial relaxation induced by activators of various potassium channels (K_{ATP}, BK_{Ca} and K_V) and vasoconstriction evoked by inhibition of K_V channels of PCAs. The study also aimed to determine the effects of PVAT on PCA contraction to K_V inhibition in the absence or presence of COX inhibitor and a TXA_2 receptor antagonist.

Chapter 2

EFFECTS OF HYPERGLYCAEMIA AND PERIVASCULAR ADIPOSE TISSUE ON FUNCTION OF PORCINE CORONARY ARTERY

2.1 INTRODUCTION

2.1.1 HYPERGLYCAEMIA AND VASCULAR REACTIVITY

Hyperglycaemia accompanying diabetes may perturb arterial tone and increase contractility by its effects on vascular smooth muscle or endothelium (MacKenzie *et al.*, 2008; Node *et al.*, 2009). Oxidative stress is involved in altered vasodilatation and contraction in hyperglycaemia associated with diabetes (Donmez *et al.*, 2014). Excessive superoxide (O_2^-) may be produced by vascular tissue in hyperglycaemia from a number of sources including nicotinamide adenine dinucleotide hydrogen phosphate (NADPH) oxidase, NO synthase as well as due to deficiency of endogenous anti-oxidants or tetrahydrobiopterin (BH_4) (an essential co-factor needed for NO synthesis by NO synthase) (Liu *et al.*, 2002).

The resulting O_2^- may impair vasorelaxation, as seen in a study using guinea pig aortic segments which showed that incubation of the vessels with glucose (50 mM) for 6 hours resulted in impaired endothelium-dependent dilatation to adenosine-triphosphate (ATP) and acetyl-choline. The vasodilatation was restored by treatment with superoxide dismutase (SOD) (a scavenger of O_2^-), indicating that O_2^- produced during hyperglycaemia may have impaired the vasodilatation (Dorigo *et al.*, 1997).

O_2^- may alter the function of vascular smooth muscle K^+ channels resulting in abnormal responses during hyperglycaemia and has been linked to impaired dilatation of aortae, mesenteric and cerebral arteries of diabetic rats in response to ATP-sensitive potassium channel (K_{ATP}) openers (Liu *et al.*, 2002). In contrast, the large-conductance, calcium-activated potassium (BK_{Ca}) channels of vascular smooth muscle cells (VSMCs) have been found to be resistant to the detrimental effects of O_2^- and may serve to compensate for the dysfunction of other vascular K^+ channels during oxidative stress (Liu *et al.*, 2002).

Impaired regulation of voltage-activated (K_v) potassium channels in conjunction with oxidative stress may also be a contributory factor in the development of vascular dysfunction in diabetes (Liu *et al.*, 2001). Studies on isolated rat aortic smooth muscle cells demonstrated reduced whole-cell K_v current on exposure to O_2^-. Also, when rat small coronary arteries were incubated with high glucose (23 mM) for 24 hours, a reduction in 4-amino pyridine (4-AP) (a K_v inhibitor)-sensitive K^+ current density, which recovered partially with SOD, was observed in smooth muscle cells when compared to vessels exposed to a normal glucose concentration (5.5 mM). The impaired K_v activity in hyperglycaemia also led to altered functional responses whereby rat small coronary arteries exposed to high glucose showed significantly decreased constriction to graded concentrations of 4-AP. This reduction in vasoconstriction to 4-AP recovered partially with SOD treatment, indicating that there was a decrease of K_v channel contribution to resting membrane potential of smooth muscle cells of the affected vessels due to the effect of O_2^- (Liu *et al.*, 2001).

Apart from oxidative stress, other mechanisms may impair smooth muscle K^+ channel activity in vessels exposed to hyperglycaemia. Experiments with isolated rat mesenteric, small porcine coronary and human internal mammary arteries using myography demonstrated that vessels exposed to hyperglycaemia (20 mM) had significantly increased basal tone as well as enhanced contractile responses to agonists, compared to control vessels bathed in 5 mM glucose. Specifically, contractile responses to extracellular K^+ (KCl, 60 mM), exogenous angiotensin II (100 nM) and U46619 (a TXA_2 agonist) were significantly enhanced during acute hyperglycaemia which was prevented by pre-incubation with a PKC inhibitor. Patch clamp electrophysiological studies showed inhibition of the K_v current by extracellular glucose in a concentration-dependent manner, which was attenuated by PKC inhibition. These findings indicate that hyperglycaemia caused

vasoconstriction by inhibiting K_V channels through the mediation of PKC (Jackson *et al.*, 2016).

In vitro investigations were also performed using rat small coronary arteries (in the presence or absence of endothelium) exposed to high extracellular glucose (23 mM). Endothelium-independent, cyclic adenosine mono phosphate (cAMP)-mediated vasodilatation induced by isoproterenol, forskolin and papaverine (in cumulative concentrations) was significantly reduced in hyperglycaemic vessels in comparison to the normoglycaemic controls (vessels exposed to 5.5 mM glucose), which was not due to the effect of osmolality of high extracellular glucose. In hyperglycaemic arteries, inhibition of vascular K_v channels with 4-AP (3 mM) resulted in significantly greater inhibition of isoproterenol-induced vasodilatation in comparison to normoglycaemic controls, suggesting that hyperglycaemia impairs cAMP-mediated coronary vasodilatation by suppressing arterial K_v channels (Li *et al.*, 2003). Further evidence from patch-clamp electrophysiological studies using VSMCs showed that whole cell K^+ currents induced by isoproterenol were suppressed in hyperglycaemic cells, compared to normoglycaemic controls. Also, K_v inhibition with 4-AP reduced the K^+ current to a greater extent in hyperglycaemic cells compared to normoglycaemic controls. These data support the findings of functional studies which indicated that reduced opening of K_v channels of VSMCs play a prominent role in impaired non-endothelial vasodilatation provoked by cAMP during hyperglycaemia (Li *et al.*, 2003).

2.1.2 PERIVASCULAR ADIPOSE TISSUE AND VASCULAR TONE

As described in Chapter 1, perivascular adipose tissue (PVAT) forms the external covering of most blood vessels (Figure 2.1) and is being recognised as a paracrine structure secreting various vasoactive biological factors (Johan Van de *et al.*, 2014). It has been reported that PVAT produces vasoprotective relaxant factors (NO, adiponectin,

angiotensin 1-7 and prostacyclin etc.) which impact on the underlying smooth muscle by activating their K^+ channels to cause vasorelaxation whose effect is over ridden by PVAT-derived contractile factors including O_2^-, angiotensin II and thromboxane A_2 in conditions such as hypertension and obesity (Szasz *et al.*, 2012).

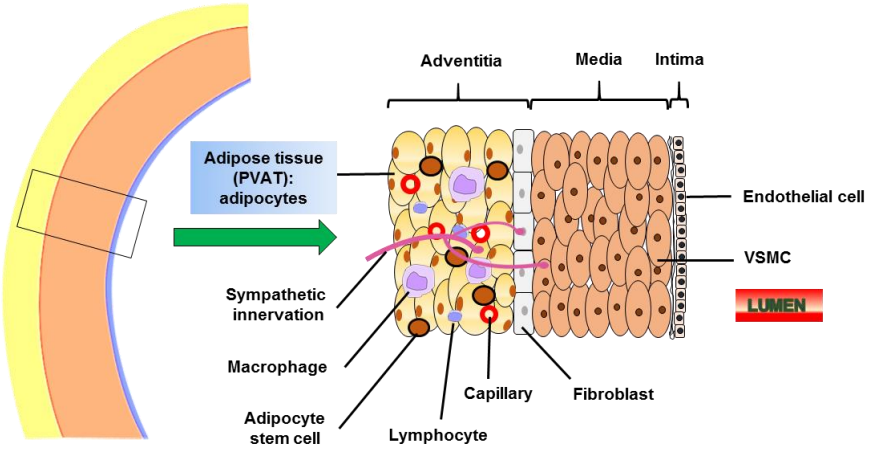


Figure 2.1 Section of a blood vessel wall. Vascular smooth muscle cells (VSMCs) constitute the tunica media, while perivascular adipose tissue (PVAT) in the adventitia contains adipocytes and other cells (macrophages, lymphocytes, adipocyte stem-cells and fibroblasts) as well as capillaries and sympathetic nerves.

Hypothesis and aims

Based on the above evidence, the present study hypothesized that perivascular adipose tissue (PVAT) and acute hyperglycaemia may perturb the contractility of porcine coronary arteries (PCAs). The work aimed to determine the influence of PVAT on basal tone and PCA contraction during exposure to high extra-cellular potassium (KCl) and cumulative U46619 (thromboxane A_2 receptor agonist). The study also aimed to determine the effect of acute hyperglycaemia on vasomotor activity of PCAs and the possible underlying mechanisms.

2.2 MATERIALS AND METHODS

2.2.1 CORONARY ARTERY DISSECTION

Porcine coronary arteries (PCAs) were used in this work as they are phenotypically closer to human arteries, compared to those derived from other animal models such as rats, mice and dogs (Swindle *et al.*, 2012). Hearts of pigs (~6 months-3 years of age) were obtained from a local abattoir (G Wood & Sons, Clipstone, Nottinghamshire) and transported on ice to the laboratory. PCA was dissected from the hearts and stored in freshly prepared oxygenated Krebs solution (composition in mM: NaCl 118, KCl 4.8, $MgSO_4$ 1.1, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 12, $CaCl_2$ 1.25) with a pH of 7.4, at 4°C overnight. Next morning, a fine dissection was carried out. Adjacent 5 mm length PCA ring segments and PVAT (0.3 g) (Malinowski *et al.*, 2008) (Figure 2.2) for addition to selected organ baths, derived from the same length of PCA, were isolated in oxygenated Krebs buffer solution. Care was taken to protect the endothelium.

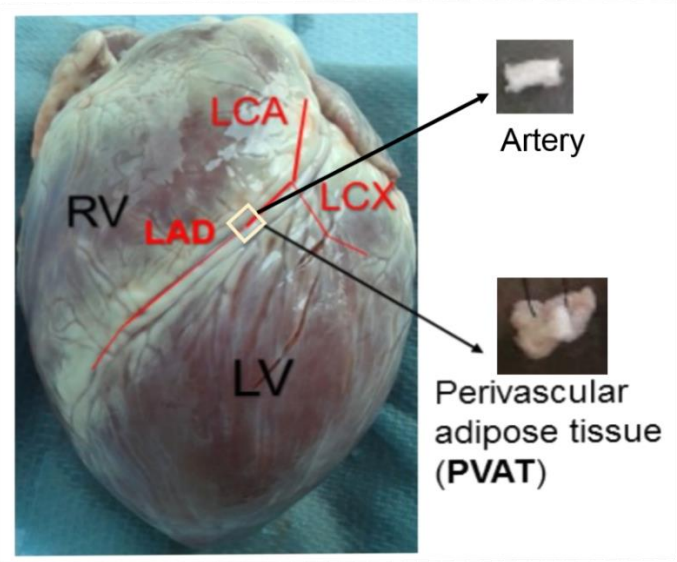
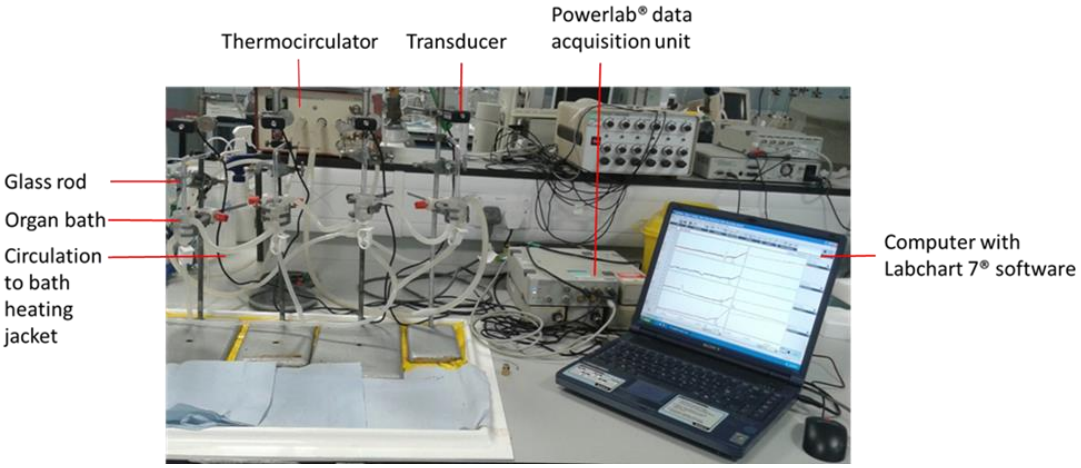


Figure 2.2 A porcine heart showing location of coronary artery and perivascular adipose tissue (PVAT). LCA, left coronary artery; LAD, left anterior descending artery; LCX, left circumflex artery; RV, right ventricle; LV, left ventricle.

2.2.2 ISOMETRIC TENSION STUDIES

In vitro studies were carried out using the isolated PCA segments in an organ bath set up, having a force transducer connected to a PowerLab® data acquisition unit (ADI instruments, LabChart 7® software) (Figure 2.3). After calibration of the unit, the cleaned PCA segments were mounted using stainless steel hooks, in the baths of the set up (Figure 2.3). Each bath contained warmed (37°C) and gassed (95% O_2 and 5% CO_2) Krebs buffer solution (5 ml), having a pH of 7.4 (Rayment *et al.*, 2007).

A)



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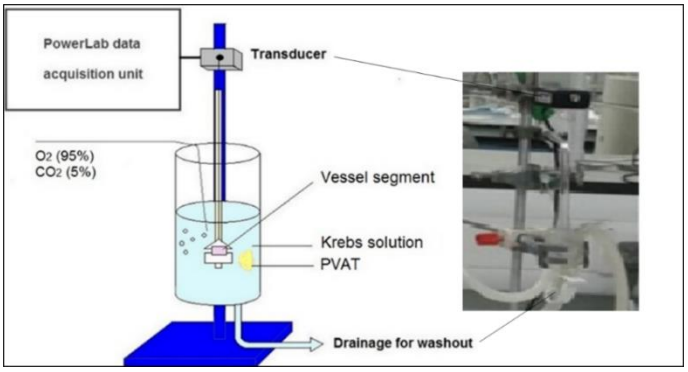

Figure 2.3 (A) An isolated tissue set up, showing four organ baths with ADI® recording equipment. (B) Cleaned vessel segment mounted for isometric tension study in an organ-bath and exposed to perivascular adipose tissue (PVAT) (0.3 g) added to Krebs solution (not to scale).

2.2.2.1 STUDY USING POTASSIUM CHLORIDE

In this study, potassium chloride was used to test the tissue's viability and to establish baseline contractility. The first two baths contained segments from one PCA while the next two baths contained segments from another PCA. At the start of the experiment, the mounted segments were tensioned carefully to 8 g and allowed to relax for 50 minutes. Then, PVAT (0.30 g) from the first PCA was added to the second bath and equal weight of PVAT from the other PCA was added to the fourth bath, the first and third baths served as controls. After 30 minutes' incubation with PVAT, the vessels were contracted with potassium-chloride (KCl) (60 mM) (Gao *et al.*, 2007), which was washed out after 15 minutes with oxygenated Krebs solution and the tissue allowed to relax for 20 minutes. Then, a second concentration of KCl (60 mM) was added to each bath, which was again washed out after 15 minutes. KCl (60 mM) was also used in this manner to establish baseline contractility of PCAs at the beginning of all other functional studies.

2.2.2.2 EFFECTS OF A THROMBOXANE A_2 AGONIST (U46619)

As hyperglycaemia and exposure to PVAT may affect the tissue responses to the thromboxane (TX) A_2 agonist U46619, one group of vessels was exposed to hyperglycaemia (22 mM) (Tune *et al.*, 2002), the other to PVAT alone and the third one to hyperglycaemia (22 mM) and PVAT together. The fourth group (exposed to 12 mM glucose in routinely used Krebs solution in the absence of PVAT) served as the control. One hour (this length of incubation in the hyperglycaemic condition was chosen because studies have shown significant vascular effects of hyperglycaemia manifesting within this period of exposure to high concentrations of glucose) (Yang *et al.*, 2009) later, all groups were exposed to the thromboxane (TX) A_2 agonist U46619 (Daray *et al.*, 2003) in cumulative concentrations (0.1 nM-1 µM).

2.2.2.3 TIME CONTROL EXPERIMENTS WITH U46619 IN THE ABSENCE AND PRESENCE OF PVAT

To assess the maintenance of tone following pre-contraction with U46619, two sets of vessels were used with a group exposed to PVAT while the other group served as control. An hour later, both groups of vessels were contracted with U46619 to achieve pre-contraction. Maintenance of the resulting tone was observed and compared in vessels in the absence and presence of PVAT.

2.2.2.4 ARTERIAL RESPONSES TO ACUTE HYPERGLYCAEMIA (22 mM)

To investigate the effect of hyperglycaemia on baseline arterial tone, the vessels were exposed to varying degrees of acute hyperglycaemia. Three adjacent vessel rings (without PVAT) obtained from a length of PCA were used. The vessels of one group were exposed to 22 mM glucose, while those of another group were exposed to 12 mM glucose plus mannitol 10 mM (osmotic control) (Seto *et al.*, 2013) and the third group were bathed in buffer solution containing 12 mM glucose only (control). The impact on vascular tone was assessed on achieving a stable contraction.

2.2.2.5 ARTERIAL RESPONSES TO ACUTE HYPERGLYCAEMIA (22 mM) IN THE ABSENCE OR PRESENCE OF SUPEROXIDE DISMUTASE

This set of experiments looked at the potential production of superoxide (O_2^-) during acute hyperglycaemia (22 mM) (as the focus here was on the effect of superoxide dismutase (SOD) on hyperglycaemia, mannitol was not used here but in the subsequent experiments). So, three groups of vessels (without PVAT) were used out of which, one group was exposed to 22 mM glucose, another to 22 mM glucose plus superoxide dismutase (SOD) (60 units/ml) (converts O_2^- to H_2O_2, which may lead to decrease in vessel tone) (Ishihara *et al.*, 2008) and the third one was

bathed in buffer solution containing 12 mM glucose only (control). The resulting change in arterial tone was compared in the groups exposed to the different conditions.

2.2.2.6 EFFECT OF MANNITOL IN THE ABSENCE OR PRESENCE OF SUPEROXIDE DISMUTASE

These experiments investigated whether mannitol caused production of O_2^-, hence a group of segments incubated with mannitol were treated with SOD. Three adjacent rings cleaned of PVAT, obtained from a length of PCA were used. The vessels of one group were exposed to mannitol (10 mM) along with glucose (12 mM), while another group was additionally incubated with SOD (60 units/ml). The third group of arterial rings were immersed in buffer solution containing 12 mM glucose only (control). The subsequent change in vessel tone was assessed on reaching a stable contraction.

2.2.2.7 EFFECT OF ACUTE HYPERGLYCAEMIA (22 mM) IN THE ABSENCE AND PRESENCE OF SUPEROXIDE DISMUTASE ALONE OR IN COMBINATION WITH CATALASE

Previous experiments had shown decrease in vessel tone following treatment with SOD of segments incubated with 22 mM glucose but not with mannitol, which may have been due to production of H_2O_2 from O_2^-, so catalase (which converts H_2O_2 to H_2O and O_2, possibly altering vessel tone) was employed in these experiments. Four adjacent cleaned vessel rings obtained from a length of PCA were used. The vessels of one group were exposed to 22 mM glucose, the second group to 22 mM glucose plus SOD (60 units/ml) while the third group was in the additional presence of polyethylene-glycated (PEG)-catalase (1000 units/ml) (Gao *et al.*, 2003). The fourth group was bathed in buffer solution containing 22 mM glucose, SOD (60 units/ml) and Tris-HCl (vehicle used to prepare

PEG-catalase). The effect on vessel tone was assessed on achieving steady-state contraction.

2.2.2.8 RESPONSES OF PORCINE CORONARY ARTERIES ON EXPOSURE TO PVAT

In order to assess the impact of PVAT on basal arterial tone, cleaned PCA segments were exposed to PVAT added to the organ bath buffer solution and the effect on tone compared to that in vessels without PVAT. Hence, two adjacent vessel rings obtained from a length of PCA were used. After equilibration of the vessels and establishment of baseline contractility with KCl (60 mM), the vessel in the second bath was exposed to isolated PVAT (0.3 g) and the subsequent change in vessel tone was assessed on achieving a stable contraction (after at least an hour).

2.2.2.9 EFFECT OF CYCLOOXYGENASE INHIBITION FOLLOWED BY ADDITION OF PVAT ON VESSEL TONE

Previous experiments indicated a consistent increase in arterial tone following exposure to PVAT, suggesting the release of a transferable contractile factor from the adipose tissue. To look into the possibility of the factor being a contractile prostanoid, a single group of arterial segments was exposed to indomethacin (10 μM) for an hour to inhibit the cyclooxygenase (COX) enzyme. Another group containing the vehicle (ethanol) used to prepare indomethacin was not employed so the effect of ethanol cannot be excluded. Then isolated PVAT was added to the buffer solution in the organ-bath. The change in tone was assessed on reaching a stable contraction and compared to the change in arterial tone which had followed exposure to the COX inhibitor.

2.2.2.10 EFFECT OF EXPOSURE TO PVAT IN THE ABSENCE AND PRESENCE OF CYCLOOXYGENASE INHIBITION

To further investigate the possible involvement of a PVAT-derived contractile prostanoid in arterial contractility, three adjacent vessel rings obtained from a length of PCA were used. After assessing baseline contractility of the vessels with KCl (60 mM), the vessels of the third group were exposed to indomethacin (10 µM) for one hour to inhibit the COX enzyme. An additional group exposed to the vehicle used to make indomethacin (ethanol) was not employed so the effect of ethanol cannot be excluded. Following this, isolated PVAT (0.3 g each) was added to the 2nd and 3rd baths and incubated with the vessels for one hour. The arterial segment in the first bath served as a control. The subsequent change in vessel tone was assessed on achieving a stable contraction (after at least an hour).

2.2.2.11 EFFECT OF PVAT IN THE ABSENCE AND PRESENCE OF THROMBOXANE A_2 RECEPTOR INHIBITION

A potential contractile prostanoid released from PVAT may be TXA_2 (Meyer *et al.*, 2013), so an arterial segment in the third bath was exposed to iodophenyl sulfonyl aminopinane (I-SAP) (10 nM) (vehicle control (ethanol) not used) for one hour to block the TXA_2 receptor. Following this, isolated PVAT (0.3 g each) was added to the 2nd and 3rd baths and incubated with the vessels for one hour. The vessel in the first bath served as control. The change in vessel tone was assessed on achieving a stable contraction.

2.2.3 NITROBLUE-TETRAZOLIUM REDUCTION ASSAY TO DETECT SUPEROXIDE ANION IN KREBS SOLUTION

To determine if superoxide was generated in the Krebs buffer solution filling the tissue-bath during hyperglycaemia, nitroblue-tetrazolium (NBT) reduction assay was performed in the absence or presence of coronary artery segments (exposed to 12 mM or 22 mM glucose) (Dehne *et al.*, 2001). Another group of vessel segments was exposed to the osmotic control (12 mM glucose plus 10 mM mannitol). In the presence of superoxide, NBT is reduced to monoformazan (NBT^+) forming a blue precipitate insoluble in aqueous solution (Goto *et al.*, 2004). To detect the amount of superoxide formed in the Krebs solution, the absorbance of the buffer solution was measured using a SpectraMax M2e microplate reader (Molecular Devices, Wokingham, Berkshire, UK). NBT (1 mg/ml) was dissolved in the Krebs solution, maintained at 37°C in the tissue-bath with continuous gassing (95% O_2, 5% CO_2) for 4 hours (duration of the pharmacological response). 200 µl of the solution was then collected from the tissue-bath chambers, placed into a 96-well micro plate and the absorbance read at 560 nm.

2.2.4 CHEMICALS

Potassium chloride (KCl), D-glucose (Fisher Scientific, Loughborough, UK); D-mannitol, superoxide dismutase (SOD), polyethylene-glycated (PEG)-catalase, nitroblue tetrazolium (NBT), indomethacin (a cyclooxygenase inhibitor) (all purchased from Sigma-Aldrich, St. Louis, MO, USA); U46619 (a thromboxane A_2 receptor agonist) (Tocris Bioscience, Bio-Techne Ltd, Abingdon, UK), iodophenyl sulphonyl aminopinane (ISAP) (TXA_2 receptor antagonist) (Cayman Chemical, Ann Arbor, MI, USA) were used in the study.

Stock solutions of KCl, D-glucose, D-mannitol, SOD and NBT were made in distilled-water while PEG-catalase was dissolved in Tris-HCl (Sigma-

Aldrich, St. Luis, MO, USA). Stock solution of U46619 was made in methanol and its subsequent dilutions were made in distilled water. Stock solutions of indomethacin and ISAP were made in ethanol, whose final bath concentration was 0.1% v/v.

2.2.5 STATISTICAL ANALYSIS

Data are expressed as mean with standard error of the mean (S.E.M) change in tension (in grams or %) or absorbance. The 'n' in the results indicates the number of individual animals. The collected data were analysed by unpaired, two-tailed, Student's *t*-test for comparison of two groups and analysis of variance (ANOVA) with Bonferroni's *post hoc* test for comparison of three or more groups using GraphPad Prism® version 7 (La Jolla, CA, USA). A p value of <0.05 was considered statistically significant.

2.3 RESULTS

2.3.1 EFFECT OF EXTRACELLULAR POTASSIUM CHLORIDE

Initially, the effect of extracellular potassium chloride (KCl) (60 mM) added to the buffer solution in the organ-baths, on cleaned porcine coronary arteries was examined. This showed that the resulting contractile responses in the segments in the presence of perivascular adipose tissue (PVAT) (0.3 g) (added to Krebs solution) were significantly reduced (mean ± standard error of the mean (S.E.M) contraction: 8.5 ± 0.5 g; n = 37) compared to responses in the segments without PVAT (controls) (mean ± S.E.M contraction: 10 ± 0.5 g; n = 37), $p<0.05$ (unpaired, two-tailed Student's *t*-test) (Figure 2.4).

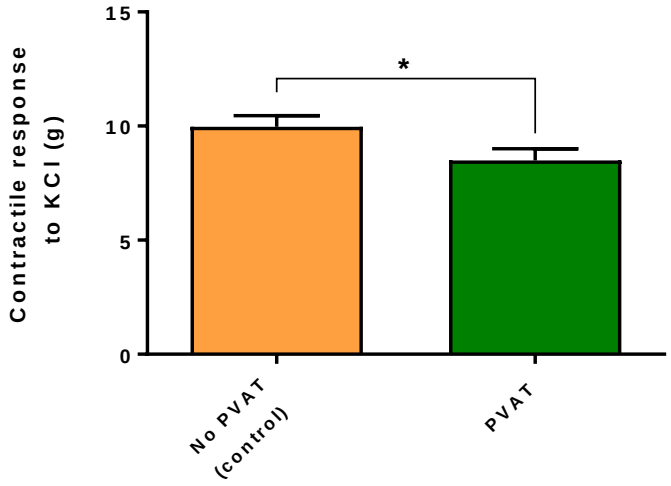

Figure 2.4 Contractile effects of 60 mM potassium chloride (KCl) in porcine coronary arterial segments, with and without perivascular adipose tissue (PVAT). The contractile responses in the segments with PVAT were significantly reduced compared to responses in the segments without PVAT. Data are expressed as mean ± standard error of the mean (S.E.M) contractile responses of 37 experiments, in grams (g), following administration of the second concentration of KCl (60 mM). *p<0.05. Error bars represent S.E.M.

2.3.2 Effects of a thromboxane A_2 agonist (U46619)

2.3.2.1 Responses of porcine coronary arteries in the presence or absence of PVAT

After establishing baseline contractility of the vessels with 60 mM KCl, one group was exposed to PVAT (0.3 g) for an hour. The vessels were then contracted with U46619 in cumulative concentrations (0.1nM-1µM). The response of vessels at 30 nM concentration of U46619 in the presence of PVAT was significantly enhanced compared to the control group (mean ± S.E.M difference between the groups: 18.7 ± 7.9) ($p<0.01$, two-way ANOVA with Bonferroni's *post hoc* test), n=7 (Figure 2.5).

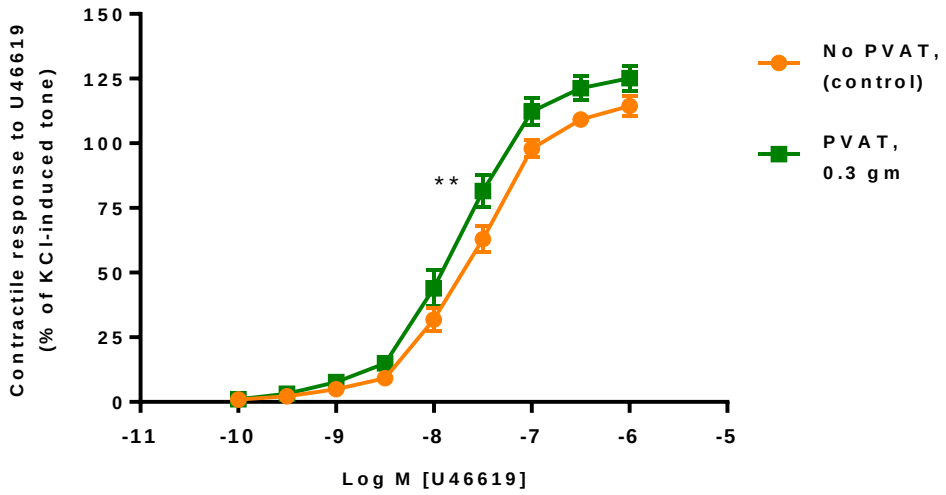

Figure 2.5 Cumulative concentration (0.1nM-1µM)-responses of porcine coronary arterial segments to U46619 with and without perivascular adipose tissue (PVAT). The contractile responses of the segments with PVAT were significantly increased at 30 nM concentration of U46619 compared to responses of the segments without PVAT. Data are expressed as mean ± standard error of the mean (S.E.M) responses of 7 experiments, in % of the KCl response. **p<0.01. Bars represent S.E.M.

2.3.2.2 RESPONSES OF PORCINE CORONARY ARTERIES EXPOSED TO HYPERGLYCAEMIA (22 mM)

After assessing baseline contractility, one group of vessels was exposed to Krebs solution containing 22 mM glucose and incubated for at least one hour. The tissue was then contracted with U46619 in cumulative concentrations (0.1nM-1µM). There was no significant difference between the responses in the group exposed to 22 mM glucose compared to the controls (glucose 12 mM) (n=7) (two-way ANOVA with Bonferroni's *post hoc*-test) (Figure 2.6).

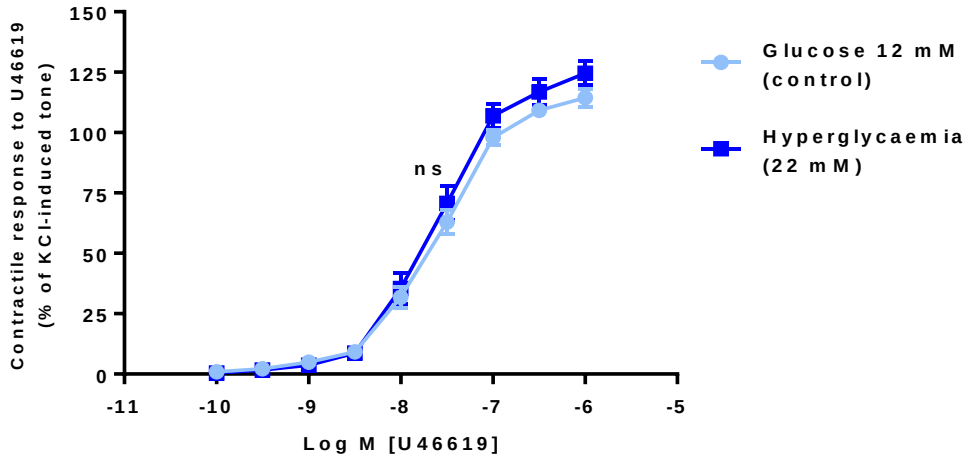

Figure 2.6 Cumulative concentration (0.1nM-1µM) - responses of porcine coronary arterial segments to U46619 exposed to acute hyperglycaemia (22 mM) compared to the control group (exposed to glucose 12 mM). The contractile responses of the segments of the hyperglycaemia (22 mM) group were not significantly different to responses of the control group. Data are expressed as mean ± standard error of the mean (S.E.M) contractile responses of 7 experiments, in % of the response to KCl. Not significant: ns. Bars represent S.E.M.

2.3.2.3 RESPONSES OF PORCINE CORONARY ARTERIES EXPOSED TO PVAT ALONE OR PVAT WITH HYPERGLYCAEMIA (22 mM)

A group of vessels was incubated with PVAT (0.3 g) alone while another group was exposed to PVAT (0.3 g) and acute hyperglycaemia (22 mM) for at least one hour. The tissue was then contracted with cumulative increments of U46619 (0.1nM-1µM). There was no significant difference between the responses in the hyperglycaemia/PVAT group compared to the vessels exposed to PVAT alone, n=7 (two-way ANOVA followed by Bonferroni's *post hoc*-test) (Figure 2.7).

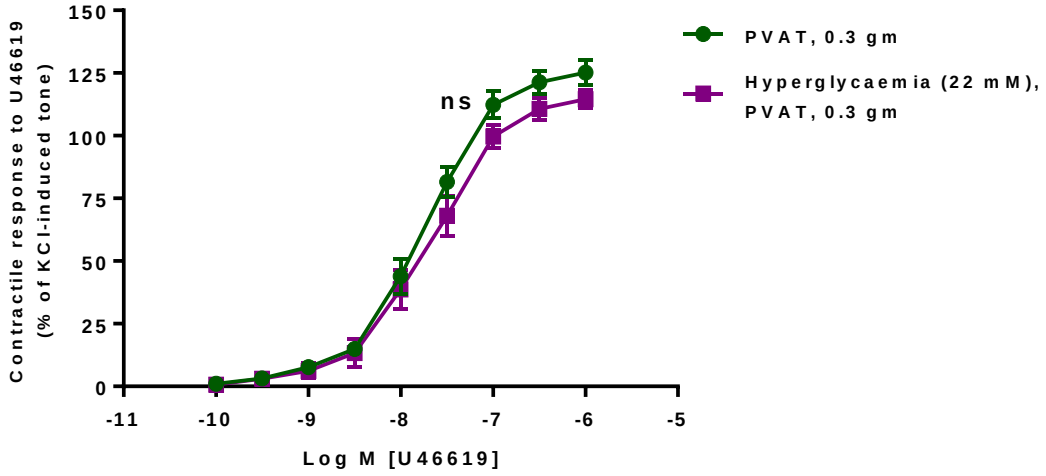

Figure 2.7 Cumulative concentration (0.1nM-1µM)-responses of porcine coronary arterial segments to U46619 exposed to perivascular adipose tissue (PVAT) (0.3 g) in the absence or presence of acute hyperglycaemia (22 mM) compared to the control group. The contractile responses of the segments of the PVAT/hyperglycaemia group were not significantly different to responses of the control group. Data are expressed as mean ± standard error of the mean (S.E.M) contractile responses of 7 experiments, in % of the KCl response. Not significant: ns. Bars represent S.E.M.

2.3.3 TIME CONTROL EXPERIMENTS WITH U46619

After reaching pre-contraction with U46619, the maintenance of vessel tone (in g tension) was assessed for an hour in the absence and presence of PVAT (Figure 2.8). There was no significant difference between the responses in the controls compared to the vessels exposed to PVAT at 120 minutes (Mean ± SEM contraction = 4.5 ± 0.3 g versus 4.4 ± 0.3 g respectively, n=9; unpaired, two-tailed, Student's *t*-test) and at 180 minutes (Mean ± SEM contraction = 4.5 ± 0.3 g versus 4.7 ± 0.4 g respectively, n = 9; unpaired, two-tailed, Student's *t*-test) and tone was consistently maintained over one hour (Figure 2.9).

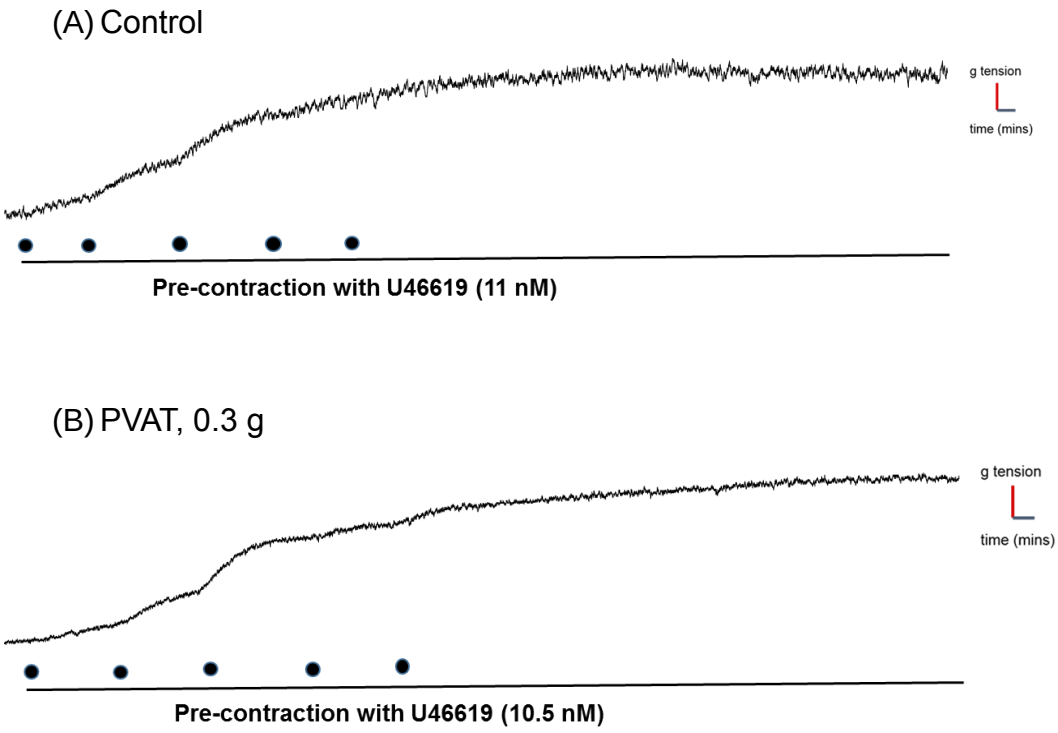

Figure 2.8 Pre-contraction of porcine coronary arteries induced by U46619 in the absence (A) and presence (B) of perivascular adipose tissue (PVAT). The subsequent maintenance of vessel tone was assessed over one hour.

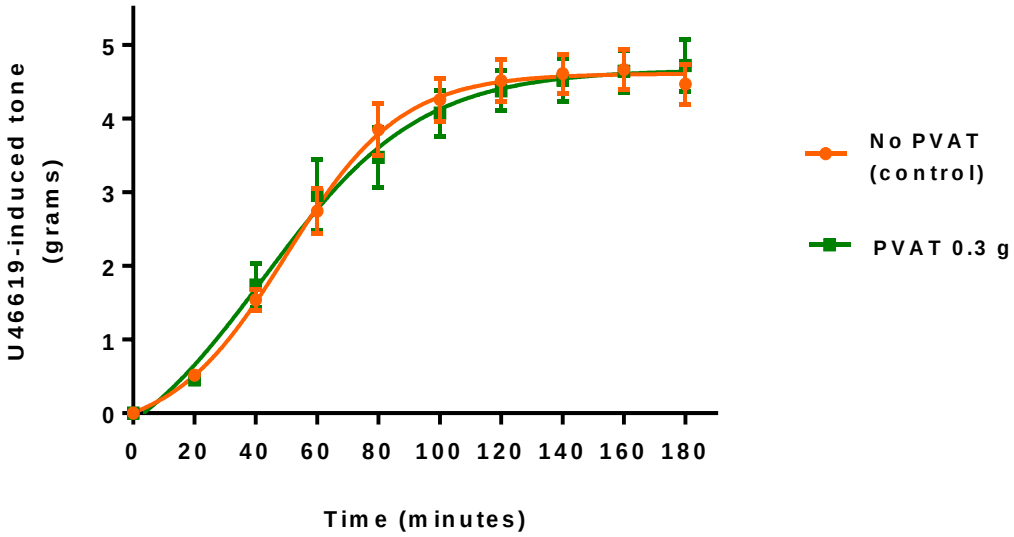

Figure 2.9 Pre-contraction of porcine coronary arterial segments to U46619 in the absence or presence of perivascular adipose tissue (PVAT) (0.3 g). The contractile responses of the two groups were not significantly different and tone was maintained for at least an hour after achieving pre-contraction. Data are expressed as mean ± standard error of the mean (S.E.M) contractile responses of 9 experiments, in grams (g) tension. Bars represent S.E.M.

2.3.4 EFFECTS OF ACUTE HYPERGLYCAEMIA ON CONTRACTILITY OF PORCINE CORONARY ARTERIES

Exposure of porcine coronary arteries (PCAs) without PVAT to acute hyperglycaemia (22 mM) and osmotic controls (mannitol 10 mM, glucose 12 mM) caused an increase in basal tone compared to the controls (exposed to 12 mM glucose alone). Analysis showed significantly increased contractile responses in vessels exposed to 22 mM glucose compared to controls (% mean ± S.E.M contraction: 7.6 ± 1.5 versus 2.8 ± 0.2 respectively), n=6, $p<0.05$, one-way ANOVA with Bonferroni's *post hoc* test (Figure 2.10). However the responses in arteries incubated with the osmotic control (mannitol 10 mM, glucose 12 mM) was similar to that in the hyperglycaemia (22 mM) group (% mean ± S.E.M. contraction: 6.9 ± 1 versus 7.6 ± 1.5 respectively), n=6, ns (not significant), one-way ANOVA with Bonferroni's *post hoc* test (Figure 2.10).

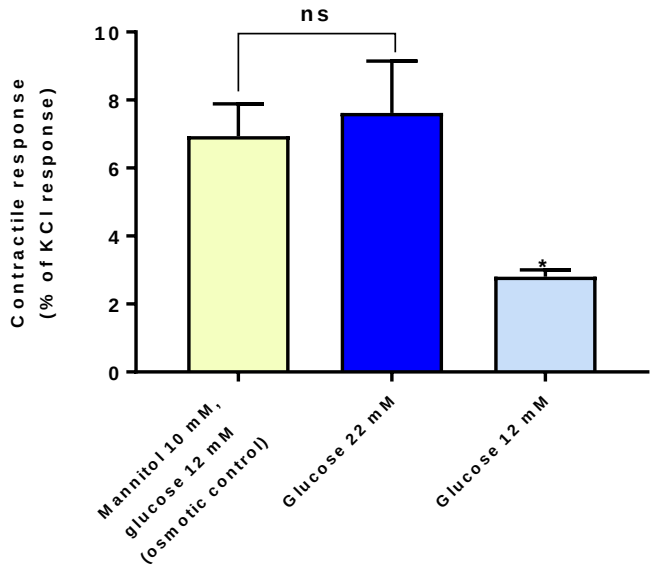

Figure 2.10 Contractile responses in porcine coronary arteries (without PVAT) following incubation with glucose (22 mM), osmotic control (mannitol 10 mM, glucose 12 mM) and control (glucose 12 mM). The responses to glucose (22 mM) and osmotic control were similarly increased compared to the control group. Data are expressed as mean ±

standard error of the mean (S.E.M) % contractile responses of 6 experiments. The error bars represent S.E.M. *p<0.05 ns: not significant.

2.3.5 EFFECTS OF HYPERGLYCAEMIA IN THE ABSENCE OR PRESENCE OF SUPEROXIDE DISMUTASE

Consistently, exposure of porcine coronary arteries (without PVAT) to glucose (22 mM) caused significantly increased contractile responses compared to controls (% mean ± S.E.M. contraction: 8.7 ± 1.3 versus 2 ± 0.2 respectively), n=6, p<0.05, one way ANOVA with Bonferroni's *post hoc* test (Figure 2.11). Interestingly, addition of superoxide dismutase (SOD) (60 units (u)/ml) led to significant decrease in the hyperglycaemia-induced vascular contractions (% mean ± S.E.M. contraction: 3.9 ± 0.7 versus 8.7 ± 1.3 respectively), n=6, p<0.05, one-way ANOVA with Bonferroni's *post hoc* test (Figure 2.11).

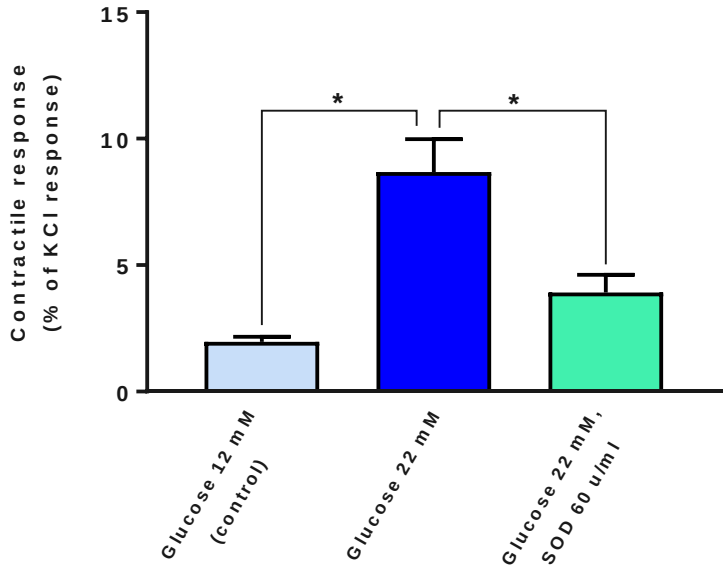

Figure 2.11 Contractile responses in porcine coronary arteries (without PVAT) following incubation with glucose (22 mM), glucose 22 mM in the presence of superoxide dismutase (SOD) 60 units (u)/ml and control (glucose 12 mM). The response to glucose (22 mM) was significantly attenuated in the presence of SOD. Data are expressed as mean ±

standard error of the mean (S.E.M) contractile responses of 6 experiments, in % of KCl-induced contraction. The error bars represent S.E.M. *p<0.05.

2.3.6 MANNITOL-INDUCED ARTERIAL CONTRACTILITY IN THE ABSENCE OR PRESENCE OF SOD

Incubation of porcine coronary arteries (without PVAT) with mannitol (10 mM) and glucose (12 mM) caused significantly increased contractile responses compared to controls (% mean ± S.E.M. contraction: 9.8 ± 0.8 versus 2.7 ± 0.5 respectively), n=5, p<0.05, one-way ANOVA with Bonferroni's *post hoc* test (Figure 2.12). However, addition of SOD (60 u/ml) did not affect the vascular contractions provoked by the combination of mannitol (10 mM) and glucose (12 mM) compared to the effect of mannitol (10 mM) and glucose (12 mM) in the absence of SOD (% mean ± S.E.M. contraction: 10.3 ± 1.3 versus 9.8 ± 0.8 respectively), n=5, ns (not significant), one-way ANOVA with Bonferroni's *post hoc* test (Figure 2.12).

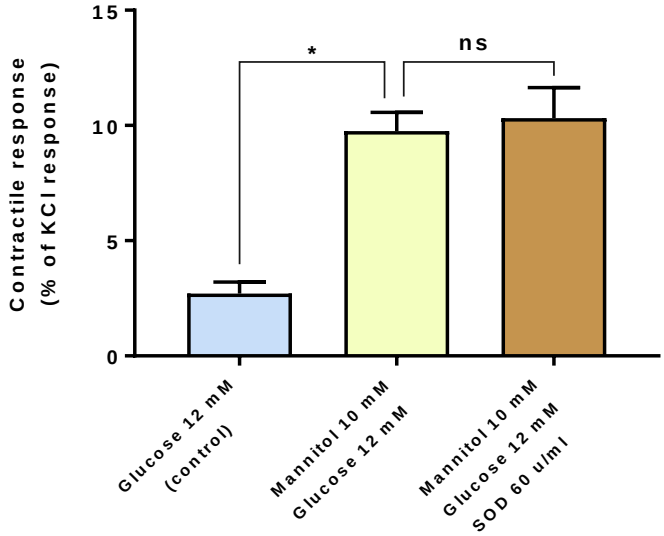

Figure 2.12 Contractile responses in porcine coronary arteries (without PVAT) following incubation with mannitol (10 mM) and glucose (12 mM) in the absence and presence of superoxide dismutase (SOD) 60 u/ml or

glucose 12 mM alone (control). The response to mannitol (10 mM) and glucose (12 mM) was not affected by the presence of SOD. Data are expressed as mean ± standard error of the mean (S.E.M) contractile responses of 5 experiments, in % of KCl-induced contraction. The error bars represent S.E.M. *p<0.05. Not significant: ns.

2.3.7 Responses to hyperglycaemia in the absence or presence of SOD and/or PEG-catalase

The contraction of porcine coronary arteries (without PVAT) on incubation with glucose (22 mM) was significantly decreased following exposure to SOD (60 u/ml) (% mean ± S.E.M. contraction: 8.7 ± 1.3 versus 3.9 ± 0.7 respectively), n=6, p<0.05, one-way ANOVA with Bonferroni's *post hoc* test (Figure 2.13). Interestingly, during the additional presence of PEG-catalase (1000 u/ml), the SOD-induced inhibition of hyperglycaemia-evoked contraction was partially reduced and no significant difference in contraction remained compared to that induced by hyperglycaemia alone (% mean ± S.E.M. contraction: 5.5 ± 0.7 versus 8.7 ± 1.3 respectively), n=6, not significant (ns), one-way ANOVA with Bonferroni's *post hoc* test (Figure 2.13). Moreover, Tris-HCl (vehicle of PEG-catalase) had no effect on reduction of hyperglycaemia-evoked contraction by SOD (Figure 2.13).

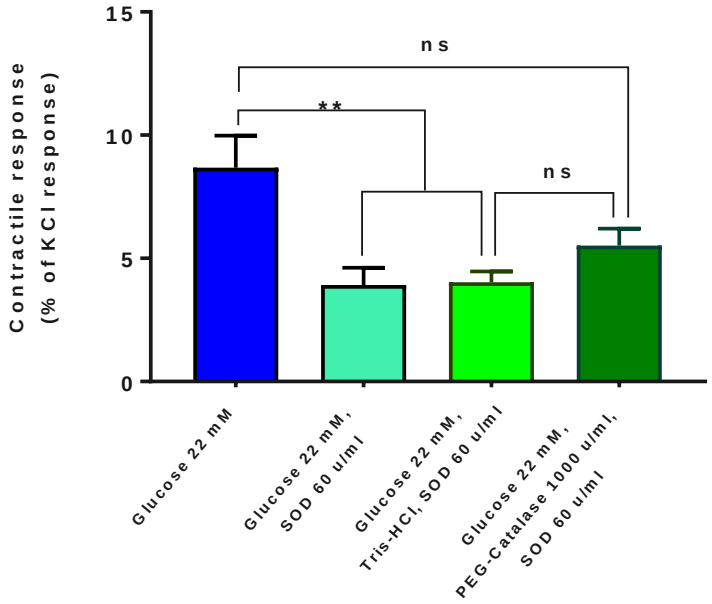

Figure 2.13 Contractile responses in porcine coronary arteries (without PVAT) following incubation with glucose (22 mM) in the absence and presence of superoxide dismutase (SOD) 60 u/ml and/or PEG-catalase (1000 u/ml). The response to glucose (22 mM) was significantly reduced by SOD. This effect was partially ameliorated with the additional presence of PEG-catalase but not with Tris-HCl (vehicle of PEG-catalase). Data are expressed as mean ± standard error of the mean (S.E.M) responses of 6 experiments, in % of KCl response. The error bars represent S.E.M. **p<0.01. Not significant: ns.

2.3.8 SUPEROXIDE DETECTION (NITROBLUE-TETRAZOLIUM REDUCTION ASSAY) IN PHYSIOLOGICAL BUFFER SOLUTION

The presence of superoxide anion (O_2^-) was determined in the physiological buffer solution incubated with PCAs (without PVAT) in triplicate. Nitroblue-tetrazolium (NBT) was reduced to formazan in the presence of O_2^-, which stained the PCA segments blue-black (Figure 2.14) at the end of the experiment. The optical density (O.D) of the buffer solution was significantly increased in the organ baths containing PCA segments exposed to hyperglycaemia (12 mM & 22 mM), compared to

the control group without PCA ($p<0.05$, n=5, one-way ANOVA with Bonferroni's *post hoc* test) (Figure 2.14).

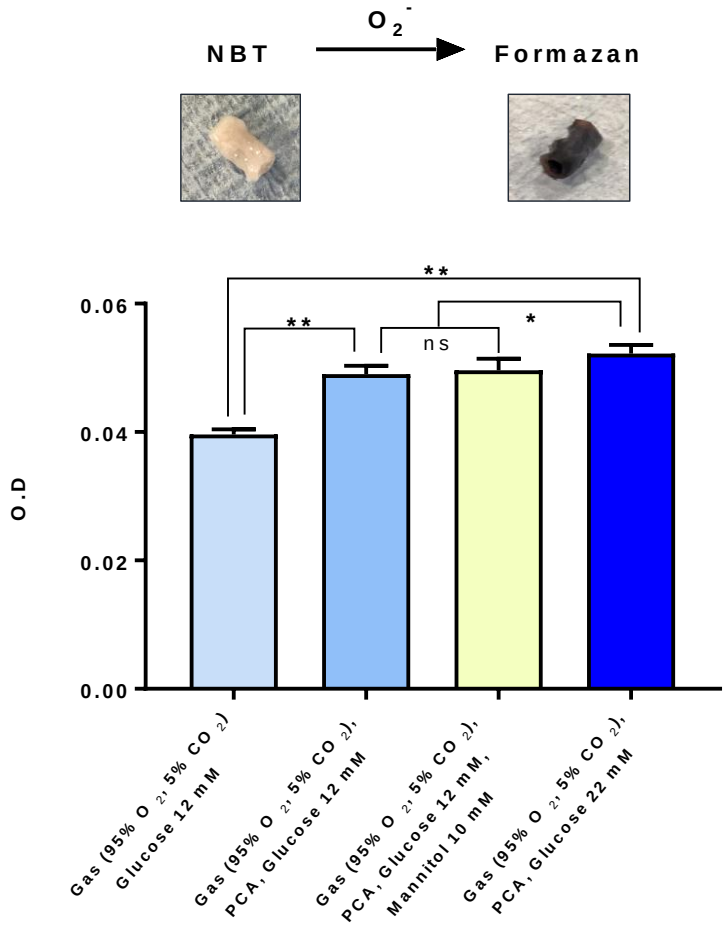

Figure 2.14 Optical density (O.D) of physiologic buffer solution gassed with 95% O_2 and 5% CO_2 during the absence or presence of porcine coronary arteries (PCAs) (without PVAT) exposed to either 12 mM glucose alone, 12 mM glucose plus 10 mM mannitol or 22 mM glucose alone. The O.D of the buffer solution containing PCAs exposed to hyperglycaemia (12 mM & 22 mM) was significantly increased compared to the control group without PCAs. The PCA segments were stained blue-black at the end of the experiment. Data are expressed as mean ± standard error of the mean (S.E.M) optical-density (O.D) of buffer solution obtained from 5 experiments. Nitroblue-tetrazolium: NBT. Superoxide: O_2^-. The error bars represent S.E.M. *$p<0.05$, **$p<0.01$.

2.3.9 Effect of PVAT on contractility of porcine coronary arteries

After establishing the baseline effect of KCl on cleaned porcine coronary arteries, addition of PVAT (0.3 g) to the buffer solution bathing one group of vessels led to a significantly increased tone compared to the controls (% mean ± S.E.M contraction: 10.9 ± 1.1 versus 1.7 ± 0.1 respectively; n = 7), $p<0.05$ (paired, two-tailed Student's *t*-test) (Figure 2.15).

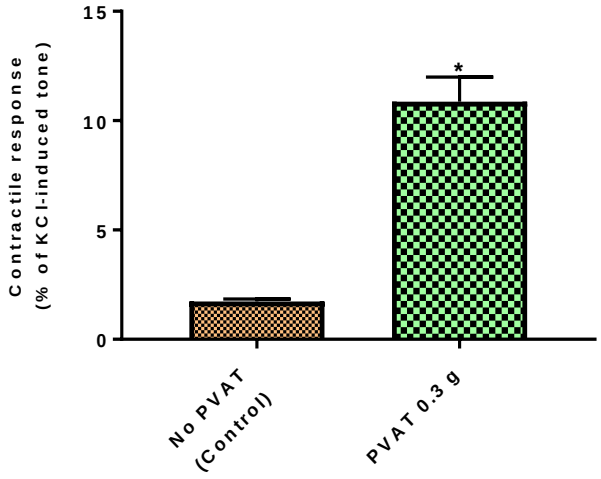

Figure 2.15 Effect of exposure to perivascular adipose tissue (PVAT) (0.3 g) on tone of porcine coronary arteries (PCAs). There was significant contraction of PCAs incubated with PVAT. Data are expressed as mean ± standard error of the mean (S.E.M) contractile responses of 7 experiments in % of KCl-induced PCA contraction. *p<0.05. Error bars represent S.E.M.

2.3.10 Impact of cyclooxygenase inhibition followed by exposure of vessels to PVAT

Incubation of porcine coronary arteries (PCAs) with the cyclooxygenase inhibitor indomethacin (10 µM) inhibited basal PCA tone (% mean ± S.E.M contraction= - 3.9 ± 0.5) which was reversed by addition of PVAT

(0.3 g) (% mean ± S.E.M contraction= 3.2 ± 0.4), n = 8, p<0.05 (paired, two-tailed Student's *t*-test) (Figures 2.16, 2.17).

Figure 2.16 Responses (in g tension) of porcine coronary arteries to indomethacin (Indo) (10 µM) followed by exposure to perivascular adipose tissue (PVAT), 0.3 grams (g). Minutes: mins.

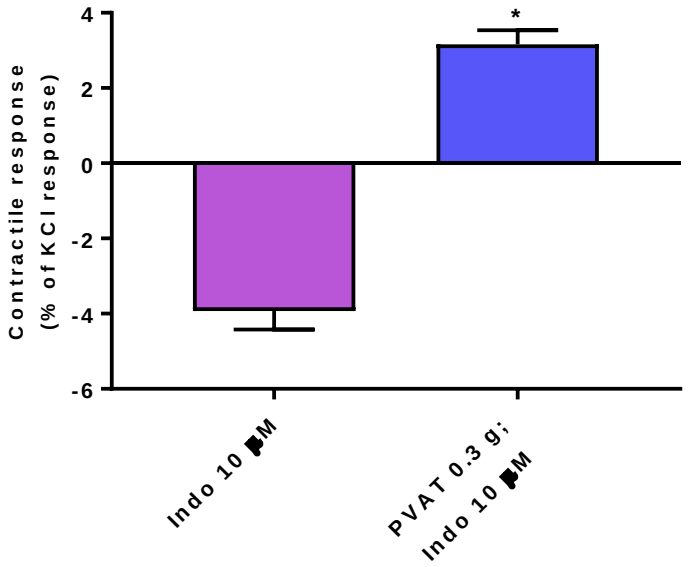

Figure 2.17 Effect of exposure to indomethacin (Indo) (10 µM) followed by perivascular adipose tissue (PVAT) (0.3 g) on tone of porcine coronary arteries (PCAs). Indo caused a decrease in basal tone which was reversed during the additional presence of PVAT. Data are expressed as mean ± standard error of the mean (S.E.M) contractile responses of 8 experiments in % of the KCl response. *p<0.05. Error bars represent S.E.M.

2.3.11 Effects of PVAT on arterial contractility in the absence or presence of a cyclooxygenase inhibitor

Exposure of arteries to PVAT (0.3 g) caused a significant contractile response compared to the controls (% mean ± S.E.M contraction: 13 ± 1.6 versus 1.8 ±0.1 respectively, n = 8), $p<0.05$ (one-way ANOVA with Bonferroni's *post hoc* test), which was attenuated during cyclooxygenase inhibition by indomethacin (% mean ± S.E.M contraction: 3.2 ± 0.4; n = 8), $p<0.05$ (one-way ANOVA followed by Bonferroni's *post hoc* test) (Figure 2.18).

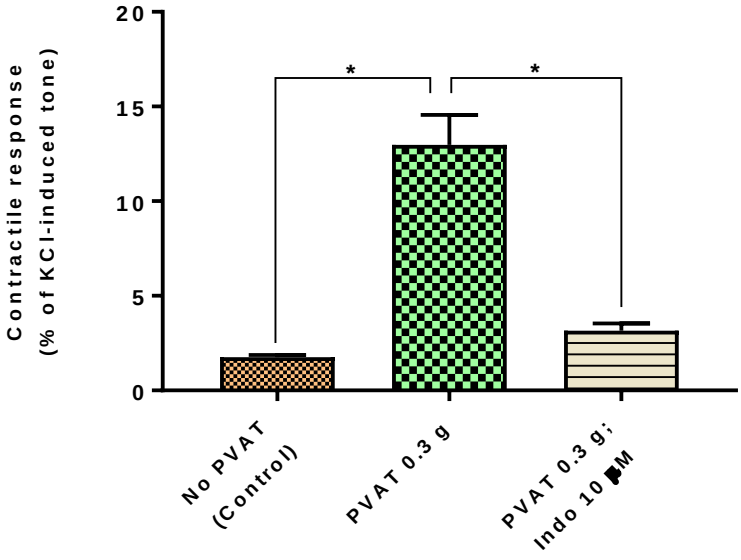

Figure 2.18 Effect of exposure to perivascular adipose tissue (PVAT) (0.3 g) on tone of porcine coronary arteries (PCAs) in the absence or presence of indomethacin (Indo) (cyclooxygenase inhibitor). There was significant contraction of PCAs incubated with PVAT, which was attenuated during cyclooxygenase inhibition. Data are expressed as mean ± standard error of the mean (S.E.M) contractile responses of 8 experiments in % of KCl-induced PCA contraction. *$p<0.05$. Error bars represent S.E.M.

2.3.12 Effects of PVAT on vessel contractility in the absence or presence of a thromboxane A_2 receptor antagonist

Exposure of vessels to PVAT (0.3 g) caused a significant contraction compared to the controls (% mean ± S.E.M contraction: 9.3 ± 1.3 versus 1.6 ± 0.1 respectively; n = 7), $p<0.05$ (one-way ANOVA with Bonferroni's *post hoc* test), which was attenuated in the presence of a thromboxane A_2 receptor antagonist (iodophenyl sulfonyl aminopinane) (I-SAP) (% mean ± S.E.M contraction: 4.9 ± 1; n = 7), $p<0.05$ (one-way ANOVA followed by Bonferroni's *post hoc* test) (Figure 2.19).

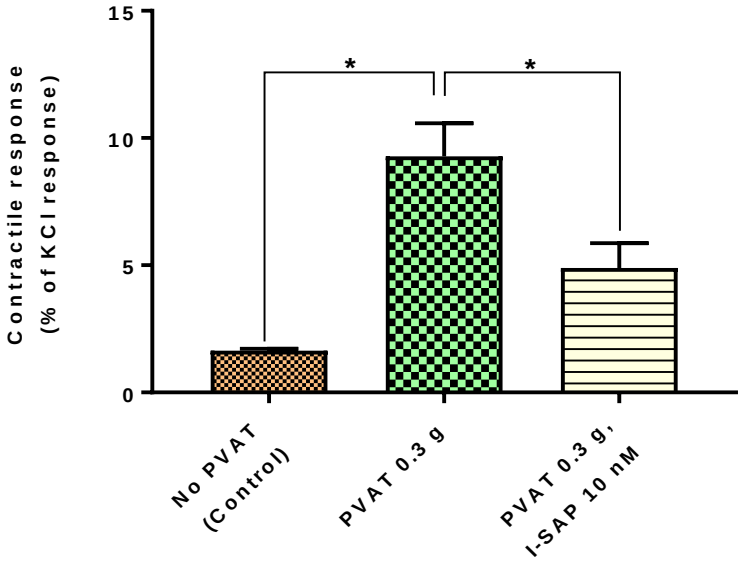

Figure 2.19 Effect of exposure to perivascular adipose tissue (PVAT) (0.3 g) on tone of porcine coronary arteries (PCAs) in the absence or presence of iodophenyl sulfonyl aminopinane (I-SAP) (10 nM) (a thromboxane A_2 receptor antagonist). There was a pronounced contraction of PCAs incubated with PVAT, which was significantly reduced during inhibition of the thromboxane A_2 receptor. Data are expressed as mean ± standard error of the mean (S.E.M) contractile responses of 7 experiments in % of the KCl response. *p<0.05. Error bars represent S.E.M.

2.4 DISCUSSION

Perivascular adipose tissue (PVAT), the exterior covering layer of most blood vessels, is receiving increasing interest as a paracrine modulator of vascular function (Gollasch *et al*., 2004). Moreover, diabetes is associated with vascular dysfunction including that of the coronary arteries (Hien *et al.*, 2016). Therefore, this study aimed to determine the effects of PVAT and hyperglycaemia on function of porcine coronary arteries (PCAs).

2.4.1 EFFECTS OF PVAT ON CONTRACTILITY OF PORCINE CORONARY ARTERY

This study showed that in PCAs with PVAT, the contractile responses to potassium chloride (KCl) (60 mM) were significantly reduced compared to the responses in PCAs without PVAT, which may be due to the release of a relaxant factor from the PVAT. Other investigators have also shown that adipocytes release a vasoactive relaxant factor (Lee *et al.*, 2009; Ozen *et al.*, 2013). A study comparing the contractile responses to KCl (60 mM) in rat aortic rings with and without intact PVAT showed a slower onset of contraction in segments with PVAT possibly due to the release of a relaxant factor from adipocytes, however the maximum tension achieved was similar in the two groups likely due to the high extracellular potassium overriding the effect of the PVAT-derived relaxant factor (Gao *et al.*, 2006). In contrast, experiments performed in rat aortae showed that the contraction to 60 mM KCl in segments with and without PVAT were not significantly different (Lohn *et al.*, 2002). The contrasting results may be due to these studies having been conducted in the aortae in rats whereas the present work was performed in coronary vessels of pig. It has been reported that PVAT in different vascular beds and species may have phenotypic differences and different adipokines, potentially altering their effects on the underlying vessel (Kennedy *et al.*, 2017).

In the present study, the contractile responses to U46619 were enhanced in the vascular segments exposed to PVAT compared to those without PVAT. This may have been due to the release of a contractile factor from PVAT, which increased the contractile effect of the TXA_2 agonist. In contrast, a study on rat mesenteric arteries with intact PVAT showed attenuation of the contractile responses to U46619. This effect was lost in the mesenteric arteries of rats who were given a high fat diet and who subsequently developed obesity (Zaborska *et al*., 2015). However the vessels in that study had intact PVAT, while the vessels in the present study had the PVAT excised and added separately to the organ baths, to exclude the possible effect of PVAT in physically impeding the diffusion of compounds into the tissue (Brown *et al.*, 2014).

In the present study, COX inhibition of cleaned PCAs led to a significant decrease in basal arterial tone. As suggested by other researchers, a contractile prostanoid originating from the endothelium may have contributed to basal tone (Ellinsworth *et al.*, 2014). The diminished basal tone which followed COX inhibition was reversed on exposure of the vessels to PVAT indicating the release of a contractile factor from PVAT (Payne *et al.*, 2012). Further experiments in the present work showed significant enhancement of tone following exposure of cleaned PCAs to PVAT. This contractile effect of PVAT was significantly diminished during inhibition of COX suggesting that a contractile prostanoid emanating from PVAT may be involved in contraction of vascular smooth muscle cells. Moreover, the PVAT-induced arterial contraction was diminished while the TXA_2 receptor was inhibited, revealing that the contractile prostanoid involved may be TXA_2 (Figure 2.20).

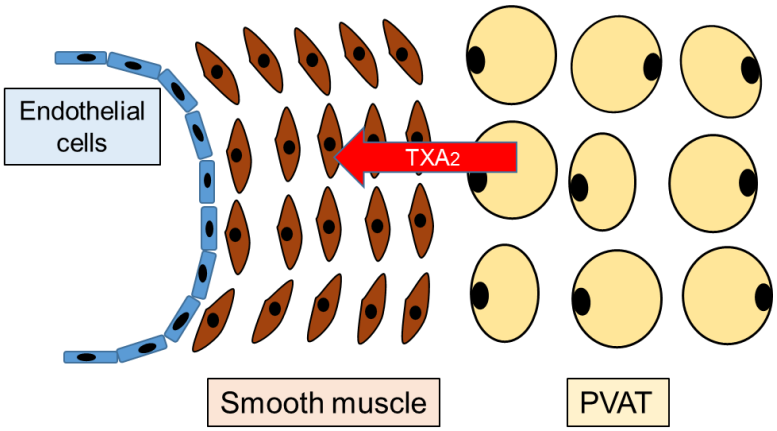

Figure 2.20 Postulated release of thromboxane A_2 (TXA_2) from perivascular adipose tissue (PVAT) of porcine coronary artery which may have contributed to constriction of the neighbouring smooth muscle.

Other reports also indicated the release of contractile factors from PVAT (Brown *et al.*, 2014). Investigations in aortae of obese male mice showed that PVAT had a contractile effect which was prevented by COX inhibition and the levels of TXA_2 in PVAT were found to be increased, pointing to TXA_2 as a PVAT-derived contractile factor (Meyer *et al.*, 2013). Experiments in porcine coronary arteries showed a contractile response on exposure of the vessels to PVAT which was inhibited by cyclooxygenase and TXA_2 (TP) receptor inhibition in female animals (Ahmad *et al.*, 2017). Also, this study documented the presence of TXB_2 (a metabolite of TXA_2) as well the TP receptor in PVAT, supporting the findings of earlier studies regarding the production of TXA_2 by PVAT (Ahmad *et al.*, 2017). However, other investigators found evidence of the presence of noradrenaline and adrenaline (Ayala-Lopez *et al.*, 2014; Gu *et al.*, 2013) as well as components of the renin-angiotensin system (RAS) including angiotensinogen and angiotensin II in PVAT (Galvez-Prieto *et al.*, 2008; Gu *et al.*, 2013).

2.4.2 ROLE OF HYPERGLYCAEMIA IN FUNCTION OF PORCINE CORONARY ARTERY

2.4.2.1 OSMOTIC EFFECT OF HYPERGLYCAEMIA

In the present study, exposure of coronary arteries to acute hyperglycaemia (22 mM) in the absence of PVAT caused a significant increase in basal tone which was similar to the effect of osmotic control (mannitol). Both glucose and mannitol are recognised as osmotic agents which may have detrimental effects on adjacent cells when their concentration increases in the extracellular fluid (Otto *et al.*, 2008). Mannitol has been reported to induce constriction of arterioles on the brain surface caused by an increase in local osmolarity (Diringer *et al.*, 2012). Likewise, high glucose concentration in the tissue-baths can increase the osmolarity around the arterial smooth muscle drawing out intracellular fluid, shrinking the smooth muscle cells and so potentially contributing to the contractile effect of hyperglycaemia on (Mager *et al.*, 2000). Hyperosmolarity may also perturb cellular function by inducing oxidative stress, inflammation and impairment of protein synthesis (Burg *et al.*, 2007).

Investigations on isolated murine arteries of different calibre (renal, mesenteric and aorta) demonstrated the vasoconstrictor effect of hyperosmolar glucose which was also present on exposure of the vessels to hyperosmolar sodium chloride and sucrose (Un *et al.*, 2013). During exposure of the vessels to hyperosmolar glucose, the endothelium-dependent relaxations to carbachol were not affected. Moreover, the contractions induced by the hypertonic solutions were markedly decreased on inhibition of the Rho kinase (ROCK) enzyme by the compound Y-27632 suggesting that ROCK was involved in the hypertonicity-induced vasoconstriction. Further evidence from this work showed that hyperosmolar glucose significantly enhanced phosphorylation of the myosin-phosphatase target protein 1, potentially

leading to vasoconstriction (Qiao *et al.*, 2014; Un *et al.*, 2013) which was attenuated by Y-27632, again supporting the role of Rho/ROCK signalling in hyperosmolarity-induced vasoconstriction (Un *et al.*, 2013).

2.4.2.2 INDUCTION OF OXIDATIVE STRESS BY HYPERGLYCAEMIA

In the current study, exposure of the vessels to acute hyperglycaemia (22 mM) led to a significant increase in basal tone of PCAs. Also, acute hyperglycaemia (22 mM) but not mannitol-induced PCA contraction in the absence of PVAT, was significantly inhibited in the presence of SOD [metabolizes superoxide (O_2^-) to hydrogen peroxide (H_2O_2)] while in the additional presence of catalase (converts hydrogen peroxide to water and molecular oxygen) the inhibition of hyperglycaemia-induced PCA contraction was partially reversed. The nitroblue-tetrazolium reduction assay also indicated the presence of O_2^- in the physiological buffer solution incubating PCA rings exposed to 12 & 22 mM glucose. These data suggest that O_2^- was produced during acute hyperglycaemia, which contributed to its contractile effect and which was acted upon by SOD to convert it to H_2O_2 leading to attenuation of vessel contraction (Brodsky *et al.*, 2002).

In the current study, acute hyperglycaemia (22 mM) did not affect the contractile response to U46619. A possible reason could be the short exposure of the tissue to the hyperglycaemic condition (an hour's incubation, followed by cumulative addition of U46619 over the duration of the pharmacological response which lasted about 4 hours). Another study incubated isolated coronary arteries for a longer period (24 hours) in 23 mM glucose, which resulted in impaired vascular K_v channel activity associated with increased vascular O_2^- production (Liu *et al.*, 2001).

In contrast, in a study which used mesenteric, small porcine coronary and human internal thoracic arteries, exposure of tissue to hyperglycaemia (20 mM) for a much shorter period (30 minutes) caused a significantly increased contractile response to U46619 in a cumulative manner

compared to the responses in vessels exposed to lower concentrations of glucose (5, 10 and 15 mM) (Jackson *et al.*, 2016). This study differs from the present work which employed larger coronary arteries and used control porcine coronary arteries treated with 12 mM glucose.

A study conducted on isolated rat aortae reported significantly increased contractility and increased tissue malondialdehyde level (an indicator of lipid peroxidation) in vessels exposed to 22 mM and 44 mM glucose compared to controls (incubated in 11 mM) glucose suggesting a role of oxidative stress in the arterial response to acute hyperglycaemia (Donmez *et al.*, 2014).

It has been reported that there is increased production of O_2^- and other reactive oxygen species (ROS) during hyperglycaemia whose formation is catalysed by the enzyme NOX. The activity of NOX may be increased by raised glucose and free fatty-acid levels accompanying diabetes through protein kinase-C activation (Hink *et al.*, 2001). Also, increased intracellular glucose diverts electrons from the transport-chain in mitochondria towards molecular oxygen, producing O_2^- (Rask-Madsen *et al.*, 2013).

The O_2^- in turn may impair vascular NO bioavailabity, potentially leading to a contractile effect (Li *et al.*, 2011). An *in vitro* study using isolated aortae of mice showed reduced endothelium-dependent vasodilatation in association with increased O_2^- and peroxynitrite ($ONOO^-$) (an oxidation product of NO) levels. Simultaneously, the tissue level of tetrahydrobiopterin (BH_4), an essential co-factor required by endothelial NO synthase (eNOS) for NO production was found to be diminished. Treatment with uric acid (an $ONOO^-$ scavenger) significantly improved aortic endothelium-dependent vasodilatation. It was concluded that ROS may inhibit endothelium-dependent vasodilatation by oxidation of NO (to $ONOO^-$) and BH_4, the later effect uncoupling eNOS towards O_2^- production instead of the manufacture of NO (Laursen *et al.*, 2001).

Another suggested mechanism is upregulation of ‘protein arginine N-methyl transferase’ and arginase enzymes, which shunt L-arginine

towards formation of asymmetric N^G, N^G-dimethylarginine (ADMA), decreasing the availability of the substrate L-arginine required for NO production by eNOS. Moreover, ADMA may compete in a direct fashion with L-arginine for interaction with eNOS, decreasing NO production further (Meziat *et al.*, 2016).

A systematic review and meta-analysis of 39 studies involving 525 healthy and 540 subjects with cardiometabolic disease showed that significant endothelial dysfunction occurred during acute hyperglycaemia compared to euglycaemic controls. This was associated with vascular smooth muscle proliferation and decreased sensitivity to NO. Oxidative stress decreasing NO bioavailability was suggested to be a principle cause of the resulting vascular dysfunction (Loader *et al.*, 2015).

Therefore, it is likely that a combination of the effect of osmotic stress and oxygen-derived free radicals contributed to contraction of PCAs during acute hyperglycaemia (22 mM) (Brocker *et al.*, 2012). The oxidative stress involved vascular production of O_2^- (Figure 2.21).

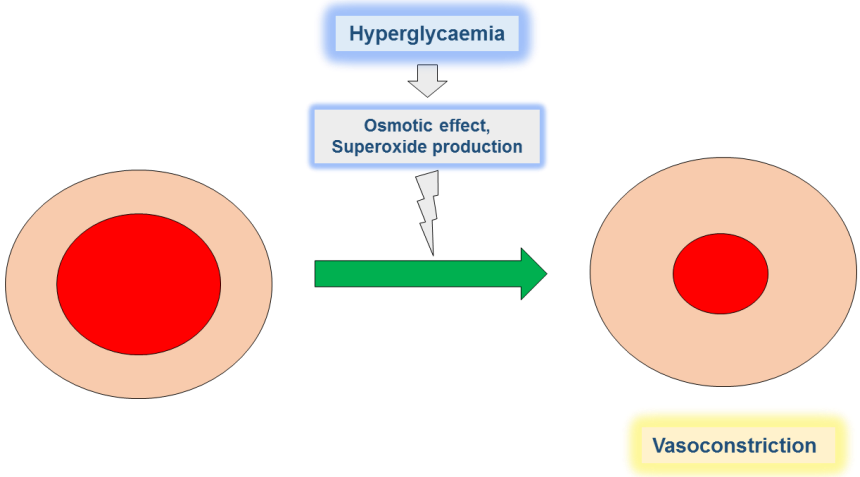

Figure 2.21 Potential effect of acute hyperglycaemia on blood vessels leading to vasoconstriction mediated by osmotic and oxidative stress.

The present study has limitations. The n numbers were small and vehicle (ethanol) control was not used in the study looking at the effects of inhibition of the COX enzyme and TXA_2 receptor on PVAT-induced vascular contraction, so the potential effect of the vehicle on the vessel

responses to these compounds cannot be excluded. However, as reported in chapter 5, ethanol did not have significant effect on vascular contractility.

In the experiments related to hyperglycaemia, the control group of vessels was exposed to a glucose concentration of 12 mM, which was a component of routinely used Krebs buffer solution. This glucose concentration falls in the *in vivo* blood hyperglycaemic range (Svensson *et al.*, 2005), but the additional glucose in the Krebs solution was needed to nourish and sustain the tissue during the duration of the *in vitro* experiment. Other investigators have also used higher glucose content in routinely used Krebs solution for *in vitro* studies (Wong *et al.*, 2014b). Another limitation is that, in the present study, the effects of hyperglycaemia on vascular contractility were investigated but its impact on smooth muscle potassium channels could not be studied.

2.4.3 SUMMARY AND FUTURE DIRECTIONS

In the present study, exposure of cleaned PCAs to PVAT significantly reduced the contractile response to KCl which may have been due to a relaxant factor released by PVAT. There was also evidence of a contractile factor originating from PVAT as incubation of PCAs with PVAT in the tissue-bath led to a significant increase in the response to U46619 and the basal tone. The latter was attenuated during inhibition of the TXA_2 receptor, suggesting that the factor may be TXA_2.

Future studies may investigate the mechanisms underlying this effect by studying the impact of TXA_2 on the membrane potential and K^+ channels of arterial smooth muscles by employing patch-clamp studies (Zavaritskaya *et al.*, 2013). Future work could also include constructing a concentration-response curve to the TXA_2 agonist U46619 in the presence of PVAT after pharmacological inhibition of specific receptors linked to potential contractile factors which could emanate from PVAT e.g. prostanoid or angiotensin II (Gu *et al.*, 2013).

The present study also demonstrated that acute hyperglycaemia (22 mM) in the absence of PVAT increased basal tone of PCAs, possibly via the vascular production of O_2^- as well as by the tissue effect of the hyper-osmolar glucose. Intensive control of glucose levels may prevent these deleterious vascular effects of hyperglycaemia in subjects with diabetes (Rask-Madsen *et al.*, 2013).

In future, the work looking at the effect of hyperglycaemia on arterial tone may be extended to include a control group of vessels exposed to glucose concentration within the normal range (e.g. 5 mM) and to incubate the vessels in high glucose buffer solution for a longer period for better simulation of the hyperglycaemic condition. The mechanism underlying the possible contractile effect of acute hyperglycaemia mediated by osmotic-stress could be investigated by employing an inhibitor of Rho-kinase to see whether it may be involved in the induction of osmotic stress. Studies could also use other assays (e.g. chemiluminescence) to elaborate the ROS which may be involved in mediating the oxidative stress during hyperglycaemia. Furthermore, the mechanisms of the effects of ROS on vascular K^+ channels and endothelial function could be investigated. Further experiments may also investigate the effects of hyperglycaemia and PVAT on the function of other arteries derived from obese and diabetic humans in comparison to healthy controls.

Chapter 3

EFFECT OF PERIVASCULAR ADIPOSE TISSUE ON NITRIC OXIDE-INDUCED RELAXATION OF PORCINE CORONARY ARTERY

3.1 INTRODUCTION

Blood vessels are surrounded by an outer layer of fat termed perivascular adipose tissue (PVAT), which is receiving increasing interest as a paracrine structure with the capacity to regulate vascular function (Szasz *et al.*, 2012). Nitric oxide (NO), a soluble gas continuously produced by endothelial cells has recently been reported to be produced by PVAT (Zaborska *et al.*, 2016). As discussed in chapter 1, evidence from work in isolated aortae (Gao *et al.*, 2007; Triggle *et al.*, 2012) and mesenteric arteries of rats showed a NOS-dependent anti-contractile effect in the presence of PVAT in young animals, whereas in older animals, this protective effect was lacking possibly due to impaired eNOS phosphorylation (Melrose *et al.*, 2015). Direct evidence of NO production in aortic PVAT of mice was reported, in which fluorescence-imaging showed basal production which was enhanced by acetylcholine and was diminished after NOS inhibition. Also, immunohistochemistry showed the expression of eNOS in PVAT. (Xia *et al.*, 2016a).

The protective effect of NO on the vasculature may be compromised if NOS is diverted from its physiological function of producing NO towards manufacture of O_2^- in dysfunctional PVAT seen in obesity and advanced age (Britton *et al.*, 2011). This may be associated with decreased bioavailability of the NOS cofactor BH_4 or its recycling enzyme dihydrofolate reductase (DHFR), reduced L-arginine or increase arginase expression, potentially leading to endothelial dysfunction and cardiovascular disease. Treatment with antioxidants including ascorbic acid and folic acid may be beneficial in this circumstance (Shi *et al.*, 2004) (Xia *et al.*, 2016a).

The above evidence indicates that NO, which performs an essential protective role in the vasculature under physiological conditions, may originate from the endothelium as well as from PVAT.

Hypothesis and aims

The present study hypothesized that PVAT derived from porcine coronary arteries (PCAs) may release NO and so aimed to determine the role of PVAT in vascular response of PCAs (with intact or disrupted endothelium) to a NO donor and a NOS inhibitor. The study also aimed to measure levels of nitrite (a stable and non-volatile product of NO) in buffer solution incubated with PCAs and PVAT by the Griess reaction and to determine the expression of endothelial-NOS (eNOS) in PVAT by Western blotting.

3.2 MATERIALS AND METHODS

3.2.1 ISOMETRIC TENSION STUDIES

PCAs and PVAT were prepared for use in experiments as described in chapter 2, exercising care to protect the endothelium. *In vitro* studies were carried out using the isolated PCA segments in an organ bath set-up.

3.2.1.1 STUDY USING A NITRIC OXIDE (NO)-DONOR IN THE ABSENCE AND PRESENCE OF PVAT

These experiments investigated the possible interaction between the NO released by sodium nitroprusside (SNP: an NO donor) and that possibly derived from PVAT. After assessing tissue viability and establishing baseline contractility of cleaned PCAs using KCl, U46619 (a thromboxane A_2 agonist) was added in incremental concentrations to achieve pre-contraction of 40-60 % of the second KCl response; the potential impact of PVAT on pre-contraction was controlled for by tailoring the step-wise contraction to U46619 to achieve comparable final pre-contraction levels in the groups of vessels with and without PVAT. This

was followed immediately by assessing the relaxant response to cumulative concentrations of sodium nitroprusside (Tanahashi et al., 1999) (SNP) (1pM-3μM) in vascular segments with and without PVAT derived from the same PCA.

3.2.1.2 EFFECT OF A NO DONOR DURING INHIBITION OF NO-SYNTHASE (NOS) IN THE PRESENCE OF PVAT

As NOS inhibition in PCA/PVAT may potentially alter the vasorelaxant responses to an exogenous NO donor (SNP), this set of experiments were conducted in the presence of PVAT (0.3 g). One group of PCAs was exposed to L-NAME (300 μM) (NOS inhibitor) for an hour while the second group served as the control. This was followed by pre-contraction (40-60% of the 2nd KCl response) of the vessels using U46619; to control for the potential effect of NOS inhibition on PCA contraction, the step-wise contraction to U46619 in the vessels with and without PVAT was tailored to achieve similar pre-contraction levels in the two groups. Finally, construction of cumulative concentration-response curves to SNP (1pM-3μM) was undertaken.

3.2.1.3 EFFECT OF NO SYNTHASE (NOS) INHIBITION ON CONTRACTILITY OF PORCINE CORONARY ARTERIES WITH INTACT ENDOTHELIUM IN THE PRESENCE OF PVAT

NO is reported to be produced continuously by the endothelium and potentially by PVAT and hence contributes to the basal dilator tone (Duncker *et al.*, 2000). It was anticipated that inhibition of NOS at the two sites may lead to a contractile response. These experiments used two groups of PCA segments whose endothelial viability was confirmed by pre-contracting with 30 nM of U46619 followed by relaxation with 1 μM of bradykinin (BK) (Xu *et al.*, 2015). Vessels which showed > 80% relaxation

to BK were used for the study. One group of vessels was treated with L-NAME (300 µM) to inhibit NOS, which was followed by addition of PVAT (0.3 g) to the organ baths containing both groups of vessels. The resulting contractile response was compared to the control vessels which were not exposed to L-NAME.

3.2.1.4 EFFECT OF NO SYNTHASE (NOS) INHIBITION ON CONTRACTILITY OF ARTERIES WITH DENUDED ENDOTHELIUM IN THE PRESENCE OF PVAT

To focus on the PVAT-derived NO, the endothelial source was excluded by de-endothelialising the PCA segments by gently rubbing their luminal surface with the distal part of a small steel forceps (Suri *et al.*, 2010). The vessels were then divided into two groups and equilibrated in warmed and oxygenated Krebs solution in organ baths for 30 minutes and then carefully tensioned to 8 g and allowed to relax for 50 minutes. The removal of functional endothelium was confirmed by absence of relaxation of vessels to BK (1µM) following pre-contraction with U46619 (30 nM). Fifteen minutes later, baseline responses of the vessels to KCl (60 mM) were assessed and then L-NAME (300 µM) was added to inhibit NOS in one group of vessels (the study group). The vessels not exposed to L-NAME served as controls. Then both sets of vessels were exposed to PVAT (0.3 g) added to the buffer solution in the baths. The subsequent change in tone (as % of the response to the 2nd KCl) in the two groups of vessels was compared on achieving a stable response (after at least an hour).

3.2.2 Measurement of nitrite (a stable product of NO) in physiological buffer solution incubated with PVAT

The level of nitrite anion (NO_2^-), a stable, non-volatile product of nitric oxide (NO), was determined in the physiological buffer solution incubated with porcine coronary arteries (PCAs) (with intact endothelium) and perivascular adipose tissue (PVAT) in triplicate. The assay was based on the Griess reaction (Bussey *et al.*, 2013).

Preparation and incubation of samples

Hearts were received on ice from the abattoir in ice-cold, oxygenated, Krebs solution. PCA (5mm length) and PVAT (0.3 gm wet-weight) were excised immediately from each heart by fine dissection in filtered (0.45 µm pore size) ice-cold, oxygenated Krebs buffer solution. Each finely dissected tissue was transferred to a sterile glass vial containing 2ml filtered and oxygenated Krebs solution containing 2% (w/v) Ficoll and antibiotics (30 u/ml penicillin & 40 µg/ml streptomycin) (Al-Shalmani *et al.*, 2011). The samples were then incubated overnight in the medium at 4°C for 16 hours.

Nitrite determination in the buffer solution

Following incubation, the physiologic buffer solution containing each sample was analysed for nitrite level using the Griess reaction. The method is an azo dye coupling reaction formed between nitrous acid (acidified nitrite), sulphanilamide and N-naphthylethylene diamine dihydrochloride (NDD).

Standard (10 mM) was prepared by adding 6.9 mg sodium nitrite salt to 10 ml freshly prepared Krebs solution. Then a dilution series of standards was freshly prepared in the range of 100µM-0µM by diluting the original standard in Krebs solution. 500 µl of sample or standard was added to a micro-centrifuge tube followed by addition of 250 µl of sulphanilamide

(2% w/v) to each tube and the contents were mixed well. Then 250 µl of NDD (0.1 % w/v) was added to the above tubes and mixed well. The tubes were incubated in the dark for 15 minutes and 200 µl of the mixed reagents were transferred from each tube to a 96-well microplate in triplicate. The optical density (O.D) was measured immediately in a microplate reader (SpectraMax M2e, Molecular Devices, Wokingham, Berkshire, UK) at 546 nm wavelength. A standard curve was constructed and hence sample values were determined using the SoftMax Pro 7 software (Molecular Devices, Wokingham, Berkshire, UK).

To ensure that the endothelial source of NO was preserved, the integrity of the endothelium of PCA segments was confirmed after the assay by suspension of the vascular segments in 5ml organ baths containing oxygenated Krebs solution maintained at 37°C. After equilibration for 30 minutes, the vessels were pre-contracted with U46619 (30nM). After achieving a steady state contraction, BK (1µM) was added to assess their endothelium-dependent relaxation. Vessels with 80% or more relaxation to BK were considered to have functional integrity of the endothelium (Vysniauskiene *et al.*, 2006a; Xu *et al.*, 2015).

3.2.3 Expression of endothelial NO-synthase in PVAT

Western blotting was used to determine the expression of endothelial nitric oxide-synthase (eNOS) in PVAT obtained from porcine coronary arteries (PCAs). Segments of PCAs (positive controls) and PVAT were extracted and stored at -80°C. Some PCA segments were manually de-endothelialized (to remove the endothelial source of NO) by gently rubbing the luminal surface with the distal part of a small steel forceps (removal of the endothelium was not confirmed by functional studies), to serve as negative controls and then stored at -80°C. Samples were thawed and homogenized in lysis buffer [Tris (20 mM), ethylene glycol tetra acetic acid (EGTA) (1 mM), Triton X-100 0.1 % (v/v), sodium fluoride

(NaF, 1 mM), sucrose 320 mM, β-glycerophosphate (10 mM), pH 7.6], containing 2% (v/v) protease inhibitor cocktail (Calbiochem, USA).

The samples were centrifuged at 13,000 revolutions per minute (rpm) for 10 minutes at 4˚C, 10 µl of the supernatant of each sample was removed and Bradford test (Bradford, 1976) was used to determine its protein concentration. A stock bovine serum albumin (BSA) (2 mg/ml) solution was prepared in lysis buffer and a serial dilution (from 0.125-1.5 mg/ml) was prepared from the stock to produce a standard curve. Supernatants and standards were mixed with Bradford 1X dye reagent (Bio-Rad, Watford, Hertfordshire, UK) and distilled water, and then incubated for 5 minutes at room temperature. The samples were then transferred to a 96 well plate and the resultant absorbance was determined using SpectraMax M2e microplate reader (Molecular Devices, Wokingham, Berkshire, UK) at 595 nm. The protein concentrations of the samples were extrapolated from the BSA standard curve.

The supernatant from the remaining samples (80µl) was diluted 1:6 in solubilisation buffer (SB) 6X (composition in appendix), pH 6.8 and heated at 95°C for 5 minutes, followed by centrifugation for 1 minute at 13,000 rpm.

Electrophoresis was carried out on 4-20% Tris Glycine (Polyacrylamide gel electrophoresis (PAGE)) Precast Gel (Bio-Rad, Watford, Hertfordshire, UK) in 1X electrophoresis buffer (appendix). 1.2 µl of the molecular marker (Bio-Rad, precision plus protein) was loaded in the first well and the rest of the wells were loaded with 12 µg of protein for each sample.

The gel was electrophoresed at 175 volts for 40 minutes, at room temperature, and then transferred onto a nitrocellulose membrane in 1X transfer buffer (appendix) for 60 minutes at 100 volts. Ponceau S solution (1X) was applied to the nitrocellulose membrane to confirm transfer of

protein. The membrane was divided into two parts by cutting horizontally with a scalpel just below the 75 (kilo Dalton) (kD) mark of the protein ladder. The upper part was used to probe for eNOS (predicted molecular weight: 140 kD) while the lower portion was probed for the loading control (β-actin; predicted molecular weight: 42 kD). The Ponceaus S stain was quickly washed out with Tris-buffered saline containing Tween 20 (TBST) after which the membranes were blocked with 5% (w/v) BSA for 1 hour.

The two nitrocellulose membrane parts were incubated overnight at 4ºC with anti-eNOS (1:1000) (Cell Signalling Technology Inc., MA, USA) and anti-β-actin (1:50,000) (Sigma-Aldrich, MO, USA) primary antibodies respectively, diluted in 5% BSA, on a shaking platform. Next day, the nitrocellulose membrane blots were immediately rinsed with TBST, followed by three five-minute and three fifteen-minute washes with TBST at room temperature. Then the blots were incubated with anti-rabbit IgG (for detecting eNOS) (IRDye® 800CW, LI-COR Biotechnology, NE, USA) and anti-mouse IgG (to probe for β-actin) (IRDye® 680RD, LI-COR Biotechnology, NE, USA) secondary (2º) antibodies diluted 1:10,000 in 5% BSA, for one hour in the dark on a shaking platform. Then the 2º antibody solutions were discarded and the blots immediately rinsed with TBST followed by three five-minute and three fifteen-minute washes with TBST at room temperature, in the dark. At the end, the blots were rinsed once with distilled water and then scanned on the Odyssey® Imaging System, LI-COR Biotechnology, NE, USA using the Image Studio Software.

3.2.4 CHEMICALS AND SOLUTIONS

Pharmacological compounds (used for *in vitro* studies)

Potassium chloride (KCl) (Fisher Scientific, Loughborough, UK); N^G-nitro L-arginine methyl ester (L-NAME) (an inhibitor of NO production), SNP (NO donor) (all purchased from Sigma-Aldrich, St. Louis, MO, USA);

U46619 (a thromboxane A_2 receptor agonist) (Tocris Bioscience, Bio-Techne Ltd, Abingdon, UK).

Chemicals used for nitrite estimation (Griess reaction)

Sulphanilamide, N-naphthylethylene diamine dihydrochloride (NDD), sodium nitrite, Ficoll, penicillin, streptomycin (all obtained from Sigma-Aldrich, St. Louis, MO, USA).

Lysis buffer [Tris (20 mM), ethylene glycol tetra acetic acid (EGTA) (1 mM), Triton X-100 0.1 % (v/v), sodium fluoride (NaF, 1 mM), sucrose 320 mM, β-glycerophosphate (10 mM), pH 7.6], containing 2% (v/v) *protease inhibitor* cocktail (Calbiochem, EMD Millipore Corporation, Temecula, CA, USA).

Bradford 1X dye reagent (Bio-Rad, Watford, Hertfordshire, UK).

Western blotting solutions

- *Solubilisation buffer* (SB) 6X [24% (w/v) SDS, 30% (v/v) glycerol, 5% (v/v) beta mercaptoethanol, 2.5% (v/v) bromophenol blue, 1.5M Tris-hydrochloride], pH 6.8.
- *Electrophoresis buffer* 10X (0.19 M Tris hydrochloride, 1.9 M glycine and 35 mM SDS).
- *Tris buffered saline with tween 20* (TBST) (25mM Tris, 125mM NaCl, 0.1 % Tween 20, pH 7.6).

Antibodies used for Western blotting

Anti eNOS (Cell Signalling Technology Inc., MA, USA; catalogue (cat.) number (#) 9572) and anti-β actin (Sigma-Aldrich, MO, USA; cat. # A2228) primary antibodies; anti-rabbit IgG (LI-COR Biotechnology, NE, USA; cat. # 926-32211) (for detecting eNOS) and anti-mouse IgG (LI-COR Biotechnology, NE, USA; cat. # 926-68070) (to probe for β-actin) secondary antibodies.

Stock solutions of KCl, L-NAME and SNP were made in distilled-water. Subsequent dilutions of SNP were also made in distilled water. Stock solution of U46619 was made in methanol and its subsequent dilutions were made in distilled water.

3.2.5 STATISTICAL ANALYSIS

Data are expressed as mean with standard error of the mean (S.E.M) change in tension (in grams) or mean percentage (%) with S.E.M change in tension or concentration. The 'n' in the results indicates the number of individual animals. The collected data were entered and analysed in GraphPad Prism® version 7, by unpaired, two-tailed, Student's *t*-test or by two-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. A p value of <0.05 was considered as statistically significant.

3.3 RESULTS

3.3.1 EFFECT OF PVAT ON VASORELAXATION OF PORCINE CORONARY ARTERIES INDUCED BY A NITRIC OXIDE (NO) DONOR

After achieving the target pre-contraction of the vascular segments using U46619 (which was not significantly different between the group of vessels with PVAT compared to the controls: mean ± SEM % contraction 44.8 ± 0.7 versus 44.2 ± 1.9 respectively, p=not significant (ns); unpaired, two-tailed, Student's *t*-test), the SNP (NO donor) was immediately added in cumulative concentrations (1pM-3μM) to the organ baths, in order to construct concentration-response relaxation curves. It was observed that the response curve of the segments with PVAT (0.3 g) was potentiated compared to the control segments without PVAT, n=5 ($p<0.05$, two-way ANOVA with Bonferroni's *post hoc* test) (Figure 3.1).

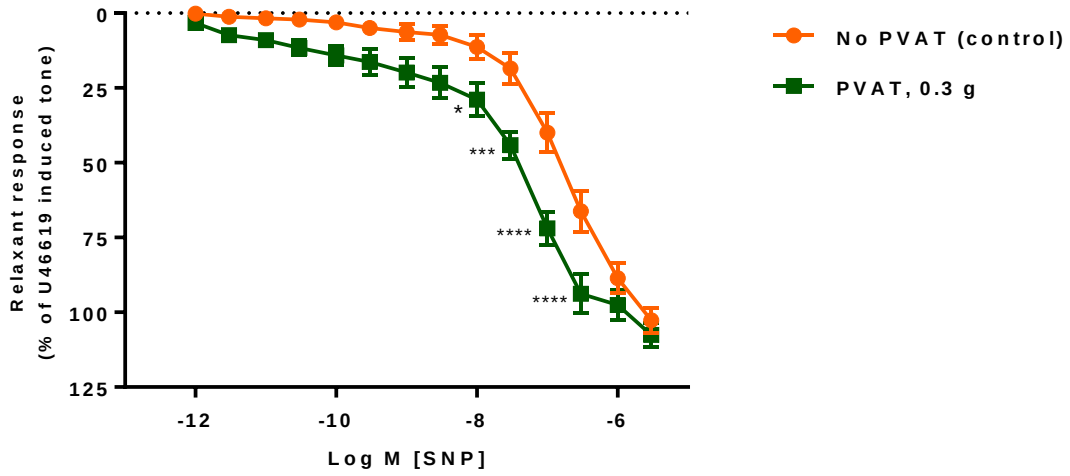

Figure 3.1 Relaxant responses to sodium nitroprusside (SNP) in porcine coronary arteries with and without perivascular adipose tissue (PVAT). The concentration-response curve in the segments with PVAT significantly shifted to the left compared to the control segments without PVAT, suggesting that the SNP-induced relaxant response was potentiated in the presence of PVAT. Data are expressed as mean ± standard error of the mean (S.E.M) relaxation responses of 5

experiments, in % of maximum U46619 induced contraction. Error bars represent standard error. *p<0.05, ***p<0.001, ****p<0.0001.

3.3.2 Relaxant response of PCAs to a NO donor in the presence of PVAT with intact or inhibited nitric oxide-synthase

The PCAs (in the presence of PVAT, 0.3 g) underwent pre-contraction with U46619 to a level which was not significantly different between the group with L-NAME compared to the control (mean ± SEM % contraction 50.2 ± 4.2 versus 41.9 ± 2.2 respectively; p=ns; unpaired two-tailed, Student's *t*-test) and were then relaxed with cumulative concentrations of SNP (NO-donor) (1pM-3μM). It was observed that the response to exogenous NO (derived from SNP) was significantly enhanced in vessels with endogenous NOS inhibition compared to the group of vessels with intact endogenous NO source, n=5 (p<0.05, two-way ANOVA with Bonferroni's *post hoc* test) (Figure 3.2).

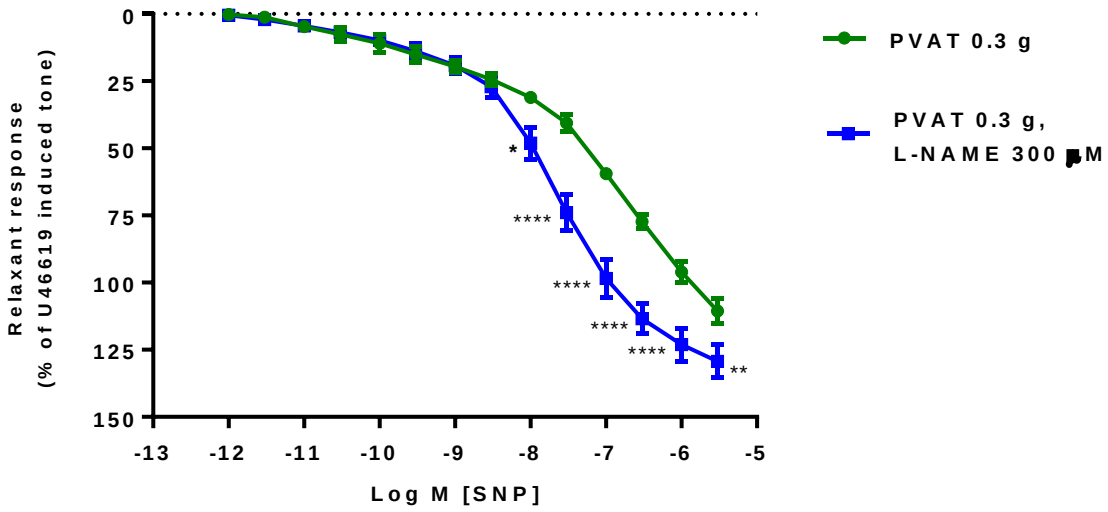

Figure 3.2 Relaxant effects of SNP in the porcine coronary arterial segments in the presence of perivascular adipose tissue (PVAT), with and without NO-synthase (NOS) inhibition (by L-NAME). The response in the presence of L-NAME shows a significant shift to the left, indicating

enhancement of the relaxant response during inhibition of endogenous NOS. Data are expressed as mean ± standard error of the mean (S.E.M) relaxation responses of 5 experiments, in % of maximum U46619 induced contraction. Error bars represent S.E.M. *p<0.05, **p<0.01, ****p<0.0001.

3.3.3 Contractile response of PCAs (with intact endothelium) in the presence of PVAT with inhibited NO-synthase

Baseline contractility of the vessels was established with KCl (60 mM) and endothelial viability of the vessels was confirmed by pre-contraction with 30 nM of U46619 followed by endothelium-dependent relaxation with 1 µM of bradykinin (BK). Vessels which showed > 80% relaxation to BK were used for the study (Figure 3.3). One group of vessels was treated with L-NAME (300 µM) to inhibit NOS, which was followed by addition of PVAT (0.3 g) to the organ baths. The resulting contractile response was compared to the control vessels which were not exposed to L-NAME. It was noted that the contractility was significantly increased in vessels in which NOS was inhibited compared to the controls (mean ± SEM contraction (%): 14.8 ± 1.8 versus 6.5 ± 0.8, n=8, p<0.05, unpaired, two-tailed, Student's *t*-test) (Figure 3.4).

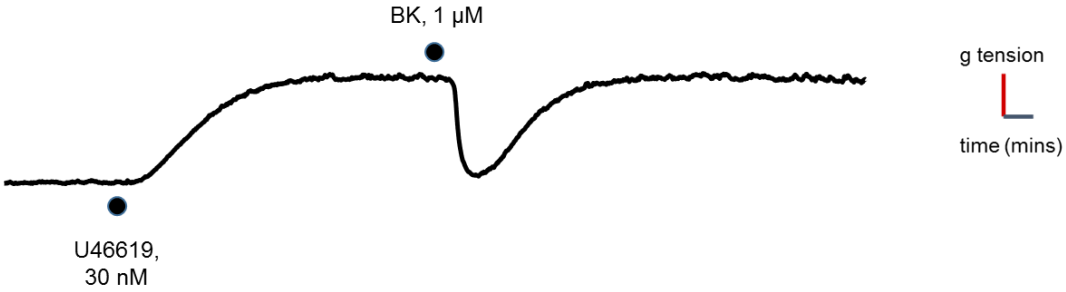

Figure 3.3 Representative trace of the responses of a PCA segment to pre-contraction with U46619 (30 nM) followed by relaxation to bradykinin (BK) (1µM), indicating viability of the endothelium. Relaxation of >80% of

the U46619-induced tone indicated viable endothelium. Grams: g; minutes: mins.

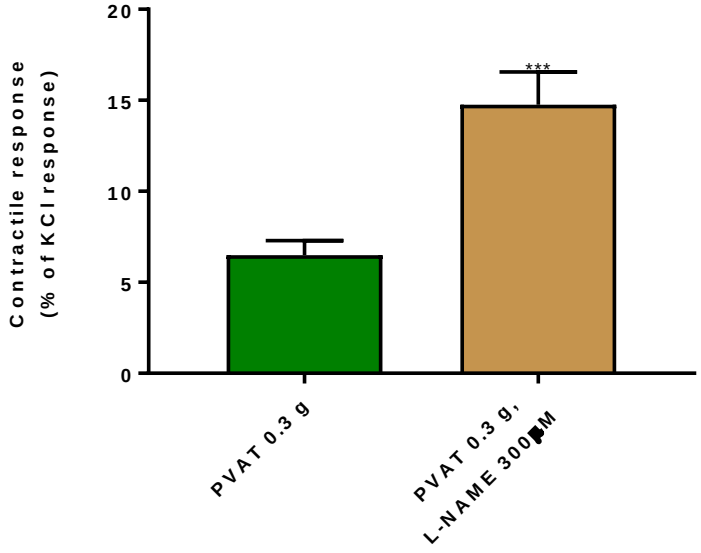

Figure 3.4 Contractile responses of porcine coronary arteries with intact endothelium in the presence of perivascular adipose tissue (PVAT), with and without L-NAME (NO-synthase inhibitor) (n=8). The mean ± S.E.M contractile responses to addition of PVAT (0.3 g) were significantly increased in vessels whose NO-synthase was inhibited compared to the controls. Error bars represent S.E.M. ***p<0.001.

3.3.4 Contractile response of PCAs (without endothelium) in the presence of PVAT with inhibited NO-synthase

In the presence of PVAT, arteries with denuded endothelium (confirmed by lack of a relaxant response to 1 µM of BK following pre-contraction of the vessel segments by treatment with 30 nM of U46619) (Figure 3.5), showed a significant contractile response following NOS inhibition (mean ± SEM contraction: 5.7 ± 0.8 %) compared to vessels with intact NOS

(3.1 ± 0.7 %) (n=4) ($p<0.05$, unpaired, two-tailed Student's *t*-test) (Figure 3.6).

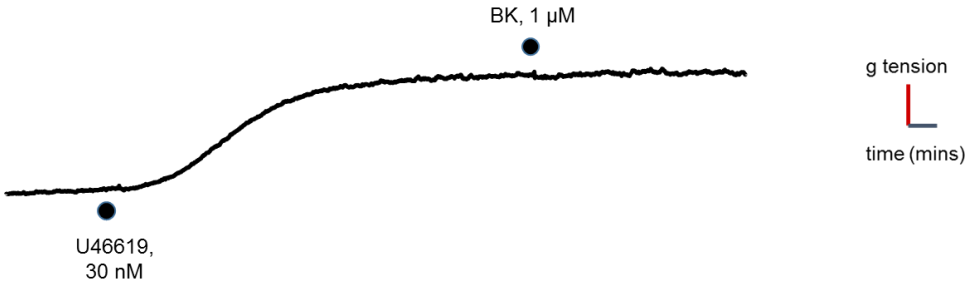

Fig 3.5 A representative trace showing pre-contraction of a PCA segment with U46619 (30 nM) followed by addition of bradykinin (BK) (1µM), which did not result in vasorelaxation, confirming removal of the endothelium. Gram: g; minutes: mins.

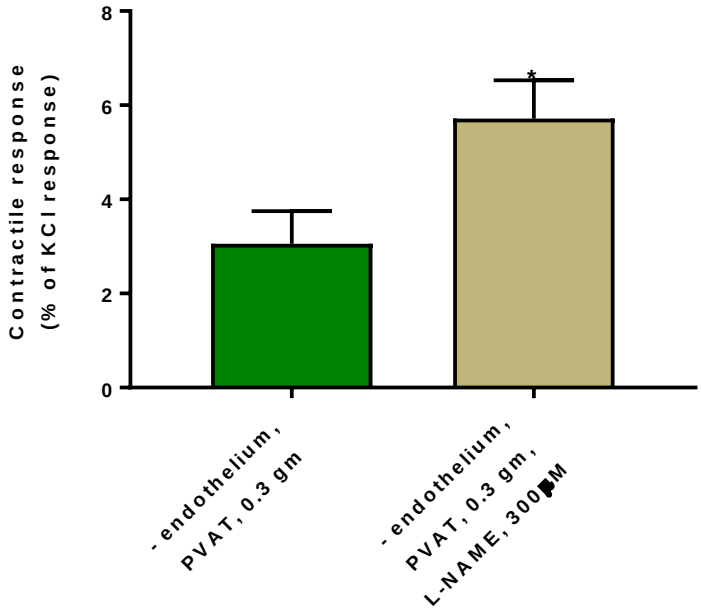

Figure 3.6 Contractile responses of porcine coronary arteries without endothelium in the presence of perivascular adipose tissue (PVAT), with and without NO-synthase inhibition with L-NAME (NO-synthase inhibitor) (n=4). The mean ± S.E.M contractile responses to addition of PVAT (0.3 g) were significantly increased in vessels whose NO-synthase was inhibited compared to the controls. Error bars represent S.E.M. *$p<0.05$.

3.3.5 NITRITE (GRIESS REACTION) IN PHYSIOLOGIC BUFFER SOLUTION INCUBATED WITH PCA (+ENDOTHELIUM) OR PVAT

Nitrite (NO_2^-) production was detected in the buffer solution incubated with PVAT (mean ± SEM concentration: 11.8 ± 1.1 pmoles/mg tissue), although the levels were significantly lower than in the solution incubated with porcine coronary arteries (PCAs) (with intact endothelium) (32 ± 2.7 pmoles/mg tissue) ($p<0.0001$; unpaired, two-tailed Student's *t*-test, n=9) (Figure 3.7).

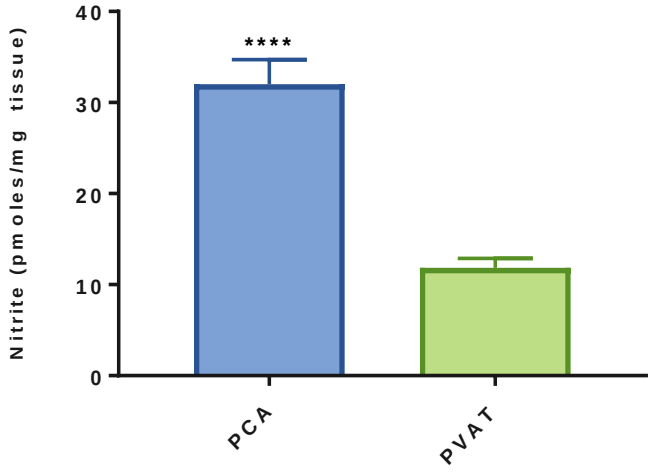

Figure 3.7 Nitrite in physiological buffer solution incubated with perivascular adipose tissue (PVAT) or porcine coronary arteries (PCAs) (n=9). The mean ± S.E.M nitrite levels were significantly greater in the buffer solution incubated with PCAs compared to the solution incubated with PVAT. Error bars represent S.E.M. ****$p<0.0001$.

Endothelial viability of PCAs used in the assay was confirmed after the procedure, in organ bath set ups by pre-contraction with U46619 (30 nM) following by relaxation to BK (1 µM) which was greater than 80 % of the U46619-induced tone (Figure 3.8).

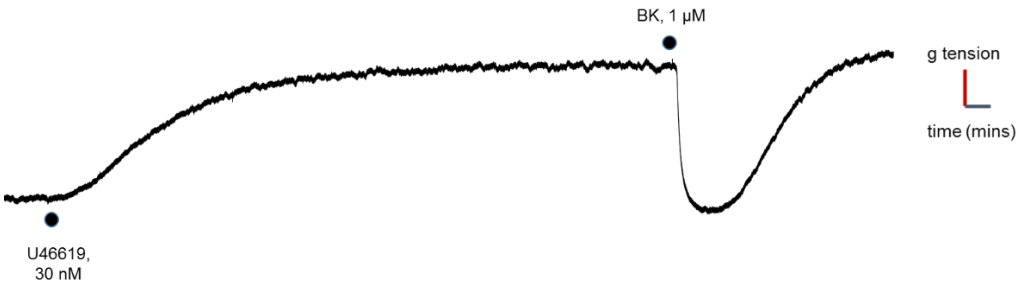

Fig 3.8 A representative trace showing pre-contraction of a porcine coronary artery (PCA) segment (used in nitrite (NO_2^-) estimation) with U46619 (30 nM) followed by addition of bradykinin (BK) (1µM), which showed >80 % relaxation, confirming viability of endothelium of the PCA used in the assay to detect NO_2^- in physiologic buffer solution incubated with the vessels.

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3.3.6 ENDOTHELIAL NO-SYNTHASE EXPRESSION IN PERIVASCULAR ADIPOSE TISSUE

Endothelial NO synthase (eNOS) expression in perivascular adipose tissue (PVAT) (n=5) was detected by Western blotting at the expected molecular weight (140 kilo Daltons (kD)). Porcine coronary arteries (PCAs) with intact endothelium (positive controls) (n=3) also showed eNOS bands in the same region, while PCAs with denuded endothelium (negative controls) (n=3) did not express the eNOS enzyme. Beta (β)-actin served as a loading control (Figure 3.9).

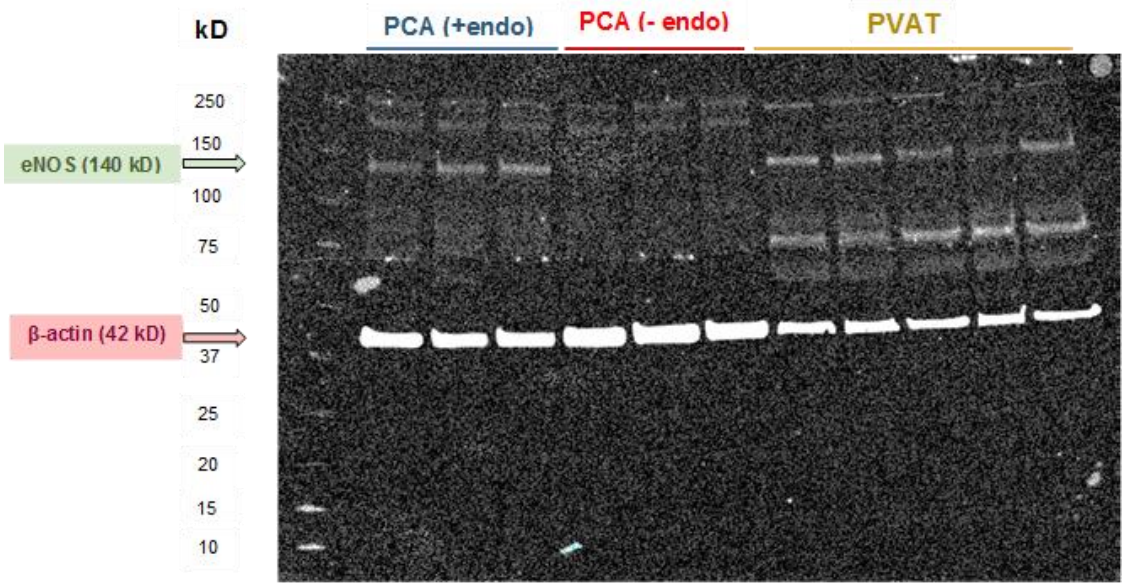


Figure 3.9 Expression of endothelial nitric oxide synthase (eNOS) in perivascular adipose tissue (PVAT) (n=5) of porcine coronary artery (PCA). PCAs with/without endothelium (n=3 each) were used as positive and negative controls respectively, while beta (β)-actin served as a loading control. +endo: endothelium intact; - endo: endothelium removed; kD: kilo Daltons.

3.4 DISCUSSION

Perivascular adipose tissue (PVAT) is increasingly recognised as being involved in paracrine regulation of vascular tone in physiological conditions where it exerts a predominant anti-contractile effect (Fernandez-Alfonso *et al.*, 2013). This function may be mediated by relaxant factors released from PVAT and recent accumulating evidence points to one of these factors to be nitric oxide (NO) (Lynch *et al.*, 2013; Zaborska *et al.*, 2016). Hence the present study aimed to determine the role of NO released by PVAT in function of porcine coronary arteries (PCAs).

3.4.1 NITRIC OXIDE (NO): A PVAT-DERIVED RELAXANT FACTOR

The present study showed that the relaxant responses of PCAs to SNP (a NO donor) in the presence of PVAT were significantly potentiated compared to the vessels without PVAT. In the presence of PVAT, de-endothelialised PCAs showed significantly increased contractile responses during inhibition of nitric oxide-synthase (NOS). Nitrite (NO_2^-) was detected in the buffer solution incubated with PVAT by the Griess reaction. Finally, the expression of endothelial-NOS (eNOS) in PVAT, a constitutive enzyme which catalyses the production of NO, was confirmed by Western blotting. These data point to the release of NO from PVAT which facilitated SNP-induced vasorelaxation, in addition to the endothelium-sourced NO (Figure 3.10) (Gil-Ortega *et al.*, 2010).

This notion is supported by work on whole perfused mesenteric vascular bed (with intact PVAT) of male mice with early diet-induced obesity, which showed significant potentiation of the relaxant response to SNP in association with increased NO bioavailability in PVAT but no increase in NO release from the endothelium. It was postulated that PVAT

undergoes adaptation to preserve vascular function via upregulating NO production in the initial stages of obesity (Gil-Ortega *et al.*, 2010).

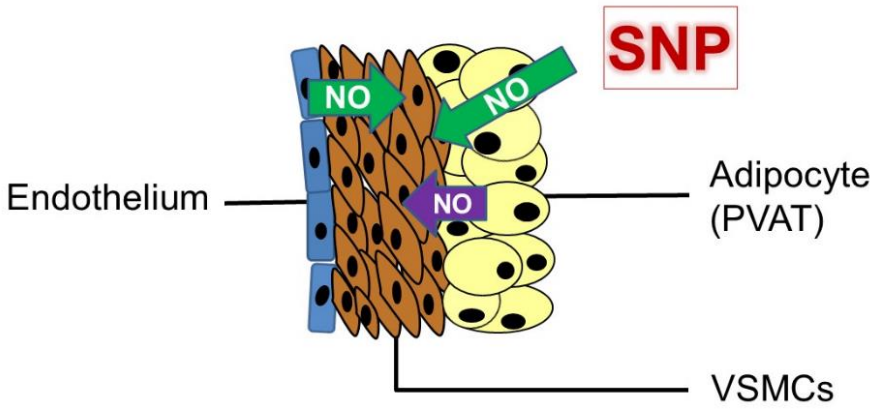


Figure 3.10 Potential sources of nitric oxide (NO) affecting vascular smooth muscle cells (VSMCs). SNP, sodium nitroprusside; PVAT, perivascular adipose tissue.

PVAT can potentially induce vasodilatation through the release of NO, adiponectin or other relaxant factors (Lynch *et al.*, 2013; Zaborska *et al.*, 2016). An investigation in male Sprague-Dawley rat mesenteric arteries showed that PVAT exhibited an anti-contractile effect, which was lost following inhibition of NOS (Zaborska *et al.*, 2016). A study on gluteal arteries of healthy humans showed that adiponectin enhanced NO bioavailability (Owen *et al.*, 2014).

Other studies also demonstrated the production of NO in PVAT (Fruhbeck, 1999). Work on mesenteric arteries (with PVAT) of rats demonstrated inhibition of vessel contractility to the TXA_2 agonist U46619 and the α-adrenoceptor agonist phenylephrine in young animals, which was reversed by pre-incubation of vessel segments with a NOS inhibitor. However, this effect was absent in older animals, suggesting that NO contributed to the anti-contractile effect of PVAT in young rats. Moreover, it was found that the expression of total adenosine monophosphate-dependent protein kinase (AMPK) (which phosphorylates eNOS) and the ratio of phosphorylated eNOS to total eNOS was significantly decreased in older animals, indicating that the reduced anti-contractile effect of

PVAT in these animals may be due to impaired eNOS phosphorylation which resulted in decreased NO production in PVAT (Melrose *et al.*, 2015).

In investigations using rat aorta, use of organ bath solution which bathed aortic rings with intact PVAT to incubate aortic rings without PVAT resulted in relaxation in the later rings (Gao *et al.*, 2007), suggesting the release of a relaxant factor from PVAT. This relaxation response was attenuated by NOS inhibition. (Gao *et al.*, 2007; Triggle *et al.*, 2012).

Studies using endothelium-denuded aortic rings of lean mice showed a significantly increased relaxant response of pre-contracted vessels to acetyl choline in the presence of PVAT, which was abrogated during NOS inhibition. Fluorescence-imaging of aortic sections exhibited NO production in PVAT which was enhanced in the presence of acetyl choline and reduced after NOS inhibition. Immunohistochemistry detected staining of PVAT with anti-eNOS antibody. These findings indicate that NO produced from PVAT contributed to vasorelaxation evoked by acetylcholine (Xia *et al.*, 2016a).

Work by the same group on aorta of mice with diet-induced obesity showed significant loss of the pro-relaxant effect of PVAT accompanied by its impaired NO production. This was confirmed by fluorescence imaging using DAF-2 DA. To identify the mechanisms involved, the group looked at the phosphorylation of eNOS in PVAT by immunohistochemistry, which was found to be reduced in animals fed a high fat diet. Moreover, superoxide (O_2^-) production was increased in PVAT which was ameliorated in the presence of L-NAME (NOS inhibitor), indicating the uncoupling of NOS from NO production towards the manufacture of O_2^-. The eNOS uncoupling was found to be due to reduced L-arginine content and induction of arginase expression in PVAT of animals fed a high fat diet (Xia *et al.*, 2016a).

3.4.2 ROLE OF NO IN MAINTENANCE OF BASAL CORONARY ARTERIAL TONE

In the present study, NO released from PVAT and/or endothelium may have contributed to maintenance of the basal coronary dilator tone, as evidenced by a significant contractile response of vessels in the presence of PVAT to NOS inhibition compared to the controls with intact NOS. Evidence from *in vivo* experiments in swine showed that NOS inhibition led to significant aortic, pulmonary and coronary arterial constriction accompanied by decreased blood flow in the respective vascular beds at rest. This suggests that vascular-derived NO contributes to dilatation in coronary and other arteries in basal conditions (Duncker *et al.*, 2000).

Investigations were performed by Quyyumi *et al.* in human subjects (who had normal coronary arteries on angiography) by giving intra-coronary infusion of L-N^G monomethyl arginine (L-NMMA) (NOS inhibitor). This led to a significant increase in coronary vascular resistance and decrease in distal coronary arterial diameter in the resting state. Acetyl choline-induced vasodilatation was inhibited by L-NMMA and these effects were ameliorated by treatment with intra-coronary L-arginine (Quyyumi *et al.*, 1995). These findings indicate that NO released from intact coronary arteries participates in maintenance of basal tone of coronary arteries.

Chu *et al* recorded coronary arterial diameter and blood flow in awake canines before and after L-NMMA infusion. There was a significant dose-dependent (5-120 mg/kg) increase in basal vasomotor tone after NOS inhibition with N-NMMA and at higher doses, blood flow was also decreased. At the highest dose, flow and acetyl choline mediated vasodilatation were also inhibited, which was reversed with L-arginine. These data are consistent with the observation that NO contributes to baseline coronary arterial tone as well as regulation of endothelium-dependent vasodilatation (Chu *et al.*, 1991). Support for this concept also comes from isometric tension studies performed by Yao *et al* using porcine ophthalmic arteries who found that in vessels with intact

endothelium, inhibition of NOS led to a significant contractile response which was attenuated by L-arginine. Another plausible conclusion is that release of NO from the vessels may be an essential protective mechanism against vasospasm (Yao *et al.*, 1991).

The above evidence from present and other studies supports the role of NO in maintenance of basal arterial tone and explains the development of vasoconstriction following inhibition of endogenous NO production. However, it is also possible that simultaneous release of a contractile factor from PVAT and/or endothelium occurred during NOS inhibition that contributed to the contractile response. A study on isolated rat middle cerebral arteries demonstrated that blocking NOS with N^G-nitro-L-arginine induced a contractile response which was significantly attenuated by inhibition of thromboxane A_2 (TXA_2) synthase as well as by antagonism of the TXA_2 (TP) receptor. This phenomenon suggests that NO regulates vascular tone by suppressing TXA_2 and therefore removal of endogenous NO facilitates vascular TXA_2 production (Benyo' *et al.*, 1998).

Exposure of cultured endothelial cells derived from bovine aorta to TXA_2 mimetics for 24 hours led to significantly increased production of the free radicals superoxide (O_2^-) and peroxynitrite ($ONOO^-$) accompanied by membrane translocation of a NADPH oxidase (NOX) subunit as well as a marked decrease in eNOS activity, NO release and NO bioactivity. Inhibition of NADPH oxidase (NOX) or TP pharmacologically or genetically abrogated the TXA_2 mimetic-induced O_2^- production and improved eNOS activity, along with NO release and activity. This work indicates that activation of NOX by TXA_2 uncouples NOS from NO production towards manufacture of reactive nitrogen species and other free radicals which are detrimental towards vascular function, predisposing to cardiovascular disease (Zhang *et al.*, 2011).

3.4.3 INTERACTION OF A NO DONOR WITH VASCULAR NO

The present study showed that in the presence of PVAT and L-NAME (an inhibitor of endogenous NO production), the vasorelaxation to SNP (an exogenous NO donor) was enhanced in PCAs. This implies that after exclusion of the endothelial source of NO, non-endothelium-dependent vasorelaxation produced by exogenous NO (derived from SNP) was more potent. These findings are consistent with results of another study on rabbit middle cerebral artery, which showed that removal of the endothelium facilitated the relaxant-response to exogenous NO derived from SNP (Gilbert *et al.*, 2001). Also, work on isolated porcine ophthalmic arteries demonstrated a significant enhancement of the relaxant response to cumulative concentrations of 3-morpholino-sydnonimine (SIN-1) (NO donor) in vessels without endothelium (Yao *et al.*, 1991).

Experiments on thoracic aortic rings of rats demonstrated significantly increased relaxant responses to SNP following denudation of endothelium and NOS inhibition. The later work suggested that absence of endogenous NO from the vasculature increases the sensitivity to exogenous NO by upregulation of the enzymatic activity of soluble guanylyl cyclase leading to increased cGMP production and hence increased vasorelaxation (Gilbert *et al.*, 2001; Moncada *et al.*, 1991b). Evidence indicates that vascular NO controls the smooth muscle sensitivity to exogenous NO by feedback regulation (Yamashita *et al.*, 2000). This phenomenon would be advantageous to patients with vascular dysfunction associated with a compromised NO production, where NO donor compounds would be of added benefit (Ignarro *et al.*, 2002).

3.4.4 CONCLUSIONS AND FUTURE WORK

The NO donor (SNP) caused relaxation of PCAs which was significantly potentiated in the presence of PVAT. NOS inhibition in PCAs denuded of endothelium led to a contractile response, which was significantly greater in the presence of PVAT. The Griess reaction showed the presence of nitrite in buffer solution incubated with PVAT and the expression of eNOS was identified in PVAT using Western blotting. These findings indicate that the PVAT of PCAs released the relaxant factor NO.

In the present study, NOS inhibition of PCAs with intact endothelium also led to a contractile response, which was significantly greater in the presence of PVAT. Moreover, the Griess reaction detected the presence of nitrite in buffer solution incubated with PCAs having a functional endothelium and Western blotting showed the expression of eNOS in PCAs. These results suggest that the vascular endothelium may also have released NO which, along with PVAT-derived NO potentially contributed to the relaxant response of PCAs mediated by the NO derived from SNP. Hence PVAT may add to the NO produced in the endothelium, facilitating a dilator tone (Bussey *et al.*, 2016).

The current study demonstrated enhanced vasorelaxation to the NO donor SNP when endogenous NO synthesis was inhibited by L-NAME. This is in accordance with the findings of other researchers who suggested a potential explanation to be increased vessel sensitivity to exogenous NO in conditions of impaired vascular NO production (Gilbert *et al.*, 2001).

It has been reported that vascular NO maintains a dilator tone by inhibiting the release of TXA_2 (Benyo *et al.*, 1998) and these factors have been found to be released from PVAT (Brown *et al.*, 2014), so future functional studies could study the potential interaction of vascular NO and TXA_2 in segments with and without PVAT. For instance, cumulative responses of coronary arteries to the TXA_2 agonist U46619 may be performed with and without inhibition of NOS in the absence or presence

of PVAT. Future work could also include fluorescence imaging to directly show the production of NO by PVAT in human vessels obtained from various vascular beds because different depots of PVAT have differences in their phenotype and the variety of paracrine factors that they release. These studies could be performed in healthy and obese individuals, investigating the effects of factors which may alter NO production such as arginine, BH_4, arginase inhibitors and oxidative stress. Immunoblotting could be performed in PVAT derived from human vessels to study the expression of total and phosphorylated eNOS in the absence or presence of NOS inhibition.

Chapter 4

IMPACT OF PERIVASCULAR ADIPOSE TISSUE ON ENDOTHELIAL FUNCTION IN THE PORCINE CORONARY ARTERY

4.1 INTRODUCTION

Blood vessels are surrounded by an outer adipose layer consisting mainly of fat cells (adipocytes), termed perivascular adipose tissue (PVAT) which is present around most blood vessels (Frontini *et al.*, 2010). Over the last two decades, it has received increasing interest as a paracrine structure secreting a number of biological factors which modulate vascular function (Gollasch *et al.*, 2004).

As described previously, these factors include vasoprotective relaxant agents (e.g. NO and adiponectin) as well as contractile and pro-inflammatory substances including reactive oxygen species, inflammatory cytokines and adipokines (Qi *et al.*, 2018). Most components of RAS, including angiotensin (II,1-7) angiotensin-converting enzyme (ACE 1) and ACE 2 have been shown to be expressed in PVAT (Gu *et al.*, 2013) (Figure 4.1). It was reported that angiotensin II produced in PVAT leads to production of adipokines and aldosterone, which may induce vasoconstriction, inflammation and oxidative stress in the underlying vascular smooth muscle cells by its action on the angiotensin II, type 1 (AT1) receptor (Wildling *et al.*, 2009) (Figure 4.1). Moreover, in PVAT, angiotensin II is metabolized to Angiotensin 1-7 which has a vasoprotective effect by stimulation of endothelial NO production, prevention of ROS production, inflammation and thrombosis (Gu et al., 2013) (Nguyen Dinh Cat *et al.*, 2011a). Perturbation of the RAS in PVAT may contribute to vascular dysfunction in obesity, hypertension and hyperaldosteronism (Nguyen Dinh Cat *et al.*, 2011c).

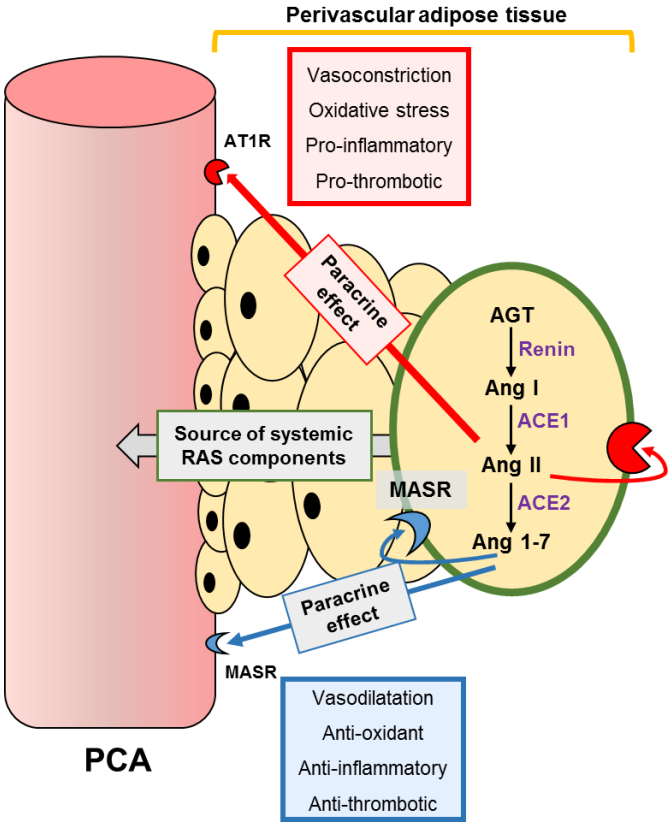


Figure 4.1 The renin-angiotensin system (RAS) in perivascular adipose tissue (PVAT) influences vascular function in a paracrine manner and may be a source of systemic RAS components. PVAT-derived angiotensin II (Ang II) and angiotensin 1-7 (Ang 1-7) have opposing effects on function of the underlying porcine coronary artery (PCA) via their specific receptors, maintaining a delicate balance in the physiological state. AGT, angiotensinogen; Ang 1, angiotensin 1; ACE1, type1-angiotensin- converting enzyme; ACE2, type2-ACE; AT1R, Ang II type1 receptor; MASR, Mas receptor. Adapted from Agabiti-Rosei *et al.*, 2018; Nguyen Dinh Cat *et al.*, 2011a.

Aside from the RAS components, PVAT has been reported to produce other adipokines and inflammatory cytokines which may impair vascular reactivity, increase local inflammation and oxidative stress, potentially perpetuating atherosclerosis and vascular disease. Animal and human studies have identified other PVAT-derived factors including leptin, resistin, chemerin, tumour necrosis factor alpha (TNF-α) and interleukin one beta (IL1β). (Payne *et al*., 2012). Apart from the production of these

factors from dysfunctional PVAT, the amount of PVAT surrounding coronary arteries on the heart surface (epicardial PVAT) increases in obesity and is positively correlated with the severity and extent of coronary artery disease (Ding *et al.*, 2009).

The adipokines and cytokines produced by PVAT present potential novel therapeutic targets in the treatment of cardiovascular disease, and future therapies may focus on suppressing the contractile and atherogenic adipokines while simultaneously enhancing vasculoprotective PVAT products such as nitric oxide (NO), adiponectin and angiotensin 1-7 (Payne *et al.*, 2012).

Factors released from PVAT may directly interact with the underlying endothelium to produce a relaxant effect in the physiological state (Brown *et al.*, 2014). PVAT can release adiponectin, leptin and angiotensin 1-7 which may act on the adjacent endothelium to increase NO production, whereas leptin can also directly stimulate the EDH response to cause vasorelaxation (Gu *et al.*, 2013). In hypertension and obesity, the release of contractile factors from PVAT may be increased which may inhibit endothelial function (Gu *et al.*, 2013). Angiotensin II and aldosterone originating in PVAT may cause vasoconstriction by binding to their specific receptors on the endothelium, while angiotensin II may in addition increase oxidative stress and inflammation (Daugherty *et al.*, 2000; Nakashima *et al.*, 2006; Nguyen Dinh Cat *et al.*, 2011a). The aforementioned evidence indicates that PVAT may release vasoactive agents with the capacity to modulate vascular endothelial function (Gollasch *et al.*, 2004).

Hypothesis and aims

It was postulated that PVAT may interfere with function of the underlying endothelium through release of vasoactive mediators and thus this study aimed to investigate the role of PVAT and exogenous angiotensin II in endothelium-dependent relaxation of porcine coronary arteries induced

by bradykinin. In addition, the study aimed to determine the presence of angiotensin II and expression of type-1 angiotensin-converting enzyme (ACE 1) in PVAT by ELISA and Western blotting respectively.

4.2 METHODS AND MATERIALS

4.2.1 ISOMETRIC TENSION STUDIES

PCA segments with or without PVAT were excised taking care to protect the endothelium. *In vitro* experiments were carried out using the isolated PCA segments in organ bath set-ups.

4.2.1.1 EFFECTS OF VASORELAXATION WITH BRADYKININ IN THE ABSENCE OR PRESENCE OF PVAT [ALONE OR TOGETHER WITH AN ANGIOTENSIN II, TYPE 1 RECEPTOR ANTAGONIST (CANDESARTAN)].

As described in the introduction, angiotensin II released from PVAT may potentially affect endothelial function, so these experiments determined if vascular angiotensin II, type 1 (AT1) receptor inhibition (using candesartan) impacted on the effect of PVAT on the PCA segments. So, three groups of PCA rings were used, the first one consisted of cleaned PCAs (controls) while the second and third groups had intact PVAT. The third group was exposed to the angiotensin II, type 1 (AT1) receptor antagonist candesartan (1 µM) (Seeger *et al.*, 2001) for an hour. This was followed by obtaining pre-contraction of the three groups of arteries with U46619, and then relaxation with cumulative BK (1pM-1µM) (Xu *et al.*, 2015).

4.2.1.2 ENDOTHELIUM-DEPENDENT VASORELAXATION IN THE ABSENCE OR PRESENCE OF ETHANOL

In the previous experiments, the AT1 receptor antagonist candesartan had been dissolved in the solvent ethanol. To determine if the solvent had any effect on bradykinin-induced vasorelaxation, one group of vessels was exposed to ethanol (5 µl (0.1% v/v): equal volume as that of the candesartan solution used) (Xu *et al.*, 2015), while the other group (without ethanol) comprised of control arteries. After an hour's interval, pre-contraction with U46619 was undertaken to achieve a stable tone. Then, cumulative BK (1pM-1µM) was utilized to construct concentration-response curves.

4.2.1.3 EFFECTS OF EXOGENOUS ANGIOTENSIN II ON ENDOTHELIUM-DEPENDENT VASORELAXATION WITH BRADYKININ IN THE ABSENCE OF PVAT.

To directly investigate the influence of angiotensin II on endothelial function of PCAs, two groups of cleaned vessel rings (without PVAT) were incubated with angiotensin II (50 nM) (Stanke-Labesquea *et al.*, 2000) for an hour (study group) while the first group served as a control. Pre-contraction (40-60 % of the KCl response) was obtained with U46619. Then, cumulative BK (1pM-3µM) was added to assess the relaxant responses in the two groups.

4.2.1.4 RELAXANT RESPONSES OF PCA_S TO BRADYKININ DURING INHIBITION OF NO-SYNTHASE AND CYCLOOXYGENASE (EDH RESPONSE), IN THE ABSENCE AND PRESENCE OF PVAT (ALONE OR IN COMBINATION WITH CANDESARTAN).

Apart from inducing endothelial NO (by eNOS) and prostacyclin (through cyclooxygenase) production, BK stimulates endothelium-dependent

hyperpolarization (EDH) to bring about vasorelaxation. To determine if PVAT-derived angiotensin II affected the EDH response, three groups of vessels, which were in the absence or presence of PVAT (alone or with candesartan) were incubated with L-NAME (300 µM) and indomethacin (10 µM) to inhibit the NOS and COX enzymes respectively. Then the third group was exposed to candesartan (1 µM) for an hour, to inhibit the AT1 receptor, which was followed by attainment of pre-contraction with U46619. Finally, relaxant responses (due to the EDH effect) were assessed with BK (0.1 µM) (Vysniauskiene *et al.*, 2006b). A single concentration of BK was used here because in the previous experiments angiotensin II had shown a transient and slight effect on endothelium-dependent vasorelaxation to cumulative BK. A possible explanation for this is that the AT1 receptor in vascular smooth muscle cells may be subject to desensitization with continued exposure to the angiotensin II during the longer cumulative response, attenuating its impact on vascular reactivity (Bernhem *et al.*, 2017). A potential underlying mechanism was reported in a study which used immunofluorescence labelling of the AT1 receptor whereby exposure to angiotensin II caused the membrane bound receptor to rapidly internalize into endosomal vesicles and when the angiotensin II was removed the receptor cycled back to the plasma membrane (Hein *et al.*, 1997).

4.2.1.5 EFFECT OF EXOGENOUS ANGIOTENSIN II ON THE EDH RESPONSE.

To determine the effect of exogenous angiotensin II on the EDH response in PCAs, two groups of cleaned vessel rings (without PVAT) were incubated with L-NAME (300 µM) and indomethacin (10 µM) respectively for an hour. Then pre-contraction was undertaken with U46619. When a stable tone was achieved, the second group was exposed to angiotensin II (1 nM) (Stanke-Labesquea *et al.*, 2000) for ~10 minutes after which a bolus concentration of bradykinin (0.1 µM) was added to the two baths and the responses compared. Angiotensin II was incubated with the

PCAs after the pre-contraction stage in a single lower concentration (1 nM) and for a shorter period than that used previously because in preliminary work, incubation with 50 nM angiotensin II for an hour prior to the pre-contraction phase had no effect on the EDH response. This may be due to the fact that the AT1 receptor in vascular smooth muscle cells undergoes desensitization when continuously exposed to angiotensin II at concentrations above the physiological value of 1 nM (Bernhem *et al.*, 2017).

4.2.2 Determination of angiotensin II levels in PVAT

4.2.2.1 PREPARATION OF SAMPLES

Hearts were received on ice from the abattoir and PVAT was carefully dissected from the animals of either gender in ice-cold oxygenated, Krebs solution. Samples were immediately stored at -80 °C until homogenization. Samples were thawed out and finely incised for homogenization. Potassium free phosphate-buffered saline (PBS) (0.1 M, pH 7.4, 0.5 ml) added to 0.1 g of PVAT in clean tubes along with ceramic beads. Samples homogenized using an automated homogenizer (Precellys 24, Bertin instruments, Montigney le Bretonneux, France). The homogenates transferred on ice to micro-centrifuge tubes and centrifuged at 2,500 rpm at 4°C for 20 minutes. The supernatant was then carefully aspirated with a 1 ml syringe and a 25G needle, avoiding the pellet at the bottom and layer of fat at the top. The supernatant was immediately stored at -80 °C until ELISA for angiotensin II and Lowry assay to estimate protein content of the samples were performed.

4.2.2.2 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR ANGIOTENSIN-II

Enzyme immunoassay (Jakob *et al.*, 2012) performed on PVAT homogenates in duplicate, for quantitative measurement of angiotensin-II as per kit manufacturer's instructions (Bioassay Technology Laboratory, Inc.; Korain Biotech Co. Ltd., Birmingham/Shanghai).

Principle

The assay was based on biotin double-antibody sandwich technology. Samples were added to wells pre-coated with angiotensin II monoclonal antibody and incubated. Then, anti-angiotensin II antibodies labelled with biotin were added which united with streptavidin-horse radish peroxidase (HRP) to form an immune complex. After incubation, unbound enzymes were removed from the wells by washing. After this, substrate chromogen solutions A and B were added (Figure 4.4). The solution in the wells turned blue and then yellow. The shades of the solution and the concentration of angiotensin II correlated positively.

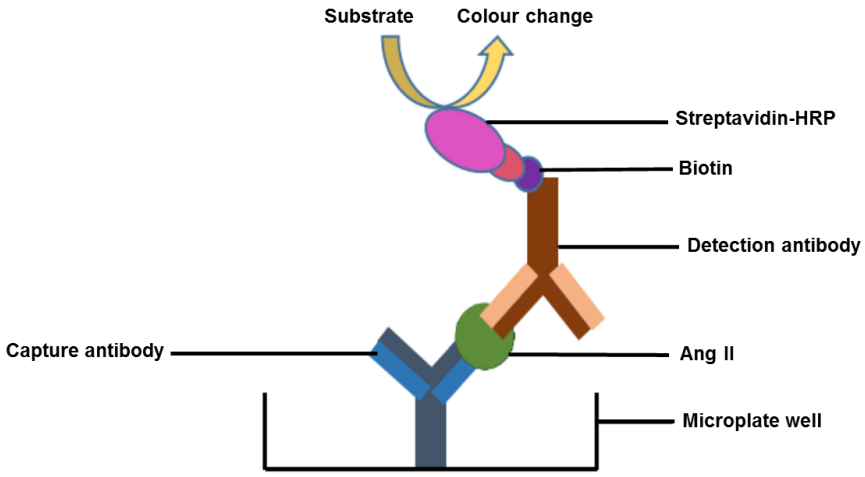


Figure 4.2 Principle of the angiotensin II enzyme linked immunosorbent assay (ELISA). Ang II, angiotensin II; HRP, Horse Radish Peroxidase.

Materials

1. Standard solution (2400 ng/l).
2. Coated ELISA plate (96 wells).
3. Standard diluent.
4. Streptavidin-HRP.
5. Stop solution.
6. Chromogen solutions A and B.
7. Washing concentrate (30 X, diluted to 1X with de-ionized water before use).
8. Anti-angiotensin II detection antibodies labelled with biotin.
9. Seal plate membrane.
10. Precision pipettes with disposable tips.
11. Microplate reader with 450 nm filter.
12. Deionized water.
13. Absorbent paper.

Preparation of standards

The standard of original concentration (2400 ng/l) was serially diluted in Eppendorf® tubes as follows:

Tube	Concentration (ng/l)	Dilution
5	1200	120 µl original standard + 120 µl standard diluent
4	600	120 µl standard number 5 + 120 µl standard diluent
3	300	120 µl standard number 4 + 120 µl standard diluent
2	150	120 µl standard number 3 + 120 µl standard diluent
1	75	120 µl standard number 2 + 120 µl standard diluent

Assay Protocol

The PVAT homogenates were analysed in duplicate as per the following protocol:

Additions	Wells		
	Blank	**Standards**	**Samples**
Deionized water	100 µl	-	-
Standards	-	50 µl	-
Samples	-	-	40 µl
Anti-angiotensin II detection antibodies	-	-	10 µl
Streptavidin-HRP	-	50 µl	50 µl

The ELISA plate was covered with a seal plate membrane, shaken gently to mix the contents, and then incubated at 37 °C for 60 minutes. After incubation, the seal plate membrane was removed carefully, liquid drained from the wells and the remainder shook off. Each well was filled with washing solution, which was drained off after 30 seconds standing. This procedure was repeated four more times following which the plate was blotted on absorbent paper.

For colour development, the following additions were carried out in sequence:

Additions	Wells		
	Blank	**Standards**	**Samples**
Chromogen A	50 µl	50 µl	50 µl
Chromogen B (photosensitive, so added in dimmed light)	50 µl	50 µl	50 µl

The plate was shaken gently to mix the contents and then incubated for 10 minutes at 37 °C in the dark for colour development. Stop solution (50 µl) added to each well to stop the reaction (blue colour of the wells' contents immediately changed to yellow at that moment). Having taken the blank well as zero, the absorbance (optical density: OD) of each well was measured using a microplate reader (SpectraMax 340 PC, Molecular Devices, Wokingham, Berkshire, UK) at 450 nm wavelength within 10 minutes of having added the stop solution. A standard curve was constructed with the angiotensin-II standards and therefore sample values were determined using the SoftMax Pro 6 software (Molecular Devices, Wokingham, Berkshire, UK).

4.2.3 LOWRY ASSAY FOR PROTEIN ESTIMATION IN PVAT

The assay was performed for samples which underwent ELISA for angiotensin II, to express the peptide as a fraction of total protein content of the sample. Sample preparation was as described for the ELISA assay. Lowry (Hartree, 1972) assay was performed on homogenates in triplicate.

Principle

The assay was a measure of phenolic tyrosine residues based on the quantification of a blue coloured copper/tyrosine molybdate complex which absorbed at 750 nm wavelength.

Solutions

1. **Lowry Solution A**: contained sodium hydroxide (NaOH) (2 g), sodium dodecyl sulphate (SDS) (1 g) and sodium carbonate (Na_2CO_3) (10 g) dissolved in 500 ml deionized-water.

2. **Lowry AB Solution:** constituted by adding 20 mls of solution A to 100 µl of 2% (w/v) sodium potassium tartrate and 100 µl of 1% (w/v) copper sulphate ($CuSO_4$).
3. **Bovine serum albumin (BSA) standards:** the original BSA solution (1 mg/ml) was serially diluted for a standard curve in 1.5 ml micro-centrifuge tubes as follows:

Tube	Concentration	BSA (µl)	Deionized-water (µl)
1	0	0	200
2	0.05	10	190
3	0.1	20	180
4	0.15	30	170
5	0.2	40	160
6	0.25	50	150
7	0.3	60	140
8	0.35	70	130
9	0.4	80	120

4. **Folin & Ciocalteu's reagent:** diluted in a ratio of 1:1 with equal volume of de-ionized water in a universal container covered with a silver foil, to protect the solution from light, the reagent being photosensitive.

Procedure:

- Lowry AB Solution and BSA standard dilutions prepared as above. In separate 1.5 ml micro-centrifuge tubes, the samples were diluted in the ratio of 1:10 by dissolving 20 µl of the sample in 180 µl of de-ionized water.

- Added 1 ml of the Lowry AB Solution to all standard and sample tubes and mixed immediately. Tubes incubated at room temperature for 10 minutes.
- Then added 100 µl of the diluted Folin & Ciocalteu's *re*agent to each tube and mixed immediately. The tubes incubated at room temperature for 60 minutes in the dark.
- The tube contents pipetted out in triplicate into a clear 96 well microplate in a volume of 200 µl per well. The optical density was measured at 750 nm wavelength using a microplate reader (SpectraMax 340 PC, Molecular Devices, Wokingham, Berkshire, UK).
- A standard curve was constructed and hence sample values were determined using the SoftMax Pro 6 software (Molecular Devices, Wokingham, Berkshire, UK).

4.2.4 Expression of angiotensin-converting enzyme I (ACE I) in perivascular adipose tissue (PVAT) by Western blotting

Samples of PVAT as well as PCA, pig and rat kidney (positive controls) were extracted and stored at -80°C. Samples were thawed and homogenized in lysis buffer [Tris (20 mM), ethylene glycol tetra acetic acid (EGTA) (1 mM), Triton X-100 0.1 % (v/v), sodium fluoride (NaF, 1 mM), sucrose 320 mM, β-glycerophosphate (10 mM), pH 7.6], containing 2% (v/v) protease inhibitor cocktail (Calbiochem, EMD Millipore Corporation, Temecula, CA, USA).

The samples were centrifuged at 13,000 rpm for 10 minutes at 4°C. Then 10 µl of each sample's supernatant was removed and Bradford test (Bradford, 1976) was used to determine its protein concentration. A stock bovine serum albumin (BSA) (2 mg/ml) solution was prepared in lysis buffer; a serial dilution (from 0.125-1.5 mg/ml) was prepared from the

stock to produce a standard curve. Supernatants and standards were mixed with Bradford protein assay 1X reagent (Bio-Rad, Watford, Hertfordshire, UK) and distilled water, and then incubated for 5 minutes at room temperature. The samples were transferred to a 96 well plate and the resultant absorbance was determined using SpectraMax M2e microplate reader (Molecular Devices, Wokingham, Berkshire, UK) at 595 nm. The protein concentrations of the samples were extrapolated from the BSA standard curve.

The supernatant from the remaining samples (80µl) was diluted 1:6 in solubilisation buffer (SB) 6X [24% (w/v) SDS, 30% (v/v) glycerol, 5% (v/v) beta mercaptoethanol, 2.5% (v/v) bromophenol blue, 1.5M Tris-hydrochloride], pH 6.8 and heated at 95°C for 5 minutes, followed by centrifugation for 1 minute at 13,000 rpm.

Electrophoresis was carried out on 4-20% Tris Glycine (PAGE) precast gels (Bio-Rad, Watford, Hertfordshire, UK) in 1X electrophoresis buffer (appendix) using a protein loading of 12 µg per well. A molecular marker (1.2 µl) (Bio-Rad, precision plus protein) was loaded in the first and/or last well. The gel was electrophoresed at 175 volts for 40 minutes, at room temperature, and then transferred onto a nitrocellulose membrane in 1X transfer buffer (appendix) for 60 minutes at 100 volts. Ponceau S solution was applied to the nitrocellulose membrane to confirm transfer of protein. The membrane was divided into two parts by cutting horizontally with a scalpel below the 75 kD mark of the protein ladder. The upper part was used to probe for angiotensin-converting enzyme, type 1 (ACE1) (predicted molecular weight: ~150 kD) while the lower portion was probed for the loading control (GAPDH) (predicted molecular weight: 36 kD). The Ponceau S stain was quickly washed out with Tris-buffered saline containing Tween 20 (TBST) after which the membranes were blocked with 5% (w/v) BSA for I hour.

The two nitrocellulose membrane parts were incubated overnight at 4ºC with anti-ACE1 (1:550) (Abcam, Cambridge, UK) and anti-GAPDH (1:30,000) (Abcam, Cambridge, UK) primary antibodies respectively, diluted in 5% BSA, on a shaking platform. Next day, the nitrocellulose membrane blots were immediately rinsed with TBST, followed by three five-minute and three fifteen-minute washes with TBST at room temperature.

The blots were incubated with anti-mouse IgG (for detecting ACE1) (IRDye® 800CW, LI-COR Biotechnology, NE, USA) and anti-rabbit IgG (to probe for GAPDH) (IRDye® 680RD, LI-COR Biotechnology, NE, USA) secondary (2º) antibodies diluted 1:10,000 in 5% BSA, for one hour in the dark on a shaking platform. Then the 2º antibody solutions were discarded and the blots immediately rinsed with TBST followed by three five-minute and three fifteen-minute washes with TBST at room temperature, in the dark. At the end, the blots were rinsed once with distilled water and scanned on the Odyssey® Imaging System, LI-COR Biotechnology, NE, USA using the Image Studio Software.

4.2.5 CHEMICALS AND ASSAY KITS

Pharmacological / bioassay compounds

Potassium chloride (KCl), sodium hydroxide (NaOH) and sodium carbonate (Na_2CO_3) were obtained from Fisher Scientific, Loughborough, UK. Bovine serum albumin (BSA), sodium potassium-tartrate, Folin & Ciocalteu's reagent, N^G-nitro L-arginine methyl ester (L-NAME) (an inhibitor of NO production), indomethacin (a cyclooxygenase inhibitor), and bradykinin (BK) (an endothelium-dependent vasodilator) were all purchased from Sigma-Aldrich, St. Louis, MO, USA.

U46619 (a thromboxane A_2 receptor agonist) and candesartan (an angiotensin II, type 1 receptor antagonist) were obtained from Tocris

Bioscience, Bio-Techne Ltd, Abingdon, UK. Sodium dodecyl sulphate (SDS) was procured from Acros Organics (distributor: Fischer Scientific, Loughborough, UK) and Copper Sulphate ($CuSO_4$) from Fisons Scientific Equipment (owned by Sanofi, Paris, France). The kit for angiotensin II ELISA was obtained from Bioassay Technology Laboratory, Inc.; Korain Biotech Co. Ltd., Birmingham/Shanghai.

Stock solutions of KCl, L-NAME, angiotensin II, BK and subsequent dilutions of BK were made in distilled-water while stock solutions of indomethacin and candesartan were made in ethanol. The concentration of ethanol in the bath-solution was 0.1% v/v and was found to have no effect on effect on endothelium-dependent vasorelaxations induced by BK.

Stock solution of U46619 was made in methanol and its subsequent dilutions were made in distilled water. Lowry Solution A (included Na_2CO_3, NaOH and SDS), $CuSO_4$, sodium potassium-tartrate and BSA were prepared in de-ionized water. Folin & Ciocalteu reagent was diluted in de-ionized water.

Lysis buffer

Tris (20 mM), ethylene glycol tetra acetic acid (EGTA) (1 mM), Triton X-100 0.1 % (v/v), sodium fluoride (NaF, 1 mM), sucrose 320 mM, β-glycerophosphate (10 mM), pH 7.6, containing 2% (v/v) protease inhibitor cocktail (Calbiochem, EMD Millipore Corporation, Temecula, CA, USA). Bradford 1X dye reagent (Bio-Rad, Watford, Hertfordshire, UK).

Western blotting solutions

- *Solubilisation buffer* (SB) 6X [24% (w/v) SDS, 30% (v/v) glycerol, 5% (v/v) beta mercaptoethanol, 2.5% (v/v) bromophenol blue, 1.5M Tris-hydrochloride], pH 6.8.
- *Electrophoresis buffer* 10X (0.19 M Tris hydrochloride, 1.9 M glycine and 35 mM SDS).

- *Tris buffered saline with Tween 20* (TBST) (25mM Tris, 125mM NaCl, 0.1 % Tween 20, pH 7.6).

Antibodies used for Western blotting

Anti ACE1 (Abcam, Cambridge, UK; catalogue (cat.) number (#) ab77990) and anti-glyceraldehyde phosphate dehydrogenase (GAPDH) (Abcam, Cambridge, UK; cat. # ab190304) primary antibodies; anti-mouse IgG (LI-COR Biotechnology, NE, USA; cat. # 925-32210) (for detecting ACE1) and anti-rabbit IgG (LI-COR Biotechnology, NE, USA; cat. # 925-68071) (to probe for GAPDH) secondary antibodies.

4.2.6 STATISTICAL ANALYSIS

Data are expressed as mean with standard error of the mean (S.E.M) change in tension/concentration or mean percentage (%) with S.E.M change in tension. The maximal relaxant response produced with the highest concentration (R_{Max}) of bradykinin and its effective molar concentration required for a half maximal response (EC_{50}) (reported as its negative logarithm: pEC_{50}) were interpolated from the individual concentration-response curves using a non-linear regression analysis. The ‘n’ in the results indicates the number of individual animals.

The data were analysed by two tailed, Student’s *t*-test for comparison of two groups and one-way analysis of variance (ANOVA) followed by Tukey’s *post hoc* test to detect significant differences between multiple groups using GraphPad Prism® version 7 (La Jolla, CA, USA). A p value of <0.05 was considered statistically significant.

4.3 RESULTS

4.3.1 EFFECTS OF PVAT ON ENDOTHELIUM-DEPENDENT RELAXATION OF PORCINE CORONARY ARTERIES IN THE ABSENCE OR PRESENCE OF AN ANGIOTENSIN II TYPE 1 (AT1) RECEPTOR ANTAGONIST (CANDESARTAN)

The pre-contracted arterial segments were relaxed with cumulative bradykinin (1pM-1µM) and the response curves were compared. It was observed that the maximal response in the segments with PVAT was significantly reduced compared to the control segments (without PVAT) (R_{Max} % values: 96.0 ± 6.4 versus 117 ± 3 respectively, n=5; $p<0.05$; one-way ANOVA), which was reversed in the additional presence of candesartan (1µM) (R_{Max} %: 117 ± 5) versus the controls, $p>0.05$; one-way ANOVA (Figure 4.3). The potency of the response was similar in the vessels exposed to PVAT alone in comparison to the group additionally exposed to candesartan (1µM) (pEC_{50}: 8.7 ± 0.2 and 8.8 ± 0.1 respectively, n=5; $p>0.05$, one-way ANOVA) (Figure 4.3).

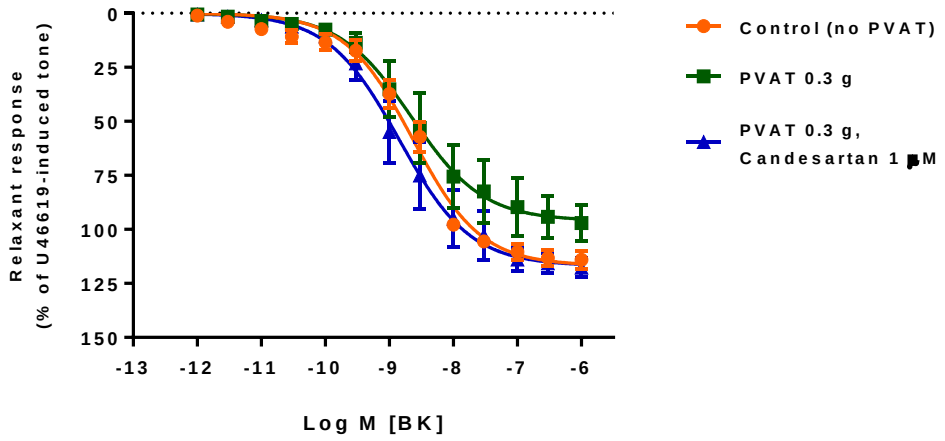


Figure 4.3 Effect of perivascular adipose tissue (PVAT) on relaxant responses to bradykinin (BK) in porcine coronary arteries with and without an angiotensin II, type 1 (AT_1) receptor antagonist (candesartan, 1µM). The maximum response of the segments with PVAT was significantly suppressed compared to that of the controls, which was

reversed in the presence of candesartan. Data are expressed as mean ± standard error of the mean (S.E.M) relaxation responses of 5 experiments, in % of maximum U46619 induced contraction. Bars represent S.E.M.

4.3.2 ENDOTHELIUM-DEPENDENT VASORELAXATION TO BRADYKININ IN THE ABSENCE OR PRESENCE OF ETHANOL

The pre-contracted PCA arterial rings without PVAT underwent relaxation with cumulative bradykinin (1pM-1µM) in the absence (control group) or presence of ethanol (5 µl (0.1% v/v)) (equal to the volume of candesartan solution used which was prepared in ethanol). The potency (pEC_{50} values: 8.6 ± 0.1 versus 8.6 ± 0.1 for the control and ethanol groups respectively, n=4; p=ns; unpaired, two-tailed, Student's *t*-test) and efficacy (R_{Max} % values: 107 ± 3 of the controls versus 109 ± 4 of the ethanol group respectively, n=4; p=ns; un-paired, two-tailed, Student's *t*-test) of the responses was not significantly different between the two groups, implying that ethanol had no effect on endothelium-dependent vasorelaxation (Figure 4.4)

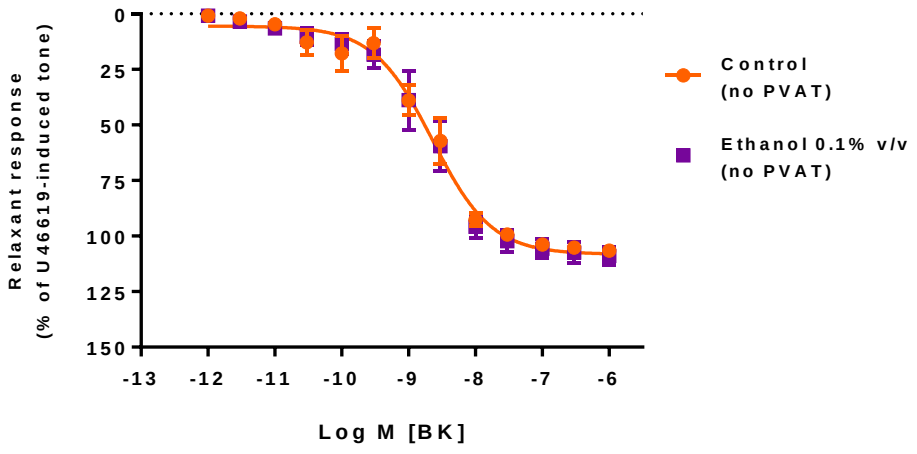


Figure 4.4 Endothelium-dependent relaxation of porcine coronary arteries (PCAs) without perivascular adipose tissue (PVAT), induced by bradykinin (BK), in the absence or presence of ethanol, which had no effect on vasorelaxation. Data are expressed as mean ± standard error

of the mean (S.E.M) relaxation responses of 4 experiments, in % of maximum U46619 induced contraction. Bars represent S.E.M.

4.3.3 Effects of exogenous angiotensin II on endothelium-dependent vasorelaxation

The maximal endothelium-dependent vasorelaxation (R_{Max}) induced by cumulative concentrations of BK (1pM-3µM) was significantly reduced (99.9 ± 2.9 %) in vessels pre-incubated with exogenous angiotensin II (50 nM) compared to response in the control vessels (116.7 ± 3.7 %) ($p<0.05$, unpaired, two-tailed, Student's *t*-test). However, the potency of the responses in the two groups of vessels were not significantly different (pEC_{50}: 8.4 ± 0.1 in vessels exposed to angiotensin II vs 8.2 ± 0.1 in control vessels respectively) (unpaired, two-tailed, Student's *t*-test) (Figure 4.5).

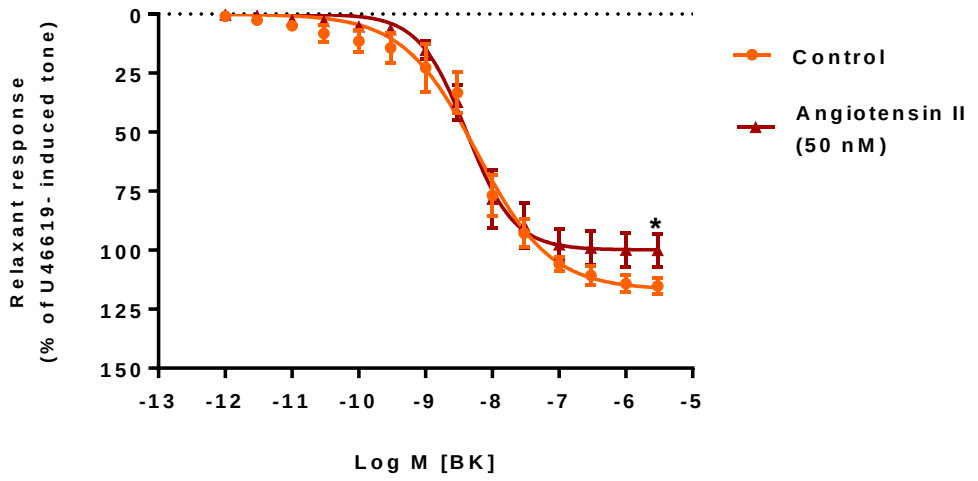


Figure 4.5 Effects of exogenous angiotensin II on the relaxant responses of porcine coronary arteries to bradykinin (BK). Relaxant effects of bradykinin in the porcine coronary arterial (PCA) segments, with and without exogenous angiotensin II. In the presence of angiotensin II (50 nM), the maximal endothelium-dependent relaxation was significantly suppressed. Data are expressed as mean ± standard error of the mean

(S.E.M) relaxant responses of 5 experiments, in % of maximum U46619 induced contraction. *p<0.05.

4.3.4 Effects of PVAT on vasorelaxation associated with EDH-type responses, in the absence or presence of an AT1 receptor antagonist

The pre-contracted vessels of the three groups, which had been treated with L-NAME (300 µM) and indomethacin (10 µM) (to inhibit the vascular NOS and COX enzymes respectively) underwent relaxation with 0.1 µM BK (EDH-type response) (a single concentration of BK was used to elicit the EDH response as in the previous experiments, PVAT and exogenous angiotensin II only had a slight and transient effect and its action on the AT1 receptor may be subject to desensitization) in the absence or presence of PVAT (either without or with the AT1 receptor inhibitor candesartan, 1 µM). It was seen that the response was significantly reduced in the presence of PVAT compared to controls without PVAT (mean ± S.E.M % relaxation: 51.5 ± 7.4 versus 82.1 ± 5.1 respectively) n=6, p<0.05, one-way ANOVA; which was reversed in the additional presence of candesartan (mean ± S.E.M % relaxation: 76.8 ± 6.1 versus 82.1 ± 5.1 in controls), n=6, p=ns, one-way ANOVA (Figure 4.6).

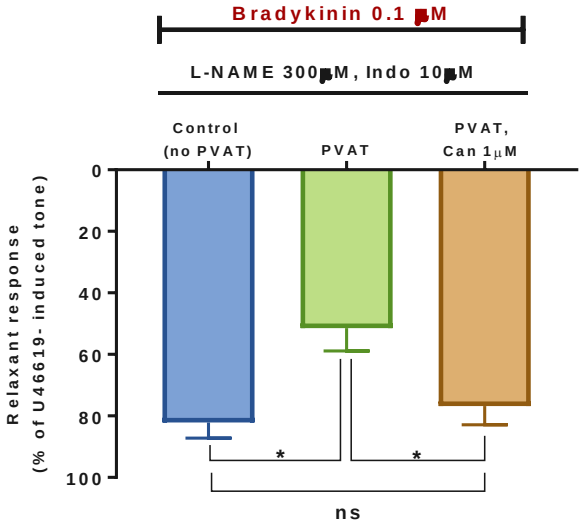


Figure 4.6 Effect of perivascular adipose tissue (PVAT) on the endothelium-dependent hyperpolarization-type (EDH) response of porcine coronary arteries (PCAs). Relaxation was induced by BK (0.1 µM) in the absence or presence of PVAT [with or without inhibition of angiotensin II, type 1 (AT1) receptor using candesartan (Can) 1 µM, in the presence of L-NAME (300 µm) and indomethacin (Indo) (10 µM)]. There was significant inhibition of vasorelaxation by PVAT, which was reversed by inhibition of the vascular AT_1 receptor. Data are expressed as mean ± standard error of the mean (S.E.M) relaxant responses of 6 experiments, in % of maximum U46619 induced contraction. *p<0.05; ns: not significant. Error bars represent S.E.M.

4.3.5 EFFECTS OF EXOGENOUS ANGIOTENSIN II ON THE ENDOTHELIUM-DEPENDENT HYPERPOLARIZATION (EDH)-TYPE RESPONSES

In the presence of L-NAME (300 µM) (NOS inhibitor) and indomethacin (10 µM) (COX inhibitor), the mean ± SEM vasorelaxation (of vessels pre-contracted with U46619) to BK (0.1µM) (endothelium-dependent hyperpolarization (EDH) response), was significantly reduced in vessels

exposed to exogenous angiotensin II (1nM) (60.9 ± 5.6 %) versus the response in control vessels (78.1 ± 4.4 %) (Figures 4.7, 4.8) ($p<0.01$, paired, two-tailed, Student's *t*-test) (Figure 4.8).

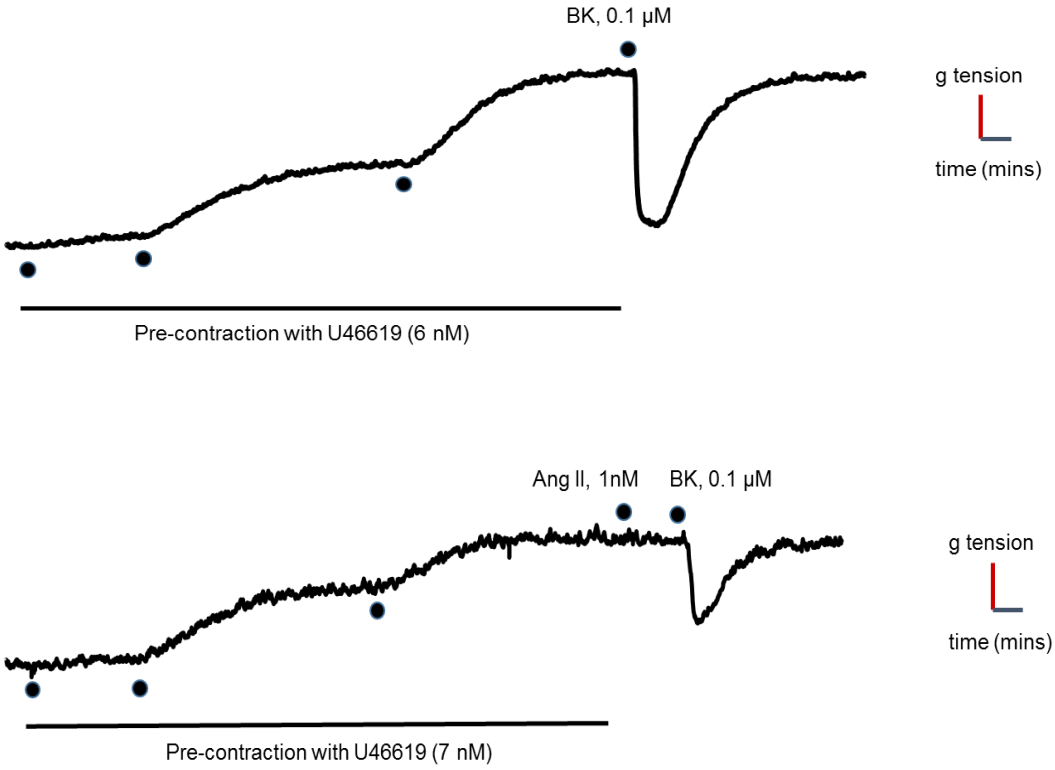


Figure 4.7 Representative traces of the reactivity of PCA segments to pre-contraction with U46619 followed by relaxation to bradykinin (BK) (0.1µM) during NO-synthase and cyclooxygenase inhibition (EDH response), in the absence (top) and presence (bottom) of angiotensin (Ang) II (1 nM), indicating inhibition of the EDH response by exogenous angiotensin II. Grams: g; minutes: mins.

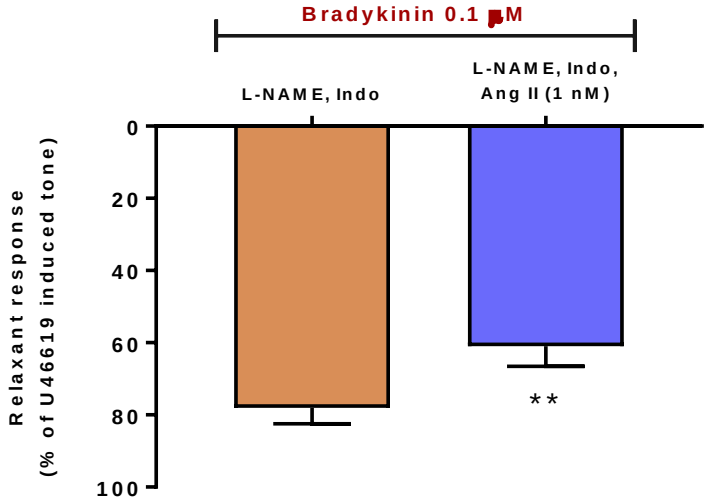


Figure 4.8 Effect of exogenous angiotensin II (Ang II) on the endothelium-dependent hyperpolarization (EDH) response. Relaxant effects of bradykinin (BK) in the presence of L-NAME (300 μm) and indomethacin (Indo) (10 μM) (EDH response) in the porcine coronary arterial segments, with or without exogenous angiotensin II, 1 nM. In the presence of angiotensin II, there was significant inhibition of vasorelaxation. Data are expressed as mean ± standard error of the mean (S.E.M) relaxant responses of 5 experiments, in % of maximum U46619 induced contraction. **p<0.01; paired, two-tailed, Student's *t*-test. Error bars represent S.E.M.

4.3.6 ANGIOTENSIN II LEVELS IN PVAT

In the present study, functional investigations showed that angiotensin II originating from PVAT may have inhibited the endothelium-dependent relaxation of PCAs. Moreover, exogenous angiotensin II also had the same effect on the vessels. To clarify these findings, angiotensin II levels were determined by enzyme linked immunosorbent assay (ELISA) in PVAT derived from PCAs of either gender. Interestingly, this showed the presence of angiotensin II in PVAT and higher mean ± standard error of the mean (S.E.M) levels in females (289 ± 32 pg/mg protein) compared to that in males (158 ± 28 pg/mg protein), n=6, ($p< 0.05$; unpaired, two-tailed, Student's *t*-test) (Figure 4.9).

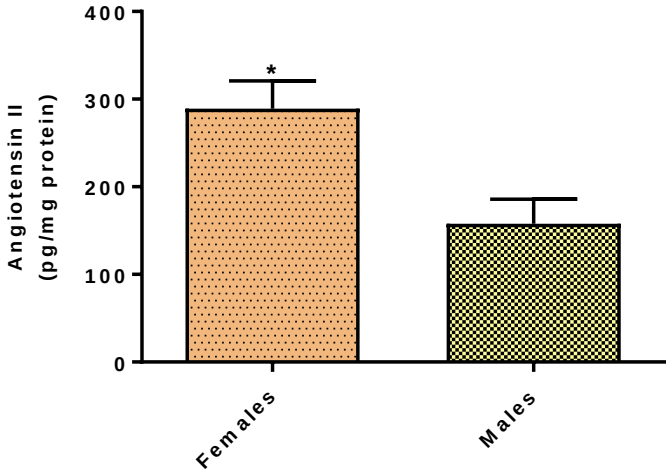


Figure 4.9 Angiotensin II levels in perivascular adipose tissue (PVAT) obtained from porcine coronary arteries of either gender. Data are expressed as mean ± standard error of the mean (S.E.M) angiotensin II level in picograms (pg), expressed as a fraction of total protein content in miligrams (mg) of PVAT obtained from six female and six male animals. *$p<0.05$; unpaired, two-tailed, Student's *t*-test. Error bars represent S.E.M.

4.3.7 Expression of Angiotensin-Converting Enzyme, type 1 (ACE1) in perivascular adipose tissue

Angiotensin-converting enzyme, type 1 (ACE1) expression in perivascular adipose tissue (PVAT) (n=7) (obtained from animals of unspecified gender) was investigated by Western blotting, which showed multiple bands in the higher molecular weight region (predicted molecular weight 150 kD) corresponding to the area of the blot treated with anti-ACE1 primary antibody. Pig kidneys (PK) were positive controls (n=3), which also showed bands within the same region. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a loading control, with its bands evident at the expected 36 kDa region (Figure 4.10).

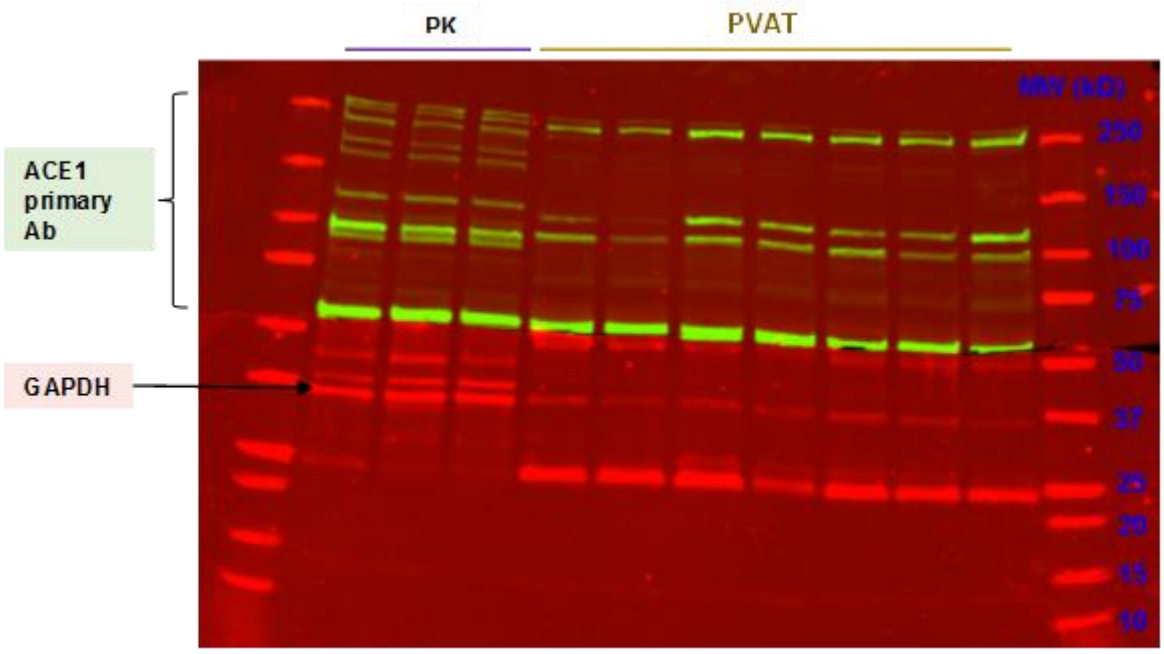


Figure 4.10 Expression of protein bands on the blot treated with angiotensin-converting enzyme, type 1 (ACE1) primary antibody (Ab) in perivascular adipose tissue (PVAT) (n=7). Pig kidney (PK) (n=3) was used as positive control, while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a loading control. kD: kilo Daltons.

Expression of protein bands in PVAT (n=4) corresponding to the ACE primary antibody (Ab) was again shown by Western blotting to be present in the higher molecular weight region, with use of rat kidney (RK), pig kidney (PK) and porcine coronary artery (PCA) as positive controls (Figure 4.11). Part of the blot (75-250 kD region) which contained the positive controls but was not exposed to the ACE1 primary Ab did not show any bands. GAPDH, serving as a loading control, was expressed at the expected 36 kDa region (Figure 4.11).

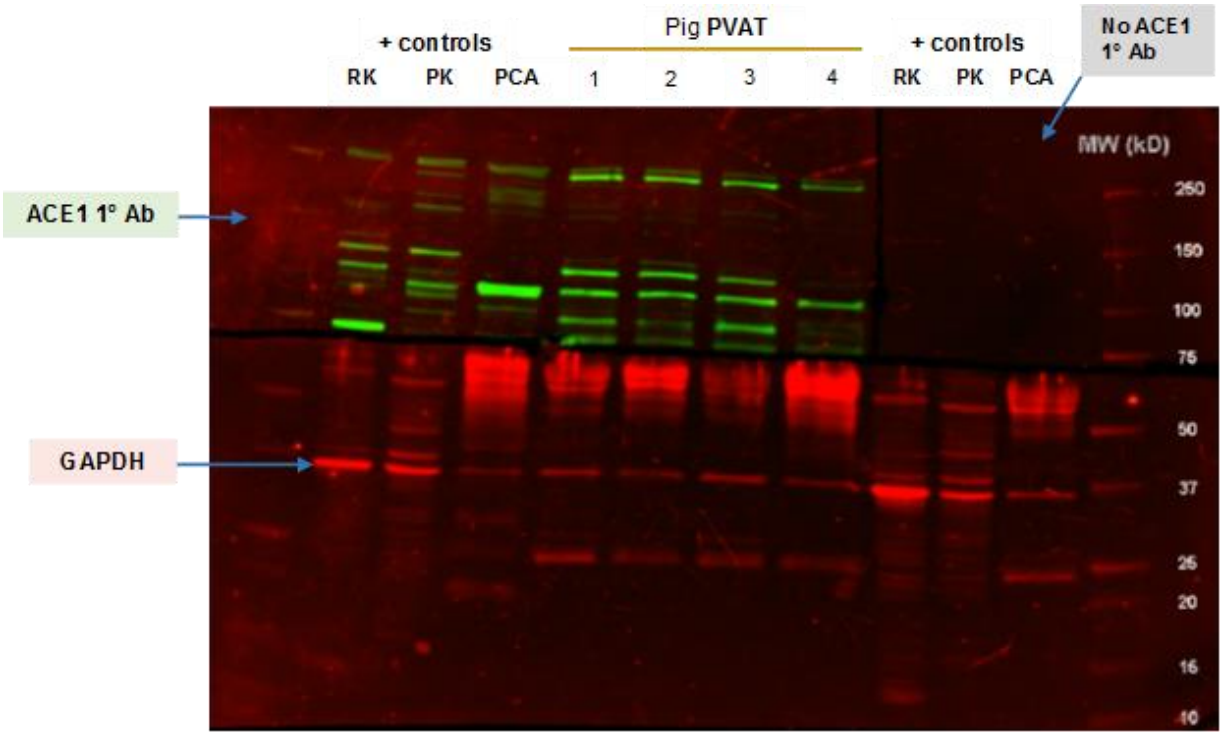


Figure 4.11 Expression of protein bands on the blot treated with angiotensin-converting enzyme, type 1 (ACE1) primary (1°) antibody, in perivascular adipose tissue (PVAT) (n=4). Rat kidney (RK), pig kidney (PK) and porcine coronary artery (PCA) were used as positive (+) controls. A portion (top right side) of the nitrocellulose membrane contained the + controls but was not exposed to the ACE1 primary antibody (Ab), where no bands were seen. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a loading control. kD: kilo Daltons.

4.4 DISCUSSION

Over the last two decades, accumulating evidence has indicated that perivascular adipose tissue (PVAT), the exterior layer of most blood vessels, has a paracrine effect on vascular tone which may also be mediated by alteration of endothelial physiology (Gollasch *et al.*, 2004). Therefore, this study aimed to determine the effect of PVAT on endothelial function of porcine coronary arteries (PCAs).

4.4.1 Role of PVAT in endothelium-dependent arterial relaxation

In PCAs used in the present study, the BK-induced relaxant responses in the segments with PVAT were significantly reduced compared to responses in the control segments without PVAT. This suggests that a contractile factor from PVAT inhibited endothelium-dependent vasorelaxation. Work in rat mesenteric arteries identified O_2^- as a PVAT-derived contractile factor, which was sensitive to inhibition by SOD and apocyanin (an NADPH oxidase inhibitor), indicating that O_2^- generation in PVAT is catalyzed by NADPH oxidase (Gao *et al.*, 2006).

Further evidence from the current study indicated that the vasorelaxation to BK in vessels with PVAT was also significantly reduced compared to the vessels without PVAT, in the presence of L-NAME (a NOS inhibitor) and indomethacin (a COX inhibitor). This would imply that PVAT inhibited EDH-mediated vasorelaxation possibly through the release of an adipocyte-derived contracting factor (ADCF) such as reactive oxygen species or angiotensin II (Brown *et al.*, 2014). A recent study showed that PVAT inhibited endothelium-dependent relaxation of male rat aortas, but through inhibition of NO production (Lee *et al.*, 2014). Other investigations on mesenteric arteries in female rats (with ovaries removed) highlighted decreased endothelium-dependent relaxation

mediated by reactive oxygen species (ROS) originating from PVAT (Wang *et al.*, 2014).

4.4.2 PVAT RELEASED ANGIOTENSIN II WHICH INHIBITED ENDOTHELIAL FUNCTION OF PORCINE CORONARY ARTERIES

In this study, the arteries with PVAT exposed to candesartan [an angiotensin II, type I (AT1) antagonist] showed significant reversal of the PVAT-associated suppression of maximal relaxation to BK. Furthermore, in the absence of PVAT, exogenous angiotensin II significantly reduced the maximum endothelium-dependent vasorelaxation. This points to angiotensin II as the potential contractile factor originating from PVAT, which inhibited endothelium-dependent vasorelaxation (Miao *et al.*, 2012). Moreover, in the present study, during AT1 receptor inhibition, the suppressed EDH response was reversed in vessels with PVAT and exogenous angiotensin II suppressed the EDH response in cleaned PCAs. These data imply that PVAT-derived angiotensin II, acting as an ADCF, may also have inhibited the EDH response.

In the present study, ELISA showed the presence of angiotensin II in PVAT. Angiotensin II was also detected by ELISA in the plasma of pigs, to investigate the potential association between elevated angiotensin II levels and vascular dysfunction (Jakob *et al.*, 2012). Angiotensin II was measured in plasma of anephric patients by other techniques including high-pressure liquid chromatography (HPLC) and radio-immunoassay (RIA), which showed a small but definite presence of angiotensin II and other angiotensins, suggesting that extra-renal tissues contribute to angiotensin production (Wilkes *et al.*, 1991). An *in vitro* study which used human and porcine coronary arteries, measured angiotensin II levels in organ-bath fluid by the RIA technique, after treatment of the vessel rings with angiotensin I in increasing concentrations. Angiotensin II was

detected by this technique, the production of which was dependent on ACE and chymase (Van Den Brink *et al.*, 1999).

In the current study, Western blotting was inconclusive, showing multiple non-specific bands on the part of the blot (75-250 kD region) treated with the ACE1 primary antibody of angiotensin-converting enzyme 1 (ACE1) in PVAT. The enzyme ACE1 produces the octapeptide angiotensin II by cleaving the inactive decapeptide angiotensin I (Bader *et al.*, 2008). Purified ACE1 has been reported to be a single polypeptide of around 150-180 kDa molecular mass (Bernstein *et al.*, 2013) and ACE1 derived from porcine lung was found to have a molecular mass of ~180 kDa, as determined by polyacrylamide gel electrophoresis (Chen *et al.*, 2010). Healthy human subjects were reported to have high (170 kDa) and low (65 kDa) molecular weight ACE1 isoforms, while untreated patients with mild hypertension mainly express low molecular weight (90 and 65 kDa) ACE1 in body fluids including urine (Hattori, 2000).

In comparison, in the current study, the blot showed multiple non-specific bands principally in the lower and higher parts of the range (75-250 kDa) in PVAT as well as in the positive controls (PCA, pig and rat kidneys), which may represent isoforms of ACE1. A study using Western blotting on kidney tissue of mice also demonstrated high (190kDa) and low (65 kDa) molecular weight isoforms of ACE1 (Ribeiro *et al.*, 2015). Moreover, the molecular mass of ACE1 may vary due to differences in its glycosylation, with potential heterogeneity in the oligosaccharides attaching to surface of the peptide (Baudin *et al.*, 1997; Sturrock *et al.*, 2004).

The present findings of ELISA support the results of isometric tension studies implicating angiotensin II as a putative PVAT-derived contractile factor that interfered with endothelium-dependent vasorelaxation. Other investigators examined the effect of PVAT-derived angiotensin II in augmentation of contraction to nerve stimulation in rat mesenteric arteries. They confirmed the expression of angiotensin II, angiotensin I-converting enzyme and angiotensinogen in mesenteric PVAT and

showed that treatment of the vessels with the AT1 receptor blocker candesartan reduced the PVAT-mediated augmentation of vessel contraction to nerve stimulation (Lu *et al.*, 2010). Our study suggested that PVAT of PCAs may have released angiotensin II, which caused a contractile effect and so inhibited endothelium-dependent vasorelaxation evoked by BK.

An *ex vivo* study on isolated mesenteric artery of male Wistar rats, which had previously been treated with an angiotensin II infusion to induce hypertension, showed significantly reduced EDH-mediated vasorelaxation (Dal-Ros *et al.*, 2009). Evidence from another work on isolated mesenteric arteries of hypertensive male rats demonstrated significantly reduced endothelium-dependent relaxations to acetyl-choline in the absence and presence of NOS and COX inhibition, the later indicating that the EDH-mediated relaxation response were impaired. These derangements were ameliorated after treatment with an AT_1 blocker as well as an angiotensin-converting enzyme inhibitor, pointing to involvement of angiotensin II in reducing the endothelium-dependent relaxant response (Goto *et al.*, 2000).

Gender differences have been demonstrated in endothelium-dependent vasorelaxation as well as in the expression and functioning of the renin-angiotensin-system (RAS) (Hilliard *et al.*, 2013; Wang *et al.*, 2014). In pre-menopausal females, ovarian hormones facilitate endothelium-dependent vasorelaxation and the vasodilator limb of RAS [ACE2/Angiotensin 1-7)/AT2R], while, with loss of protective effect of ovarian hormones after menopause and in males, the balance shifts towards the pressor limb of RAS [ACE1/Angiotensin II/AT1R] (Hilliard *et al.*, 2013).

In the present study, the angiotensin II levels were higher in PVAT of females. This may be due to the fact that the animals used in this study included older females (up to 3 years of age). These older animals may have diminished ovarian hormonal protection and therefore a dysfunctional PVAT swinging towards the pressor limb of RAS. Also,

upregulation of the pressor limb of RAS in these females may have occurred due to the estrus cycle: angiotensin II levels are higher in the luteal phase which lasts for majority (13-15) of the 24 days' cycle (O'Donnel *et al.*, 2014; Soede *et al.*, 2011).

In the present study, endothelium-dependent vasorelaxation of PCAs may have been inhibited due to the effect of angiotensin II issuing from PVAT (Figure 4.12). The possible underlying mechanisms may include inhibition of the NO-signalling pathway, production of ROS, induction of inflammation or inhibition of calcium-activated potassium channels in the endothelium (Minami *et al.*, 1995; Nakashima *et al.*, 2006). The suppressed endothelium-dependent relaxation was ameliorated after treatment with the AT1 receptor antagonist candesartan, which supports the use of this treatment modality (systemically and locally) in endothelial dysfunction associated with impaired PVAT physiology in obesity (Moon *et al.*, 2004) (Xia *et al.*, 2016b). Other studies have shown alternative mechanisms of inhibition of endothelium-dependent vasorelaxation by PVAT, including through inhibition of NO production in male rat aortas (Lee *et al.*, 2014) and by the effect of ROS in female rat mesenteric arteries (Wang *et al.*, 2014).

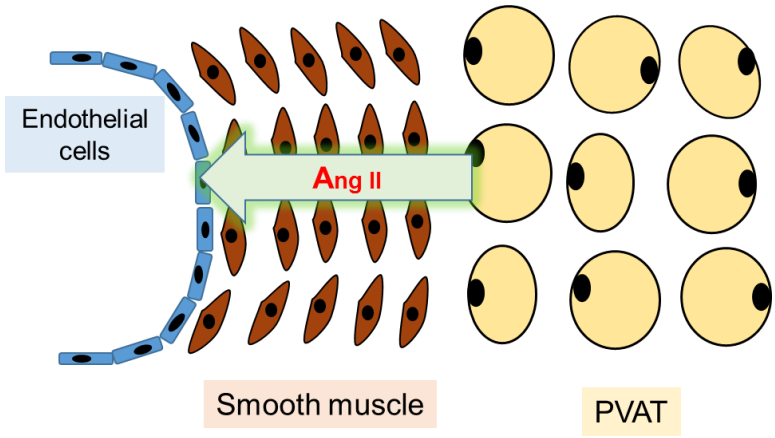


Figure 4.12 Potential impact of angiotensin II (Ang II) released from perivascular adipose tissue (PVAT) on the endothelial cells to inhibit their function, in porcine coronary artery.

The present study has limitations. The n numbers are small and only expression of two components of RAS, namely angiotensin II and ACE 1 in PVAT was investigated. The peptide bands observed during Western blotting showed expression at multiple non-specific sites (Bernstein *et al.*, 2013). This could be investigated further with Western blotting using PVAT from the pig and other animal models.

4.4.3 SUMMARY AND FUTURE DIRECTIONS

PVAT inhibited the maximal endothelium-dependent vasorelaxation induced by BK. The inhibition was reversed when AT1 receptor was blocked. PVAT also inhibited the vasorelaxation resulting from endothelium-dependent hyperpolarization (EDH), which was ameliorated during AT1 receptor blockade. Exogenous angiotensin II also inhibited the maximal endothelial and EDH-mediated vasorelaxations. Angiotensin II was detected in PVAT, whose production in this tissue may potentially have been catalysed by the ACE 1 enzyme. However, the expression of this enzyme could not be confirmed in the present study. These data lend support to the hypothesis that PVAT may have generated angiotensin II which inhibited the endothelium-dependent vasorelaxation induced by BK thus modulating vascular tone.

To further investigate the area, the work may be extended by performing functional studies looking at the effect of physiological concentrations (1 nM) of exogenous angiotensin II incubated with pre-contracted PCAs for a shorter period (10 minutes) (to avoid the potential desensitization of the vascular AT1 receptor by exposure to higher concentration of angiotensin II for a longer period) followed by undertaking endothelium-dependent relaxant responses to cumulative BK, comparing the results with that in a control group of vessels not exposed to angiotensin II.

In the present work, Western blotting was inconclusive and showed multiple non-specific bands which may have been due to variable glycosylation of the protein, possibly enhanced by storage of the tissue

in the buffer solution containing 12 mM glucose. The Western blotting procedure may be repeated by using physiological concentrations (5 mM) of glucose in the buffer solution to ascertain the presence of ACE1 protein bands at the predicted molecular weight. Furthermore, future studies incorporating isometric-tension studies, immunoblotting and immunohistochemical imaging are needed to confirm the presence of angiotensin II, ACE1, the AT1 receptor and aldosterone (which are parts of the vasoconstrictor limb of RAS) in coronary artery PVAT. The counterbalancing vasodilator arm of RAS whose effect should dominate in the physiological state and which is composed of angiotensin 1-7, ACE 2, MAS and AT2 receptors may also be looked for in the PVAT of coronary artery (Huang Cao *et al.*, 2017) (Agrawal *et al.*, 2016).

As inhibition of the AT1 receptor in the present study improved the impaired endothelium-dependent vasorelaxation in PCAs, use of AT1 receptor blockade may be of therapeutic benefit in endothelial dysfunction evoked by PVAT-derived angiotensin II (Bader *et al.*, 2008).

Chapter 5

EFFECTS OF PERIVASCULAR ADIPOSE TISSUE ON VASCULAR POTASSIUM CHANNELS

5.1 INTRODUCTION

Hypertension, in association with obesity, is a significant burden on health care costs (Boden, 2008; Wang *et al.*, 2011). These co-morbidities are linked to familial factors, sedentary life style and an excessive caloric intake (Zimmet *et al.*, 2001) . In the year 2000, about 1 billion adults were estimated to have hypertension globally, with the figure predicted to increase to 1.56 billion by 2025 (Joffres *et al.*, 2013). In the UK, about 30 % of adults were reported to be hypertensive (Falaschetti *et al.*, 2009).

Hypertension is often asymptomatic and can insidiously lead to cardiovascular dysfunction including coronary artery disease, heart failure, cerebrovascular and renal disease (Gollasch, 2012; Loh *et al.*, 2018). The underlying mechanisms involved in development of hypertension in obesity include increased free fatty acid production and insulin resistance, sodium retention, reduced endothelial NO production, increased leptin as well as ROS synthesis. As a result, vasodilatation is reduced and development of hypertension is promoted (Inoguchi *et al.*, 2000). Furthermore, obesity is accompanied by increase in the overall blood volume, sodium retention, leptin levels and sympathetic nerve activity, increasing the blood pressure (Fortuño *et al.*, 2003; Hall *et al.*, 2001). However, obesity is also associated with dysfunction of PVAT, which secretes a number of vasoactive molecules, and whose potential role in impaired vascular reactivity has not received much attention (Oriowo, 2015).

Perivascular adipose tissue (PVAT) is reported to produce an adipocyte-derived relaxing factor (ADRF), which may cause vasodilatation by opening K^+ channels in vascular smooth muscle (Galvez *et al.*, 2006; Lohn *et al.*, 2002; Oriowo, 2015). Studies on rat aorta and the mouse mesenteric artery indicated that adiponectin released from PVAT causes vasodilatation through its action on voltage dependent K^+ (K_v) channels (Fésüs *et al.*, 2007).

As described previously, other potential ADRFs including H_2S (Fang *et al.*, 2009), angiotensin 1-7 (Gao *et al.*, 2007) palmitic acid methyl ester (Lee *et al.*, 2011) and prostacyclin (Chang *et al.*, 2012). The ADRF response may also be brought about by activation of the ATP-sensitive potassium channel (K_{ATP}) and calcium-dependent K^+ channel (K_{Ca}) (Lohn *et al.*, 2002) (Gao *et al.*, 2007).

PVAT releases contractile factors which can potentially inhibit the function of vascular K^+ channels, as evidenced by a study in Ossabaw swine, which showed that coronary PVAT had an anti-contractile effect and inhibited K_{Ca}, K_v and K_{ATP} channels (Noblet *et al.*, 2015). Vasopressor components of the renin-angiotensin system (RAS) including angiotensin II and aldosterone have been shown to be expressed in PVAT and angiotensin II may induce vasoconstriction by impairing the function of vascular K_V channels (Rainbow *et al.*, 2009) potentially contributing to vascular dysfunction in hypertension and obesity (Nguyen Dinh Cat *et al.*, 2011c).

Experiments in aortae of obese mice demonstrated a pro-contractile effect of PVAT which was sensitive to inhibition by a COX inhibitor. This was accompanied by increased level of the contractile prostanoid TXA_2 in PVAT which may act by inhibiting vascular K_v channels (Meyer *et al.*, 2013). Moreover, it was observed that in hypertension, the loss of anti-contractile effect of PVAT could be reversed by treatment with Kv channel activators, suggesting a potential role for these channels in development of hypertension in PVAT dysfunction (Zaborska *et al.*, 2017). Further support for the crucial role of K_v channel inhibition in development of increased vascular resistance in conjunction with PVAT comes from studies in murine and rat models of hypertension and obesity, in which Kv inhibition with 4-aminopyridine led to loss of the anti-contractile effect of PVAT (Tano *et al.*, 2014).

The evidence from the above studies indicates that PVAT can potentially influence the function of K^+ channels located in the underlying vessel through the mediation of paracrine contractile and relaxant factors.

Hypothesis and aims

Based on the above evidence, the present study hypothesized that PVAT may modulate the function of potassium channels of PCAs. Therefore, functional studies were performed which aimed to investigate the influence of PVAT on relaxation induced by activators of various potassium channels (K_{ATP}, BK_{Ca} and K_V) and constriction evoked by inhibition of K_V channels of PCAs. The study also aimed to determine the effects of PVAT on PCA contraction to K_V channel inhibition in the absence or presence of COX and TXA_2 inhibitors.

5.2 METHODS AND MATERIALS

5.2.1 FUNCTIONAL STUDIES

Coronary arterial segments and PVAT was prepared for isometric tension studies in organ-bath set-ups as described previously.

VASCULAR EFFECTS OF POTASSIUM CHANNEL MODULATORS IN RELATION TO PVAT

ATP-SENSITIVE POTASSIUM CHANNEL (K_{ATP}) AGONIST STUDY

As mentioned, PVAT may release factors which activate vascular smooth muscle K_{ATP} channels, resulting in increased efflux of potassium and smooth muscle cell hyperpolarization resulting in vasorelaxation (Lohn *et al.*, 2002). Hence experiments in this study investigated the effect of isolated PVAT on relaxant responses of PCAs (of both genders) to cumulative Pinacidil (a K_{ATP} activator). After establishing baseline contractility of the vessel segments with 60 mM KCl as described previously, U46619 was added in increasing concentrations (1-9.6 nM) to achieve pre-contraction to 40-60 % of the second KCl response. This was followed immediately by assessing the relaxant responses to increasing concentrations of pinacidil (10 nM-10 µM) in vascular segments with and without PVAT derived from the same PCA.

LARGE-CONDUCTANCE, CALCIUM-DEPENDENT, POTASSIUM CHANNEL (BK_{Ca}) AGONIST STUDY

As discussed previously, BK_{Ca} channels of vascular smooth muscle may be activated by adipocyte-derived relaxant factors to elicit vasodilatation, so these set of experiments investigated the effect of PVAT on relaxation of PCAs to the BK_{Ca} activator NS1619. The two groups of PCA segments (in the absence or presence of isolated PVAT respectively) were pre-contracted with U46619 (4-18 nM) to a level of 40-60 % of the second

KCl response to achieve a stable tone. Then NS1619 was added to the organ baths in cumulative concentrations (0.1 pM -1 µM) and the relaxant responses compared in segments with PVAT to those without PVAT (control).

BK_{Ca} CHANNEL AGONIST AND HYDROGEN SULPHIDE (H_2S) DONOR STUDY

Because the gaseous mediator H_2S may be manufactured in PVAT and can contribute to activation of BK_{Ca} channels, this study investigated the effect of exogenous H_2S on tone of pre-contracted PCAs (with or without PVAT) relaxed with EC_{50} concentration of the BK_{Ca} activator NS1619 used in the previous study. After achieving pre-contraction with U46619 (6-34 nM), 0.1 nM of NS1619 was added to the baths containing vascular segments without PVAT (control) and those with PVAT. The experiments were then extended by adding sodium hydrogen sulphide (NaHS) (an H_2S donor) (1 mM) and the relaxant responses were compared in the two segments.

VOLTAGE-DEPENDENT POTASSIUM CHANNEL (K_V) AGONIST STUDY

As was reported before, the K_v channels in the vascular smooth muscle can also be a target of adipocyte-derived paracrine factors potentially altering vessel tone. So, for these experiments, two vascular segments were obtained from each PCA and the initial steps were as in the above study. After attaining pre-contraction of the tissue with U46619 (7.5-18.5 nM), the compound L364,373 (a K_V channel agonist) was added to the baths containing the segments with and without PVAT, in cumulative concentrations (0.1 nM-10 µM), to elicit a relaxant response.

K_V CHANNEL ANTAGONIST STUDY

Inhibition of K_v channels of vascular smooth muscle will lead to depolarization of the cells resulting in vasoconstriction. PVAT-derived vasoactive factors which target the K_v channels may alter the

aforementioned response. So, this study looked at the effect of isolated PVAT on the response to K_V inhibition of PCAs. For this purpose, PCAs obtained from female animals were used and the initial steps were same as in the above study. Thirty minutes after the end of the response to the second concentration of KCl (60 mM), the K_V channel antagonist, 4-aminopyridine (4-AP) (1 mM), was added to the vascular segments from the same PCA in the presence and absence of PVAT. The resultant contractile responses in the two segments were compared.

K_V CHANNEL ANTAGONIST AND AGONIST STUDY

This study investigated whether K_V channel inhibition affected the relaxant response of PCAs to Kv channel activation either in the absence or presence of PVAT. PCAs were obtained from animals of unspecified gender, the first two baths containing vascular segments without PVAT while the third and fourth baths contained segments with PVAT from the same PCA. Thirty minutes following the initial steps involving KCl additions as in the previous study, 4-AP (a K_V channel blocker; 1 mM) was added to the second and fourth segments to ascertain whether it prevented the subsequent L364,373-induced relaxant response in segments without and with PVAT respectively.

After incubation with this compound for at least one hour and noting the resulting contractile response, U46619 (in absence of 4AP: 9-24 nM; in presence of 4AP: 1.4-6 nM) was added to attain pre-contraction to a level of 40-60 % of the second KCl response. This was followed by addition of L364,373 (a K_V channel agonist) in cumulative concentrations (0.1nM-3µM). The resulting relaxant responses in vascular segments without PVAT, but in the absence and presence of 4-AP (1 mM) were compared with each other. Also, the relaxant responses in the segments which were in the presence of PVAT but in the absence or presence of 4-AP (1 mM) were compared with each other.

STUDY USING A K_V CHANNEL ANTAGONIST WITH/WITHOUT CYCLOOXYGENASE INHIBITION

Previous experiments showed that inhibition of K_v channels of PCA segments in the presence of PVAT led to a significantly increased contractile response compared to segments without PVAT. This may have been due to the release of a contractile factor, possibly a prostanoid, from PVAT which augmented the closure of K_v channels. Hence, in this study cyclooxygenase was inhibited in a group of vessels with PVAT and the effect compared to the controls. Vessels obtained from hearts of animals of unspecified gender were used in this study. Three adjacent 5 mm vessel rings obtained from a length of PCA were prepared. Then the vessel in the third bath was exposed to indomethacin (10 µM) for one hour to inhibit the cyclooxygenase enzyme. Following this, isolated PVAT (0.3 g each) was added to the 2nd and 3rd baths and incubated with the vessels for one hour. The arterial segment in the first bath served as a control. Then 4AP (1 mM) was added to all the tissue baths and subsequent change in vessel tone was assessed on achieving a stable contraction (after at least an hour).

STUDY USING A K_V CHANNEL ANTAGONIST WITH/WITHOUT THROMBOXANE A_2 RECEPTOR INHIBITION

The previous study demonstrated that the enhanced contractile response to Kv inhibition in vessels exposed to PVAT was significantly attenuated during cyclooxygenase inhibition. This finding pointed to the release of a contractile prostanoid from PVAT which may have augmented the closure of vascular K_v channels. To investigate if the prostanoid involved is TXA_2, a TXA_2 receptor antagonist (ICI192605) was used. Four groups of vessels were utilised as per the following plan: the arterial segment in the third bath was incubated with ICI192605 (1 µM: 5 µl) (Mueed *et al.*, 2008; Tazzeo *et al.*, 2003) for one hour to block the TXA_2 receptor, while the vessel in the fourth bath was exposed to ethanol (5 µl) (0.1 % v/v) (vehicle control). Following this, isolated PVAT (0.3 g each) was added to the 2nd - 4th baths and incubated with the vessels for one hour. The

vessel in the first bath served as control. Then 4-AP (1 mM) was added to all the tissue baths and subsequent change in vessel tone was assessed on achieving a stable contraction (after at least an hour).

5.2.2 CHEMICALS

Potassium chloride (KCl) was obtained from Fisher Scientific. Indomethacin (a cyclooxygenase inhibitor), pinacidil (K_{ATP} channel agonist), NaHS (H_2S donor) and 4-amino pyridine (4-AP) (a K_V channel antagonist) were obtained from Sigma-Aldrich. U46619 (a TXA_2 receptor agonist), NS1619 (BK_{Ca} channel agonist), L364,373 (a K_V channel agonist) and ICI192605 (a TXA_2 receptor antagonist) were obtained from Tocris Bioscience.

Stock solutions of KCl, NaHS and 4-AP were freshly made in distilled-water. Stock solution of U46619 was made in methanol and its subsequent dilutions were made in distilled water. Stock solutions of pinacidil, NS1619 and L-364,373 were made in dimethyl sulfoxide (DMSO) (its concentration in the tissue bath was 0.1 % v/v and both study and control groups were exposed to compounds dissolved in the same amount of solvent). Stock solutions of indomethacin and ICI192605 were made in ethanol whose final bath concentration was 0.1 % v/v (functional studies employing ethanol as a vehicle control showed it to have no significant effects on vascular reactivity). After formulation, all the solutions except KCl, were stored at -20 ºC.

5.2.3 STATISTICAL ANALYSIS

Data are expressed as mean with standard error of the mean (S.E.M) change in tension (in grams) or mean percentage (%) with S.E.M change in tension. The ‘n’ in the results indicates the number of individual animals. The collected data were entered and analyzed in GraphPad Prism® version 7 (La Jolla, CA, USA), by unpaired, two-tailed, Student’s

t-test for comparison of two groups and analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test for comparison of more than two groups. A p value of <0.05 was considered statistically significant.

5.3 RESULTS

5.3.1 EFFECTS OF MODULATORS OF VASCULAR POTASSIUM (K) CHANNELS

5.3.1.1 K_{ATP} CHANNEL AGONIST (PINACIDIL) STUDIES

Porcine coronary arteries (PCAs) of female animals

After achieving the target contractile responses of the vascular segments with and without PVAT with U46619, pinacidil was immediately added in cumulative concentrations (10nM-10μM) to the organ baths, in order to construct concentration-response relaxation curves. It was observed that there was no significant difference between the relaxation-response curve of the segments with PVAT and that of the control segments (n=4) (two-way ANOVA with Bonferroni's *post hoc* test) (Figure 5.1).

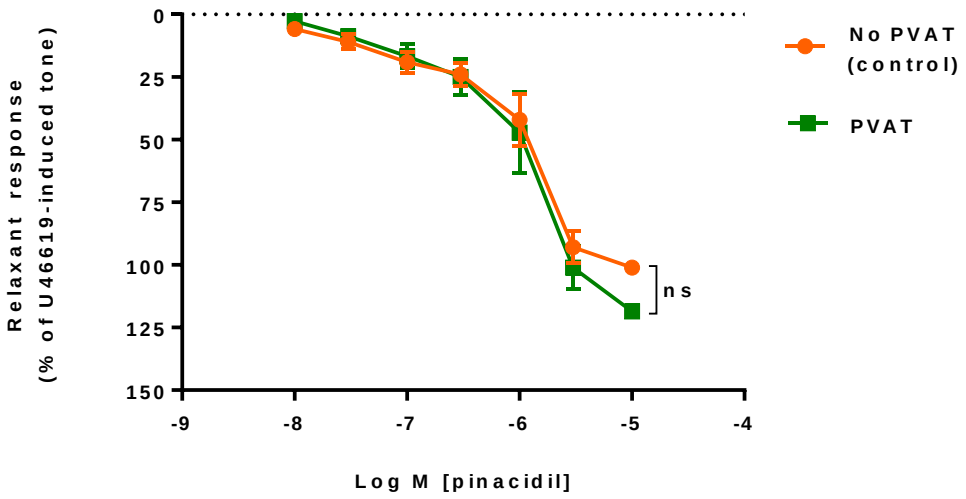


Figure 5.1 Responses in PCAs showing relaxant responses to pinacidil, with and without perivascular adipose tissue (PVAT), in female animals. The responses in the vascular segments with and without PVAT showed no difference. Data are expressed as mean ± standard error of the mean (S.E.M) relaxant responses of 4 experiments, in % of maximum U46619 induced contraction. The bars represent S.E.M. Not significant: ns.

Porcine coronary arteries (PCAs) of male animals

After achieving the target contractile responses of the vascular segments with and without PVAT with U46619, pinacidil was immediately added in cumulative concentrations (10 nM-10 μM) to the organ baths, in order to construct concentration-response relaxation curves. It was observed that the relaxant response corresponding to 10 μM pinacidil concentration was significantly enhanced in segments exposed to PVAT compared to the control segments (mean ± S.E.M difference between the groups: 24.6 ± 6.4) ($p<0.05$, two-way ANOVA with Bonferroni's *post hoc* test, n=6) (Figure 5.2).

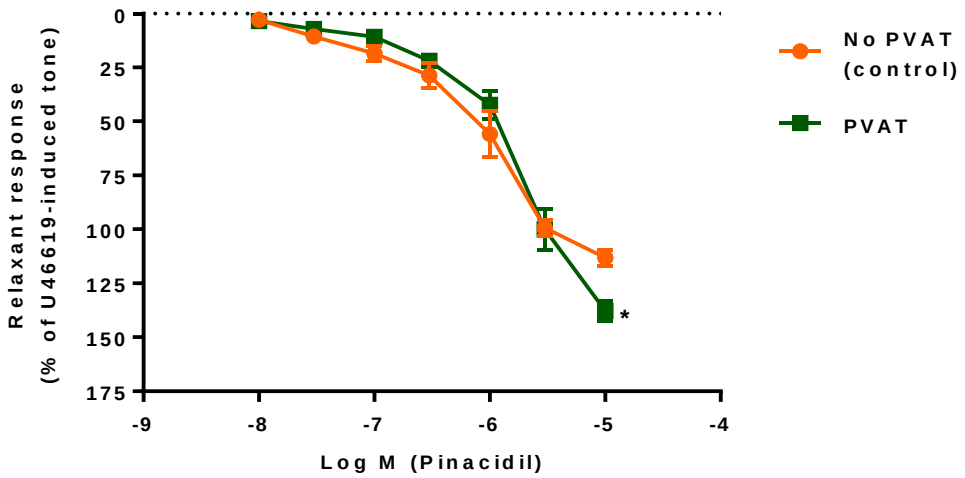


Figure 5.2 Responses in porcine coronary arteries showing relaxant effects of pinacidil, with and without perivascular adipose tissue (PVAT) in male animals. The response at 10μM pinacidil concentration was significantly increased in the segments with PVAT compared to the control group. Data are expressed as mean ± standard error of the mean (S.E.M) relaxant responses of 6 experiments, in % of maximum U46619 induced contraction. The bars represent S.E.M. *$p<0.05$.

5.3.1.2 BK_{Ca} CHANNEL AGONIST (NS1619) STUDY

Porcine coronary arteries (PCAs) of female animals

After achieving the target contractile responses of the vascular segments with and without PVAT with U46619, NS1619 (a large-conductance, calcium-dependent, potassium (BK_{Ca}) channel agonist) was added to the organ baths in cumulative concentrations (0.1pM-0.1μM) in order to construct concentration-response relaxation curves. There was no significant differences between the relaxant responses to NS1619 in the segments with PVAT and the control segments (n=4) (two-way ANOVA with Bonferroni's *post hoc* test) (Figure 5.3).

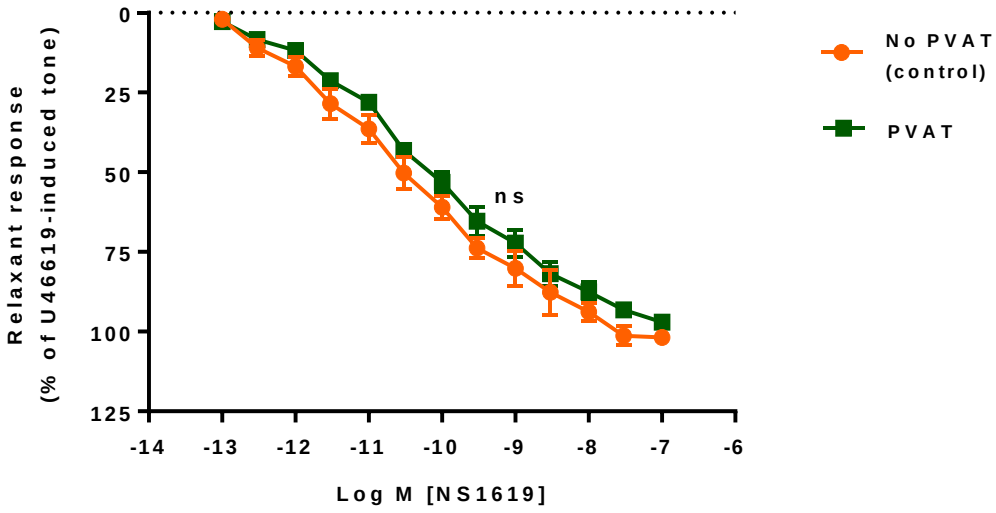


Figure 5.3 Concentration-responses to NS1619 (0.1pM-100nM) in porcine coronary arteries with and without perivascular adipose tissue (PVAT) in female animals. Data are expressed as mean ± standard error of the mean (S.E.M) relaxant responses of 4 experiments, in % of maximum U46619 induced contraction. The bars represent S.E.M. Not significant: ns.

Porcine coronary arteries (PCAs) of male animals

The pre-contracted PCA segments were exposed to the BK_{Ca} channel activator NS1619 in cumulative concentrations (0.1pM-1µM) in order to construct concentration-response curves. It was noted that there was no significant difference between the relaxant responses to NS1619 in the segments with PVAT and the control segments (n=5) (two-way ANOVA with Bonferroni's *post hoc* test) (Figure 5.4).

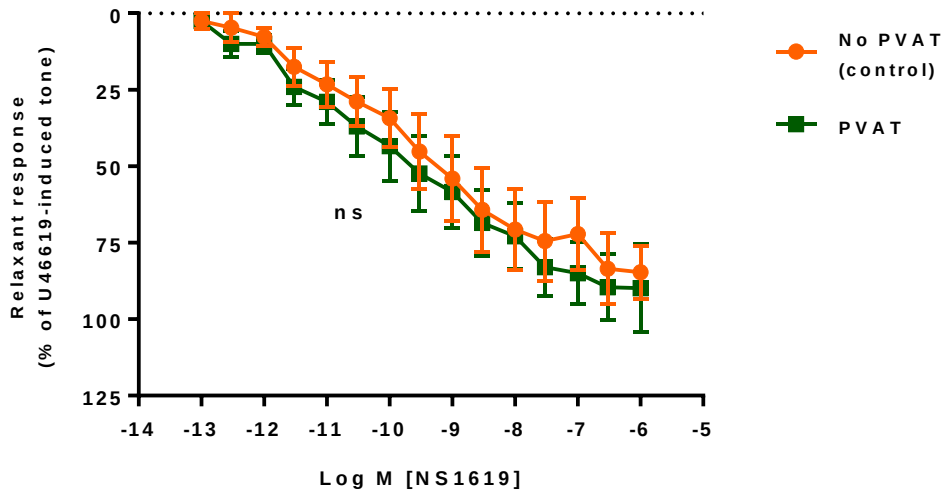


Figure 5.4 Concentration-responses to NS1619 (0.1pM-1µM) in PCAs with and without perivascular adipose tissue (PVAT) in male animals. Data are expressed as mean ± standard error of the mean (S.E.M) relaxant responses of 5 experiments, in % of maximum U46619 induced contraction. The bars represent S.E.M. Not significant: ns.

BK_{Ca} agonist (NS1619) and H_2S donor (NaHS) study

In the previous NS1619 study, gender differences in the relaxant responses were not seen. So, in the present study involving the additional use of NaHS, vessels from both female and male animals were used without focusing on any particular gender. After achieving pre-contraction with U46619 (40-60 % of KCl response), EC_{50} concentration (0.1 nM) of NS1619 was added to the baths containing vascular segments without PVAT (control) and those with PVAT (Figure 5.5). As H_2S may be

released by PVAT and can potentially interfere with activity of vascular BK_{Ca} channels, the experiments were extended by adding NaHS (an H_2S donor) (1 mM) to the buffer solution in the organ baths. The resulting response in the PCA segments with PVAT were compared to control segments without PVAT (Figure 5.5).

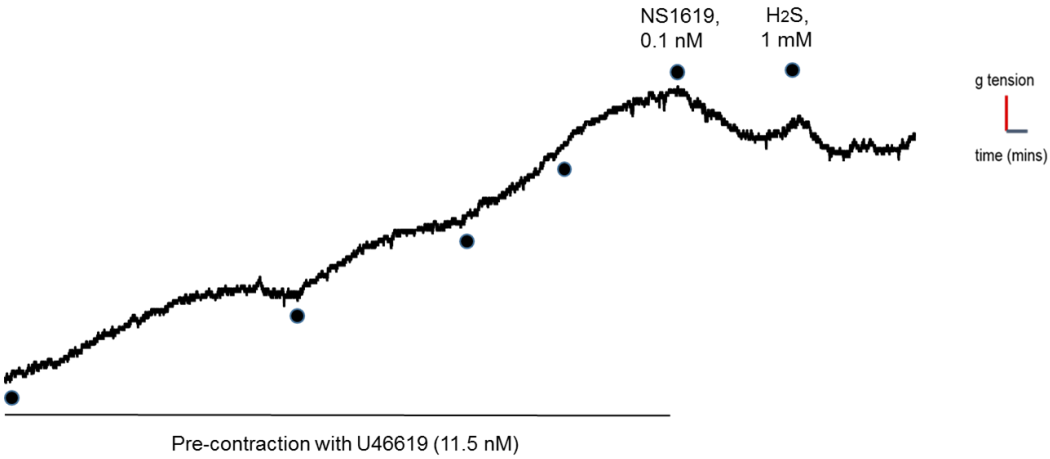


Figure 5.5 Lab Chart® representative trace showing the pre-contraction response (in grams (g) tension) to U46619 followed by relaxant responses to EC_{50} concentration (0.1 nM) of NS1619 and then to sodium hydrogen sulphide (NaHS) (1 mM) in a porcine coronary arterial segment.

Effect of EC_{50} concentration of the BK_{Ca} agonist (NS1619)

The experiments showed that the relaxant responses to EC_{50} concentration (0.1 nM) of the BK_{Ca} agonist NS1619 in the segments with PVAT were not significantly different (mean ± standard error of the mean (S.E.M) relaxation: 10 ± 0.7 %; n = 5) to those in the segments without PVAT (mean ± S.E.M relaxation: 9.3 ± 0.7 %; n = 5) (unpaired, two-tailed, Student's *t*-test) (Figure 5.6). This showed that in these vessels, PVAT did not potentiate the relaxant effects of NS1619, consistent with the previous concentration-response experiments.

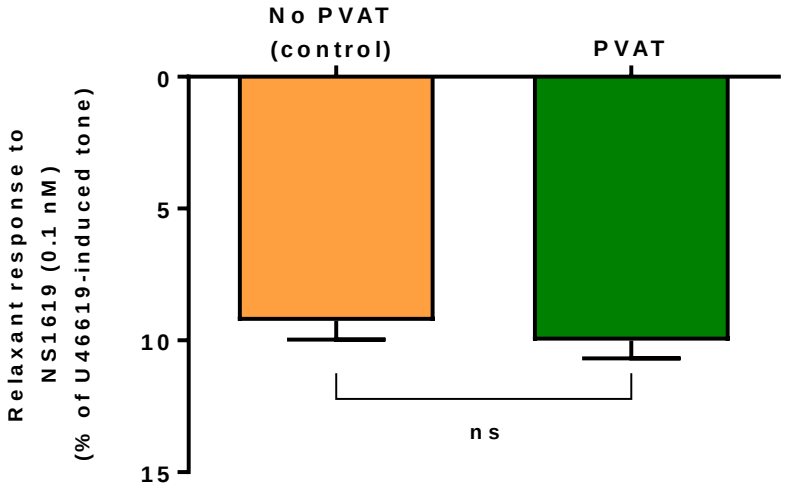


Figure 5.6 Relaxant effects of EC_{50} concentration (0.1 nM) of NS1619 in porcine coronary arterial segments, with and without perivascular adipose tissue (PVAT). Data are expressed as mean ± standard error of the mean (S.E.M) relaxant responses of 5 experiments, in percentage (%) of U46619 induced pre-contraction. The error bars represent S.E.M. Not significant: ns.

Effect of an H_2S donor (NaHS)

It was observed that the relaxant responses to sodium hydrogen sulphide (NaHS) (an H_2S donor) (1mM) were not significantly different in segments with PVAT (mean ± standard error of the mean (S.E.M) relaxation: 8.1 ± 1 %; n=4) to those in the segments without PVAT (mean ± S.E.M relaxation: 7.3 ± 0.9 g; n=4) (unpaired, two-tailed, Student's *t*-test) (Figure 5.7). These data suggest that PVAT did not interfere with exogenous H_2S-induced relaxation of porcine coronary artery in the presence of a BK_{Ca} agonist.

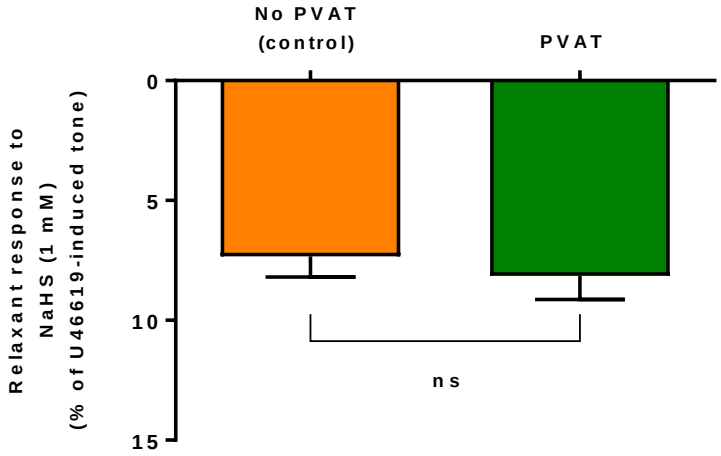


Figure 5.7 Relaxant effects of sodium hydrogen sulphide (NaHS) (an H_2S donor) (1 mM) were not significantly different in vascular segments with perivascular adipose tissue (PVAT) compared to those without PVAT. Data are expressed as mean ± standard error of the mean (S.E.M) relaxant responses of 4 experiments, in percentage (%) of U46619-induced pre-contraction. The error-bars represent S.E.M. Not significant: ns.

5.3.1.3. EFFECT OF A K_V CHANNEL AGONIST (L364,373)

PCAs obtained from female animals were used for this study. After attaining the target pre-contractile responses of the vascular segments with and without PVAT, L364,373 was added to the baths in cumulative concentrations (0.1nM-10µM) in order to construct concentration-response curves. It was observed that there was no significant difference between the relaxant responses in the segments with PVAT and the control segments (p=not significant, two-way ANOVA with Bonferroni's *post hoc* test, n=4) (Figure 5.8).

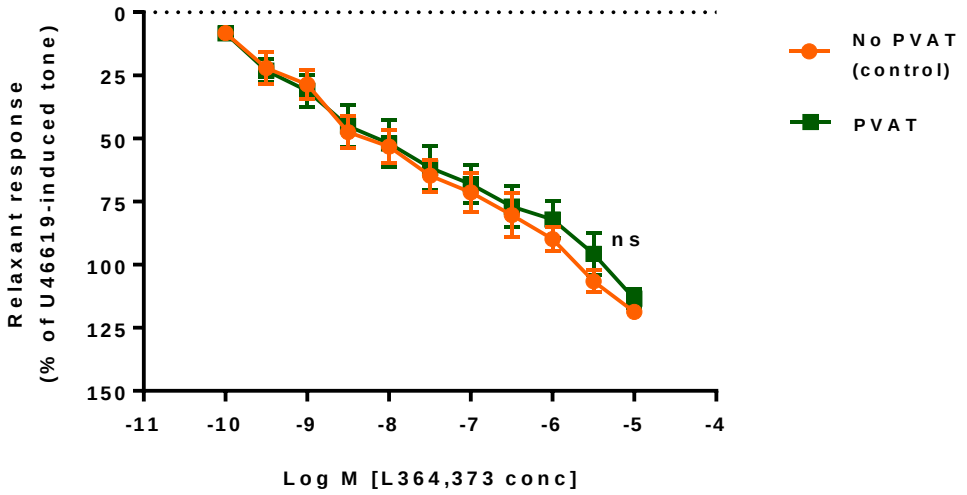


Figure 5.8 Concentration-responses to L364,373 (0.1nM-10μM) in porcine coronary arteries with and without perivascular adipose tissue (PVAT) in female animals. Data are expressed as mean ± standard error of the mean (S.E.M) relaxant responses of 4 experiments, in % of maximum U46619-induced contraction. The bars represent S.E.M. Not significant: ns.

5.3.1.4 EFFECT OF A K_V CHANNEL ANTAGONIST (4-AMINOPYRIDINE)

PCAs derived from female animals were also used in this study. Thirty minutes after the second KCl response, the K_V channel antagonist 4-aminopyridine (4-AP) (1 mM) was added to the vascular segments from the same PCA in the presence and absence of PVAT. Interestingly, a higher contractile response with fluctuations in tone was seen in segments exposed to PVAT compared to the controls (Figures 5.9, 5.10). Following the K_V channel inhibition with 4-AP (1 mM), exposure of the vessels to a TXA_2 receptor agonist (U46619) in low concentration (1 nM) showed a prompt contractile response, with increased intensity in the presence of PVAT (0.3 g) which was accompanied by prominent fluctuations in vessel tone (Figure 5.11).

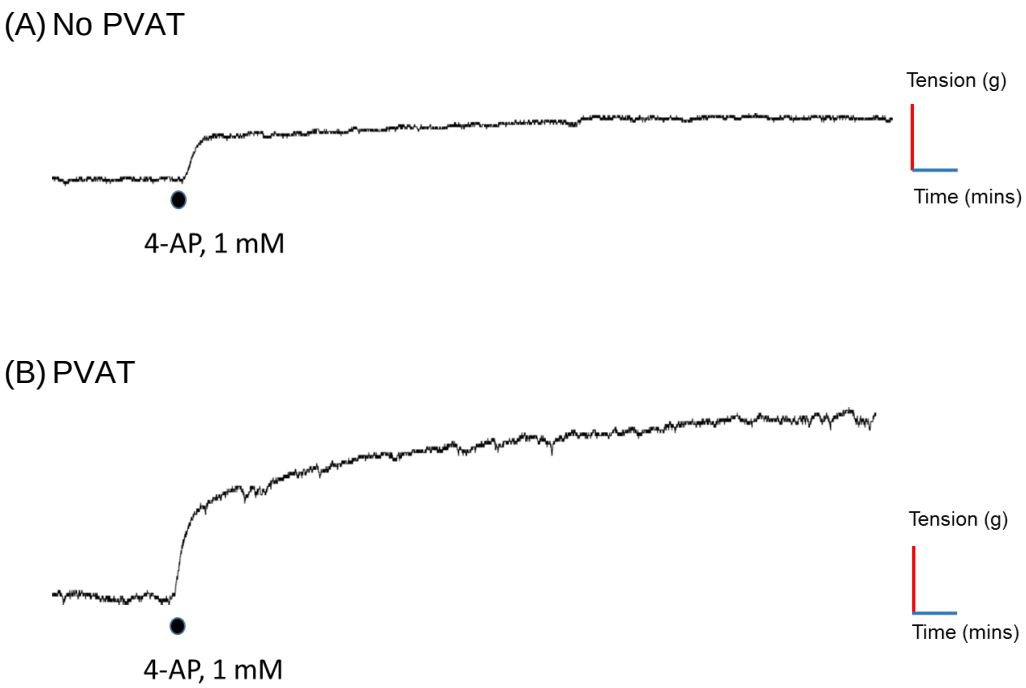


Figure 5.9 Lab Chart® representative traces showing the contractile effects of 4-aminopyridine (4-AP) (1 mM) (in grams (g) tension) on porcine coronary arteries of in the absence (A) and presence (B) of perivascular adipose tissue (PVAT). The response to inhibition of vascular K_V channel by 4-AP was significantly enhanced in vessels exposed to PVAT.

Analysis of the response following the addition of 4-AP showed that the mean ± standard error of the mean (S.E.M) contraction (g tension) was significantly higher in the segments with PVAT (3 ± 0.8) compared to the segments without PVAT (control) (1 ± 0.2), n= 5, $p<0.05$ (unpaired, two-tailed, Student's *t*-test) (Figure 5.10). These data suggest that K_V inhibition had an amplified effect in the presence of PVAT.

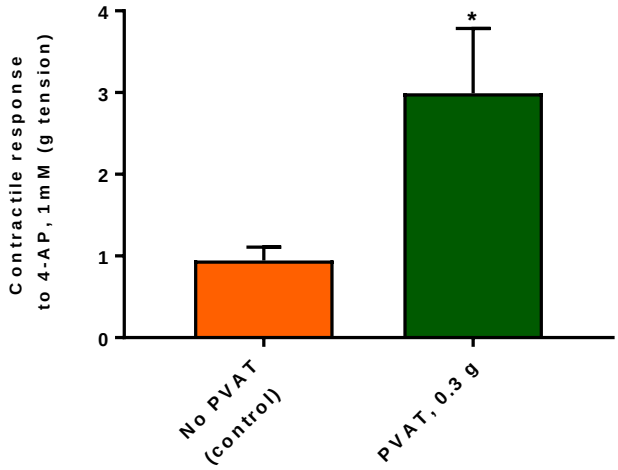


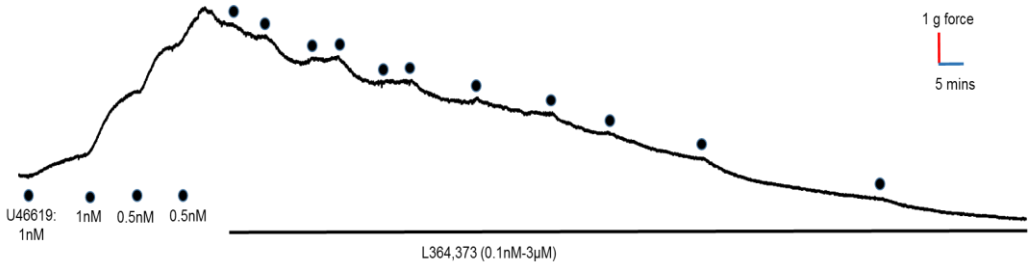
Figure 5.10 Contractile effects of 4-aminopyridine (4-AP) in porcine coronary arteries with and without perivascular adipose tissue (PVAT), obtained from female animals. Data are expressed as mean ± standard error of the mean (S.E.M) contractile responses of 5 experiments in gram (g) tension. n= 5. The error bars represent S.E.M. *p<0.05.

5.3.1.5. EFFECT OF A K_V CHANNEL AGONIST (L-364,373) IN THE PRESENCE OF A K_V CHANNEL ANTAGONIST (4-AP)

Initial steps were the same as in the earlier 4-AP study. The vascular segments (derived from animals of un-specified gender) were incubated with 4-AP (1 mM) for at least one hour, after which U46619 was added in incremental concentrations to achieve pre-contraction to a level of 40-60 % of the second KCl response. Then L364,373 (a K_V channel activator) was added to the segments with and without PVAT in cumulative concentrations (0.1nM-3µM) to construct concentration-response curves (Figure 5.11). The resulting relaxant responses in the two groups of segments without PVAT (in the presence and absence of 4-AP respectively) were compared with each other (Figures 5.12). Also the responses in the two groups of segments with PVAT (in the presence and

absence of 4-AP respectively) were compared with each other (Figure 5.13)

(A) No PVAT:



(B) PVAT:

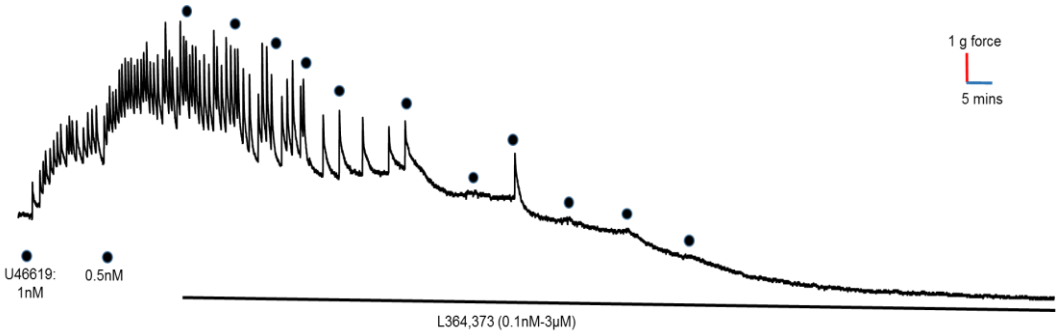


Figure 5.11 Lab Chart® representative traces showing the contractile responses to U466119 (thromboxane A_2 agonist) and relaxant effects of L364,373 (voltage-dependent K^+ channel activator) in grams (g) force following pre-incubation with 4-aminopyridine (1mM) in the absence (A) or presence (B) of perivascular adipose tissue (PVAT) (0.3 g).

It was noted that following the addition of 4-AP and U46619, force-oscillations of the trace occurred particularly in the segments with PVAT (Figure 5.11). In the relaxant phase mediated by the K_V agonist L364,373, the oscillations in tone decreased in amplitude and frequency and ceased towards the end of the concentration-response (Figure 5.11).

Porcine coronary arteries (PCAs) in the absence of PVAT

Following incubation with 4-AP (1mM) and after reaching the target contractile responses of the vascular segments with U46619, L364,373 was added to the organ baths in cumulative concentrations (0.1nM-3 µM) to construct concentration-response curves. It was observed that there was no significant difference in the relaxant responses in the segments exposed to 4-AP, compared to those in the control group (p=not significant, two-way ANOVA with Bonferroni's *post hoc* test, n=5) (Figure 5.12).

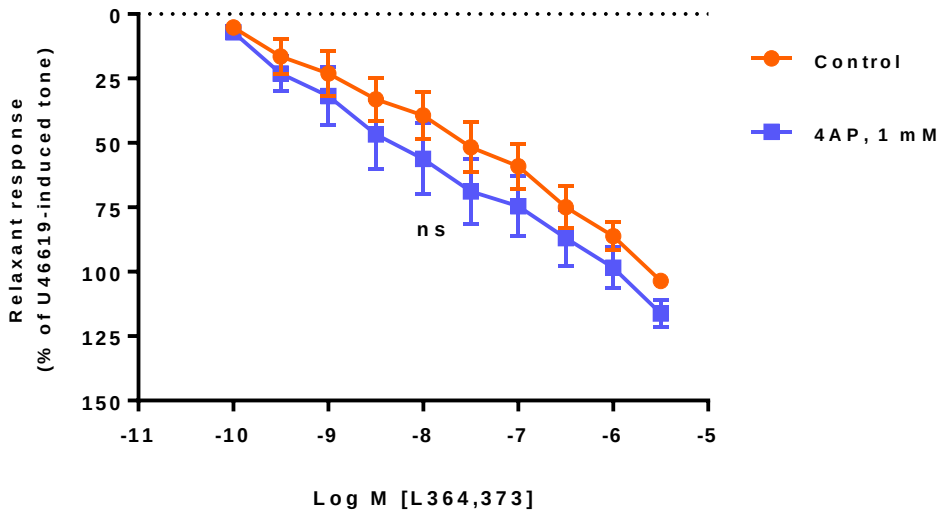


Figure 5.12 Concentration-response curves to L364,373 (0.1nM-3µM) in porcine coronary arteries following incubation with 4-aminopyridine (4-AP) in the absence of perivascular adipose tissue (PVAT). Data are expressed as mean ± standard error of the mean (S.E.M) relaxant responses of 5 experiments, in % of maximum U46619 induced contraction. The bars represent S.E.M. Not significant: ns.

Porcine coronary arteries (PCAs) in the presence of PVAT

The relaxant responses in the segments exposed to 4-aminopyridine (4-AP) (a K_V channel inhibitor) were not significantly different compared to those of the control group (two-way ANOVA with Bonferroni's *post hoc* test, n=4) (Figure 5.13).

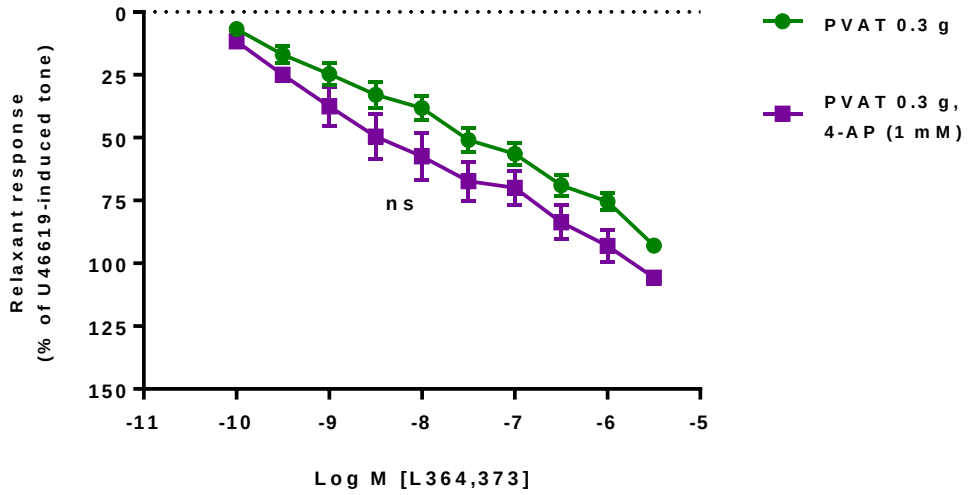


Figure 5.13 Relaxant responses to L364,373 (0.1nM-3µM) in porcine coronary arteries in the presence of perivascular adipose tissue (PVAT), after treatment of one group with 4-aminopyridine (4-AP). Data are expressed as mean ± standard error of the mean (S.E.M) responses of 4 experiments, in % of maximum U46619 induced tone. The bars represent S.E.M. Not significant: ns.

5.3.2 EFFECTS OF VASCULAR K_V CHANNEL INHIBITOR IN THE ABSENCE AND PRESENCE OF CYCLOOXYGENASE INHIBITION

It was observed consistently that there was a significantly increased contractile response to 4-AP (1 mM) in vessels incubated with PVAT (0.3 g) compared to vessels without PVAT (mean ± S.E.M % contraction 25.6 ± 4.5 versus 11.4 ± 1.9 respectively, $p<0.05$, n=6, one way-ANOVA with Bonferroni's *post hoc* test) and which was significantly attenuated in the

group of vessels which had their cyclooxygenase enzyme inhibited with indomethacin (mean ± S.E.M % contraction 13.6 ± 3, $p<0.05$, n=6, one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test) (Figure 5.14). These data indicate that PVAT potentiated the inhibition of vascular K_V channels by 4-AP which was absent during cyclooxygenase blockade. This implies that PVAT released a contractile prostanoid which contributed to vascular contractility through inhibition of vascular K_V channels.

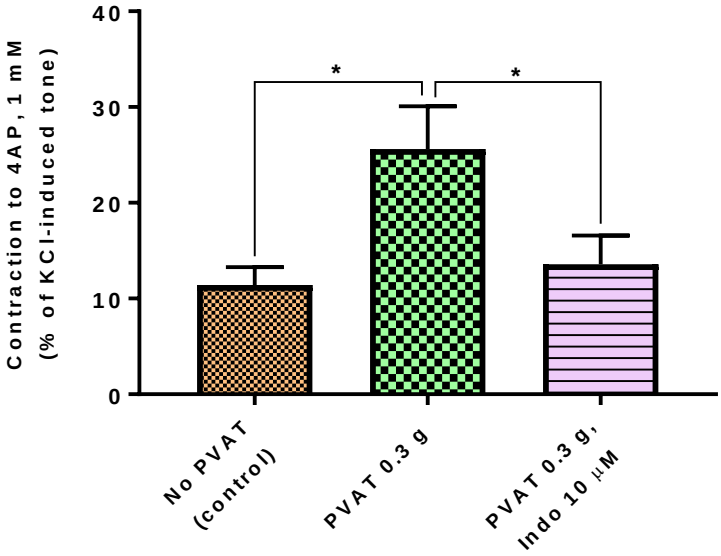


Figure 5.14 Contractile responses in porcine coronary arteries following incubation with 4-aminopyridine (4-AP) (1 mM) in the absence or presence of perivascular adipose tissue (PVAT) with/without inhibition of cyclooxygenase with indomethacin (10 μM) (Indo) in animals of unspecified gender. Data are expressed as mean ± standard error of the mean (S.E.M) contractile responses of 6 experiments, in % of KCl induced contraction. The error bars represent S.E.M. *$p<0.05$.

5.3.3 EFFECTS OF VASCULAR K_V CHANNEL INHIBITOR IN THE ABSENCE AND PRESENCE OF THROMBOXANE A_2 RECEPTOR ANTAGONIST

It was observed that there was a significantly increased contractile response to 4-AP (1 mM) in vessels incubated with PVAT (0.3g) compared to vessels without PVAT (mean ± SEM % contraction: 65.77 ± 8.6 versus 18.61 ± 3.3, $p<0.05$, n= 5, one-way ANOVA with Bonferroni's *post hoc* test) (Figure 5.15). This PVAT-evoked enhancement of vasoconstriction was significantly attenuated in the group of vessels which had their thromboxane A_2 (TXA_2) receptor inhibited with ICI192,605 (1 µM) (mean ± SEM % contraction 27.8 ± 3.4, $p<0.05$, n=5, one-way ANOVA with Bonferroni's *post hoc* test) but not in the presence of the vehicle used to dissolve ICI192,605 (ethanol) (mean ± SEM % contraction 50.3 ± 12.3, p=ns, n=5, one-way ANOVA with Bonferroni's *post hoc* test) (Figure 5.15). These data indicate that PVAT potentiated the closure of vascular K_v channels by 4-AP which was absent during inhibition of the TXA_2 receptor. This implies that PVAT released the contractile prostanoid TXA_2 which contributed to vascular contractility through closure of K_V channels.

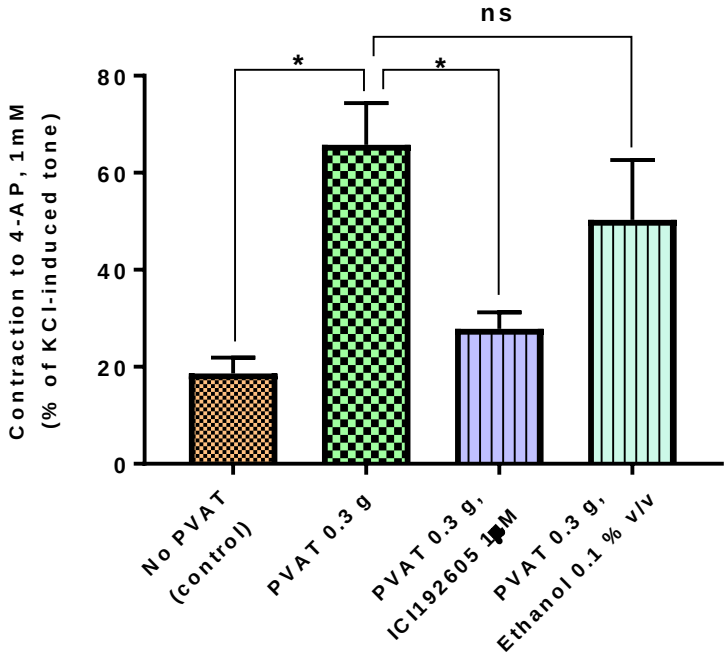


Figure 5.15 Contractile responses of porcine coronary arteries following incubation with 4-aminopyridine (4-AP) (1 mM) in the absence or presence of perivascular adipose tissue (PVAT) with or without inhibition of the thromboxane A_2 (TXA_2) receptor with ICI192,605 (1 µM) in animals of unspecified gender. PVAT enhanced Kv inhibition by 4-AP, which was attenuated in the presence of ICI192,605 but not when ethanol (vehicle used to dissolve ICI192,605) was present. Data are expressed as mean ± standard error of the mean (S.E.M) contractile responses of 5 experiments, in % of KCl induced contraction. The error bars represent S.E.M. *$p<0.05$.

5.4 DISCUSSION

Perivascular adipose tissue (PVAT), the exterior layer of most blood vessels, is being increasingly recognised as a paracrine modulator of vascular function (Gollasch *et al.*, 2004). Traditionally, pharmacological studies dissected off the adherent adipose tissue, ignoring its potential influence on vascular control (Szasz *et al.*, 2012). PVAT has been found to enhance the activity of vascular potassium channels causing a beneficial relaxant effect (Rajsheker *et al.*, 2010). Therefore, this study aimed to determine the effects of PVAT on function of potassium channels of porcine coronary arteries (PCAs).

In the current study, responses of porcine coronary artery (PCA) to agonists of K_{ATP}, BK_{Ca} and K_V channels confirm the presence and activity of these K^+ channels in PCA. Moreover, a prompt contractile response to K_V inhibition (with 4-AP) indicates that K_V channels are active in PCAs in maintaining vessel tone under basal conditions.

5.4.1 IMPACT OF PVAT ON POTASSIUM CHANNELS OF PORCINE CORONARY ARTERY

In the present work, studies using K^+ channel activators in PCAs showed that in vessels derived from male animals, PVAT enhanced the relaxant effect of pinacidil (a K_{ATP} channel agonist), indicating that PVAT may have released a relaxant factor which potentiated the opening of K_{ATP} channels. Potential factors which may have been involved include NO (identified in chapter 3 of the present work) acting through its second messenger cGMP and the vasorelaxant prostanoid prostacyclin (PGI_2) which binds to the IP receptor on vascular smooth muscle cells to promote cAMP production and activation of K_{ATP} channels (Zaborska *et al.*, 2017).

However, in the present study, PVAT did not affect the action of L-364,373 (a K_V channel agonist) and NS1619 (BK_{Ca} channel agonist) in

mediating vasorelaxation, suggesting that PVAT did not interfere with the opening of vascular K_V and BK_{Ca} channels. In contrast, other studies indicate augmentation of the opening of vascular BK_{Ca} channels by putative PVAT-derived relaxant factors, increasing the K^+ efflux, leading to smooth muscle cell (SMC) hyperpolarization, decreased calcium concentration in vascular SMCs and hence vasorelaxation (Lynch *et al.*, 2013) through candidate PVAT-derived relaxant factors involved including NO, adiponectin and H_2S (Greenstein *et al.*, 2009; Schleifenbaum *et al.*, 2010; Szasz *et al.*, 2012).

The present study was extended to see the effect of an H_2S donor (NaHS) in the presence of the BK_{Ca} agonist NS1619 on vasorelaxation of PCAs. This showed no difference in vasorelaxation with and without PVAT, indicating that exogenous H_2S did not interfere with the relaxant responses in the presence of PVAT (which is another potential source of H_2S) compared to control segments. However, other researchers have found that H_2S released from PVAT acted as a vasorelaxant under physiological conditions and increased phosphorylation of vascular BK_{Ca} to facilitate their activation (Sitdikova *et al.*, 2014). Possibly, PVAT used in the present study did not release H_2S and so was unable to modulate the BK_{Ca} channel activity of PCAs. In contrast, other investigations in rat aorta found that PVAT released H_2S which caused vasorelaxation in isolated vessels (Fang *et al.*, 2009).

In the present study, K_V inhibition had an amplified effect in the presence of PVAT with significant enhancement of the contractile responses to 4-AP (1 mM) and generation of force-oscillations which were augmented by addition of U46619 and which ceased with addition of cumulative concentrations of L364,373 (a K_V channel agonist). These findings point to the release of a contractile factor from PVAT which potentiated the closure of vascular K_V channels [causing increased depolarization of vascular smooth muscle cells (VSMCs)], the effect of which was abolished on opening of vascular K_V channels by L364,373 (resulting in

hyperpolarization of VSMCs and hence vasorelaxation) (Gollasch *et al.*, 2004).

In experiments on aorta of female rats, 4-AP enhanced the contractile responses to 5-hydroxytryptamine in the presence of PVAT (Al-Jarallah *et al.*, 2016). A study on PCAs showed the development of force-oscillations with a high concentration of 4-AP (10 mM), which were potentiated by addition of U46619 (also blocks K_V channels) and were inhibited by diltiazem (antagonist of voltage-dependent Ca^{2+} channels) and tetraethyl-ammonium chloride (TEA, an inhibitor of K_{Ca} channels) (Shimizu *et al.*, 2000). In the later study, the force-oscillations were seen with a higher concentration of 4-AP (10 mM) in the absence of PVAT, whereas the present study showed similar effect with a lower 4-AP concentration of 1 mM in the presence of PVAT.

As per the above evidence, a likely mechanism for the upstroke of force-oscillations could be 4-AP induced VSMC depolarization which caused increase in intracellular Ca^{2+} by opening voltage-dependent Ca^{2+} channels. This effect was further potentiated by U46619 which also added to K_V channel blockade. This in turn activated K_{Ca} channels as a negative feedback, leading to hyperpolarization of VSMCs. This closed voltage-dependent Ca^{2+} channels, decreasing intracellular calcium concentration leading to down stroke of the force-oscillations (Cogolludo *et al.*, 2003; Omote *et al.*, 1994).

In the present study, K_v antagonism with 1 mM 4-AP caused a contractile response but was insufficient to prevent L364,373-induced relaxation in porcine coronary arteries. This can be explained by the fact that the concentration used here falls in the range of half-block concentration of 4-AP (0.2-1.1 mM) (Nelson *et al.*, 1995). Addition of a higher concentration of 4-AP in the experiments may attenuate L364,373-induced vasorelaxation. Furthermore, this work has identified TXA_2 as a contractile factor originating from PVAT which mediated its contractile effect by inhibiting vascular K_V channels and whose impact was attenuated by the TXA_2 receptor antagonist ICI192,605.

Based on findings of the present study, a postulated mechanism is presented here in which thromboxane A_2 mediated the contractile effect of perivascular adipose tissue (PVAT) by potentiating the closure of Kv channels in vascular smooth muscle cells leading to vasoconstriction (Figure 5.19). Antagonism of TXA_2 production by PVAT and its vascular receptor may be novel therapeutic targets in vascular disease.

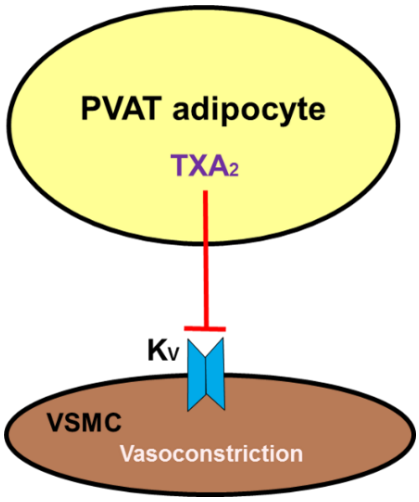


Figure 5.16 Postulated mechanism of increased tone of porcine coronary artery (PCA) under the influence of perivascular adipose tissue (PVAT) based on findings of the present study. Inhibition of voltage-activated potassium channel (K_v) in vascular smooth muscle cells (VSMCs) by thromboxane A_2 (TXA_2) released from PVAT caused decreased efflux of potassium cations out of the VSMCs leading to their depolarization. This precipitated contraction of VSMCs culminating in vasoconstriction.

The present study has limitations. The n numbers were small and vehicle control was not used in the study looking at the effect of cyclooxygenase inhibition by indomethacin (dissolved in ethanol) on the PVAT-induced amplification of the contractile response to 4-AP. However, the vehicle ethanol was employed in the next set of experiments using the thromboxane A_2 receptor antagonist (ICI192,605) where it showed no significant effect on vascular contractility.

As described, porcine coronary arteries were used as they are more similar to human arteries compared to those obtained from murine or canine models (Rodrigues *et al.*, 2005). Further investigations may be performed in human coronary arteries including patch-clamp studies to clarify the role of vascular K^+ channels in the influence of PVAT on arterial function.

5.4.2 SUMMARY AND FUTURE DIRECTIONS

In the current study, responses of porcine coronary artery (PCA) to agonists of K_{ATP}, BK_{Ca} and K_V channels confirm the presence and activity of these K^+ channels in PCA. The activation of K_{ATP} channels was enhanced by PVAT (possibly by release of a relaxant factor) but it did not influence the opening of BK_{Ca} and K_V channels.

A prompt contractile response to K_V inhibition (with 4-AP) indicates that K_V channels are actively involved in maintenance of vessel tone of PCAs under basal conditions. PVAT increased the contraction of PCAs induced by K_V antagonism, as evidenced by enhancement of the contractile responses to 4-AP. Furthermore, this work has identified TXA_2 as a contractile factor originating from PVAT which mediated its contractile effect by inhibiting vascular K_V channels and whose impact was attenuated by the TXA_2 receptor antagonist ICI192605. Inhibition of vascular K_V channels with 4-AP (1 mM) in the absence or presence of PVAT did not affect the relaxant response evoked by K_V agonism with cumulative L364,373 concentrations. This suggests that 4-AP in a concentration of 1 mM did not cause a significant inhibition of vascular K_v channels.

The current study looked at the effect of PVAT on inhibition of vascular K_v channels but not on the antagonism of K_{ATP} and BK_{Ca} channels. So, future functional studies may investigate the effect of PVAT on coronary artery responses to inhibitors of K_{ATP} (e.g. glibenclamide) (Shimizu *et al.*,

2014) and BK_{Ca} (e.g. iberiotoxin) (Lee *et al.*, 2011) channels followed by electrophysiological studies to determine the associated membrane potential changes. These experiments may shed further light on the interaction of PVAT-derived factors with vascular K^+ channels in maintenance of vascular tone in the physiological and disease states such as hypertension and obesity.

Chapter 6

GENERAL DISCUSSION

6.1 IMPACT OF ACUTE HYPERGLYCAEMIA ON VASCULAR REACTIVITY

In the current work, vessels were exposed to a glucose concentration of 22 mM as a representative acute hyperglycaemic condition. This level of hyperglycaemia may occur in patients with uncontrolled diabetes and other investigators have also used a glucose amount in similar concentrations (20-23 mM) for *in vitro* vascular experiments (Jackson *et al.*, 2016; Li *et al.*, 2003).

In the present study, evidence presented in Chapter 2 showed that exposure of coronary arteries to acute hyperglycaemia (22 mM) in the absence of PVAT caused a significant increase in basal tone which was similar to the effects of osmotic control (mannitol). Mannitol has been reported to induce constriction of arterioles on the brain surface and reduce cerebral oedema through increase in extracellular osmolarity (Diringer *et al.*, 2012).

Similarly, hyperglycaemia may also increase extracellular osmolarity, contributing to its cellular effect. *In vitro* experiments were performed on cultured bovine retinal capillary endothelial cells and pericytes, exposing them to hyperglycaemia (15-25 mM), while using mannitol as osmotic control (Yu *et al.*, 2007). Exposure of the cells to hyperglycaemia led to a significantly decreased cell count, cell protein and viability, with a similar effect demonstrated in cells exposed to mannitol, indicating that osmotic stress contributed to the toxicity of glucose (Yu *et al.*, 2007). Moreover, hyperosmolar glucose caused vasoconstriction in visceral arteries, which was not dependent on the endothelium and which was sensitive to inhibition of Rho kinase (Un *et al.*, 2013). So, in the present study, osmotic stress created by hyperglycaemia may have contributed to its contractile effect on PCAs.

In the current work, as shown in Chapter 2, oxidative stress mediated by O_2^- production in the buffer solution incubated with PCAs under acute hyperglycaemic conditions may have also led to increase in vascular

contractility. There is increased production of O_2^- during hyperglycaemia whose formation is catalysed by NADPH oxidase and eNOS under the influence of protein kinase C (Hink *et al.*, 2001), as well as by diversion of electrons from the transport-chain in mitochondria towards molecular oxygen, producing O_2^- (Rask-Madsen *et al.*, 2013; Yu *et al.*, 2007). The O_2^- may reduce vascular NO bioavailabity by oxidation of NO (to ONOO) and BH_4 (the cofactor required for NO synthesis) uncoupling eNOS towards O_2^- production instead of the manufacture of NO, potentially leading to increase in vascular tone (Laursen *et al.*, 2001).

6.2 ROLE OF PVAT IN VASCULAR FUNCTION

In Chapter 2 of the current study, the contractile responses to the TXA_2 agonist U46619 were enhanced in segments with PVAT compared to the controls and there was significant increase of basal tone after exposure of cleaned PCAs to PVAT. This contractile effect of PVAT was attenuated during inhibition of the TXA_2 receptor suggesting that the release of the contractile prostanoid TXA_2 may be a contributing cause. As described previously, this finding is in line with other studies in aortae of mice and porcine coronary arteries, which have reported the production of TXA_2 in PVAT as well as its contractile effect on the adjacent vessel (Meyer *et al.*, 2013) (Ahmad *et al.*, 2017).

It was shown in Chapter 3 that the relaxant responses of PCAs to SNP (a NO donor) were significantly potentiated in the segments exposed to PVAT compared to the controls. PVAT can induce vascular smooth muscle relaxation by releasing NO and other relaxant factors (Zaborska *et al.*, 2016). In the current study, nitrite (NO_2^-, a stable, non-volatile oxidation product of NO) was detected in the physiological buffer solution incubated with PVAT and the expression of eNOS was revealed in PVAT. These data support the possibility that NO derived from PVAT acted in tandem with the NO supplied by SNP to potentiate vasorelaxation. Evidence from other investigations in mesenteric vessels in mice (Gil-

Ortega *et al.*, 2010) and aortae of rats (Victorio *et al.*, 2016) also found evidence of NO production by PVAT. Functional studies in thoracic aortae of rats showed an anti-contractile effect of PVAT (with and without intact endothelium) and NOS-inhibition enhanced the contractile response of segments (with disrupted endothelium) to phenylephrine in the presence of PVAT indicating the possible release of NO from PVAT. Western blotting confirmed the expression of eNOS and DAF-2DA imaging demonstrated the production of NO by intact PVAT (Victorio *et al.*, 2016). Work in hypercholesteraemic mice showed a protective relaxant effect of PVAT which was associated with NO production and eNOS expression in PVAT as shown by fluorescence microscopy and immunoblotting respectively (Baltieri *et al.*, 2018).

In Chapter 4, isometric tension studies suggested that an adipocyte-derived contractile factor (ADCF) possibly angiotensin II, released from PVAT may have impaired endothelium-dependent relaxation and the EDH-type arterial response. Multiple ADCF candidates have been reported including reactive oxygen species, adipokines (e.g. nefastin) and components of the renin-angiotensin system (Almabrouk *et al.*, 2014). The current study showed the presence of angiotensin II in PVAT, which supports the findings of isometric tension studies implicating angiotensin II as a potential PVAT-derived contractile factor that interfered with the endothelium-dependent relaxation response. Other investigators confirmed the expression of angiotensin II in PVAT and demonstrated beneficial effect of treatment with the AT_1 receptor blocker candesartan in reducing the PVAT-mediated contraction to nerve stimulation (Lu *et al.*, 2010). A study in rats with angiotensin II-induced hypertension showed significantly reduced EDH-mediated vasorelaxation of mesenteric arteries (Dal-Ros *et al.*, 2009) and work in mesenteric arteries of hypertensive rats demonstrated reduced EDH-response which was ameliorated with use of an AT_1 blocker and ACE inhibitor (Goto *et al.*, 2000) supporting the findings of the current study.

As presented in Chapter 5, studies using K^+ channel activators in PCAs showed no effects of PVAT on L-364,373 (a K_V channel activator) and NS1619 (BK_{Ca} channel activator)-induced vasorelaxation. These findings suggest that PVAT did not influence the opening of K_V and BK_{Ca} channels. However, the present work showed enhancement of the relaxant responses to Pinacidil (K_{ATP} channel activator) in males, suggesting the release of a factor (potentially NO or prostacyclin) from PVAT which enhanced the opening of vascular K_{ATP} channels (Almabrouk *et al.*, 2014).

In the present study, experiments showed that PVAT significantly enhanced the contractile responses of PCAs to K_v inhibition and caused oscillations in tone which were increased by the TXA_2 agonist U46619 and which resolved following K_V channel opening by L364,373. These results indicate the release of a contractile factor from PVAT which potentiated the closure of vascular K_V channels, increasing depolarization of smooth muscle cells, the effect of which was abolished on opening of K_V channels resulting in hyperpolarization and ultimately vasorelaxation (Rainbow *et al.*, 2009). Furthermore, this work has shown that the enhancement of 4-AP-induced contraction by 4-AP was significantly reduced following the inhibition of the TXA_2 receptor. This suggests that TXA_2 released from PVAT caused a contractile effect on PCAs by inhibiting their smooth muscle K_v channels (Cogolludo *et al.*, 2003). The activity of vascular K_v channels may be modulated by several other vasoactive factors including NO, endothelin and angiotensin II (Hayabuchi *et al.*, 2001; Shimoda *et al.*, 1998; Tanaka *et al.*, 2006).

Experiments on rat pulmonary arteries using the patch-clamp technique showed that the TXA_2 analogue U46619 inhibited the K_v channel current in smooth muscle cells, increased intracellular Ca^{+2} concentration and so caused vasoconstriction by binding to the TP receptors. These effects were significantly attenuated following treatment of the tissue with PKC inhibitors, suggesting that TXA_2 inhibits the function of arterial K_v channels with the support of the PKC intermediate (Cogolludo *et al.*,

2003). These data, support the findings of our work, which found that TXA_2 (derived from PVAT) augmented the inhibition of arterial K_V channels, leading to increased contractility.

6.3 SUMMARY AND FUTURE DIRECTIONS

6.3.1 Paracrine function of PVAT

The present study showed that PVAT significantly reduced contractile responses to KCl and potentiated the relaxant responses to Pinacidil and SNP in PCAs. These effects may have been due to the release of relaxant factors from PVAT, one of which was determined to be NO (Figure 6.1). The present study also points to the release of contractile factors from PVAT, as evidenced by enhancement of the contractile responses to 4-AP and demonstration of force-oscillations in the presence of PVAT, which may be related to the release of TXA_2 from PVAT. The contractile factor angiotensin II may have been released by PVAT which suppressed the endothelium-dependent relaxation response (Figure 6.1).

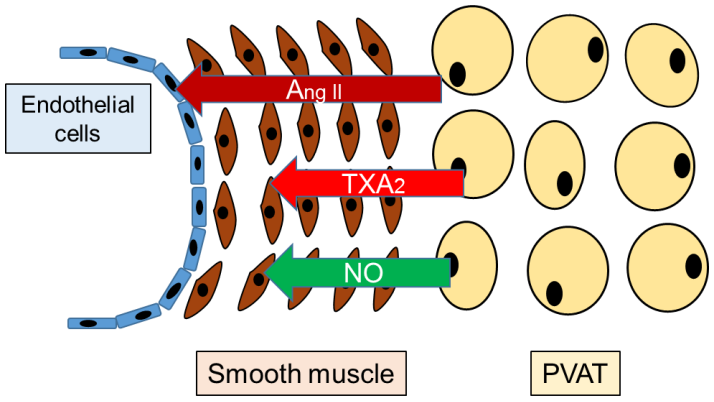


Figure 6.1 Paracrine factors released from perivascular adipose tissue (PVAT) of porcine coronary artery diffuse to the adjacent smooth muscle and endothelial cells to modulate their function. Angiotensin II, Ang II; thromboxane A_2, TXA_2; nitric oxide, NO.

6.3.2 Effects of PVAT-derived factors on vascular function in the absence or presence of acute hyperglycaemia

The current work demonstrated the contractile effect of acute hyperglycaemia on PCAs, which was likely due to the combined effect of osmotic and oxidative stress generated by high extracellular glucose (Figure 6.2). The vasoconstriction in response to acute hyperglycaemia, in combination with the effect of thromboxane A_2 released from PVAT, may override the protective vasodilator effect of PVAT-derived nitric oxide leading to vascular dysfunction (Figure 6.2). This scenario may occur in patients with poorly controlled diabetes and obesity (Emilova *et al.*, 2016). Intensive control of glucose levels and treatment of PVAT dysfunction using emerging therapeutic modalities such as arginase inhibitors and eNOS phosphorylation enhancers (which increase NO production) may mitigate these deleterious vascular effects of hyperglycaemia and thromboxane A_2 (Rask-Madsen *et al.*, 2013; Xia *et al.*, 2016b).

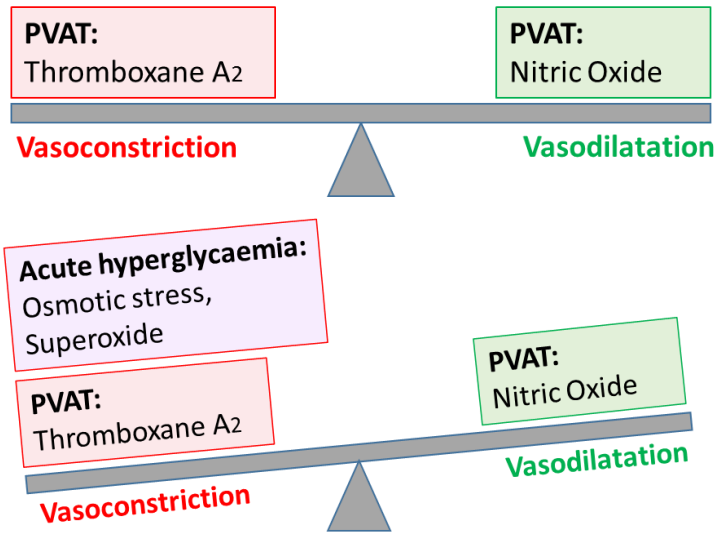


Figure 6.2 Effect of paracrine factors released from perivascular adipose tissue (PVAT) on tone of porcine coronary artery (PCA) in the absence (top) and presence (bottom) of acute hyperglycaemia. PVAT-derived thromboxane A_2 and acute hyperglycaemia caused vasoconstriction, whereas nitric oxide had an opposing effect on vascular tone.

6.3.3 Conclusions

In conclusion, the present study has demonstrated that acute hyperglycaemia (22 mM) in the absence of PVAT increased basal tone of PCAs, possibly via the vascular production of O_2^- as well as by the tissue effect of the hyper-osmolar glucose. This is in line with findings of other researchers (Su *et al.*, 2013) (Otto *et al.*, 2008), however, further experiments are needed to clarify the dual mechanism of action of acute hyperglycaemia on the tone of coronary arteries. The present work also highlights the need for intensive therapeutic control of glucose levels in diabetes to prevent detrimental effects on vascular contractility.

This study has shown that exposure of cleaned PCAs to PVAT significantly reduced the contractile response to KCl and enhanced the relaxant response to K_{ATP} channel activation which may have been due to relaxant factors released by PVAT. Chapter 3 has determined NO as a putative PVAT derived relaxant factor which may have contributed to the basal arterial tone and facilitated SNP-induced vasorelaxation in conjunction with endothelial NO, which may have an additive effect in protecting vascular function. The present work used functional studies in conjunction with quantification of a metabolite of NO (NO_2^-) and the expression of the enzyme (eNOS) in PVAT to obtain evidence of NO production in PVAT of PCAs. Further studies are required to directly look at NO production (e.g. by fluorescence imaging) in PVAT in the physiological and pathological states.

The present study also demonstrates a significant increase in basal arterial tone as well as enhancement of vasoconstriction induced by K_V inhibition on exposure to PVAT, which was attenuated by TXA_2 receptor inhibition. These findings indicate that PVAT may have released the contractile factor TXA_2, which mediated its contractile effect by inhibiting vascular K_V channels, which may serve as a therapeutic target in PVAT dysfunction. Responses of PCAs to activators of K_{ATP}, BK_{Ca} and K_V channels confirm the presence and activity of these K^+ channels in PCA. Evidence presented in Chapter 4 indicates that PVAT may have released

the contractile factor angiotensin II, which inhibited the endothelium-dependent vasorelaxation induced by BK, thus modulating vascular tone. AT1 receptor blockade may be of therapeutic benefit in endothelial dysfunction evoked by PVAT-derived angiotensin II. The present limited work indicates a paracrine contractile effect of PVAT, however future studies are required to understand the underlying mechanisms.

The findings of the present study indicate a dual paracrine role of PVAT potentially mediated by the release of the vasorelaxant NO on the one hand and the contractile factors TXA_2 and angiotensin II on the other. Hence, PVAT has the capacity to elicit a protective or a detrimental effect on the underlying blood vessel depending on its amount and patho-physiological status (Fernández-Alfonso *et al.*, 2017).

6.3.4 Future directions

Future studies could use other assays (e.g. chemiluminescence) to elaborate the ROS which may be involved in mediating the oxidative stress during hyperglycaemia as well as their effects on vascular K^+ channels and endothelial function. Direct measurement of NO and studies looking at expression of NO synthase in PVAT of various vessels (aorta, carotid, splenic and renal arteries etc.) in animal models and humans may be performed, as there are structural and functional differences between PVATs depending on the location of the vascular bed and species (PVAT mostly consists of white adipose tissue with relatively few mitochondria but in thoracic aorta of rats and mice it is composed of white as well as the thermogenic and protective brown adipose tissue with abundant mitochondria) (Gil-Ortega *et al.*, 2015) (Kennedy *et al.*, 2017).

Studies could be designed to investigate the presence of other components of the RAS in PVAT of PCAs (including angiotensinogen, angiotensin 1, angiotensin 1-7, ACE2, AT and MAS receptors) as well as their downstream impact on control of vascular tone. Furthermore, future

work could investigate the role of PVAT derived vasoactive factors including TXA_2 in functioning of K^+ channels in other porcine arteries using isometric tension and electrophysiological studies.

Experiments may be extended to study the PVAT in these contexts in humans. The volume of adipose tissue around the coronary arteries is associated with cardiovascular risk factors and atherosclerosis, so an approach could be to undertake scanning of peri-coronary adipose tissue using non-invasive ultrasound or magnetic resonance imaging to compare the amount of PVAT in healthy individuals with that in diabetics and the obese (Fernández-Alfonso *et al.*, 2017). In the latter group, the scans may be repeated following interventions such as dietary management, exercise, strict control of diabetes and pharmacological interventions (arginase inhibitors, ACE inhibitors, statins etc.) to study their potential beneficial effect on PVAT (Xia *et al.*, 2016b).

It is anticipated that research efforts in this area may unravel novel therapeutic targets in PVAT and add to the promising therapeutic modalities to correct PVAT dysfunction which are currently being investigated under experimental conditions including body weight reduction, exercise, increase of NO production by PVAT (arginase inhibitors, eNOS phosphorylation enhancers), increase of PVAT H_2S release (atorvastatin, glitazones), inhibition of RAS (ACE inhibitors, AT1 receptor antagonists), adiponectin enhancers (statins, glitazones), inhibition of inflammation (melatonin, cytokine antagonists e.g. infliximab) and adenosine monophosphate (AMP) kinase activators (metformin, resveratrol) (Xia *et al.*, 2016b). Moreover, the benefecial anti-contractile effect of PVAT involving NO release may be utilized during coronary artery bypass surgery by keeping the PVAT intact in the donor vessel to prevent development of vasospam in the graft (Zhang *et al.*, 2010).

APPENDIX

WESTERN BLOTTING SOLUTIONS

A. Lysis buffer (pH 7.6)

Contents	Concentration
Tris	20 mM
EGTA	1 mM
Triton X100	0.1 % v/v
Sodium fluoride	1 mM
Sucrose	320 mM
Beta glycero phosphate	10 mM

Table 1 Composition of lysis buffer. EGTA: ethylene glycol tetra acetic acid.

B. Protease Inhibitor Cocktail Set I (Calbiochem)

Product	Concentration (1X)	Target protease
AEBSF hydrochloride	500 µM	Serine proteases
Aprotinin, bovine lung, crystalline	150 nM	Serine proteases and esterases
E-64 protease inhibitor	1 µM	Cysteine proteases
EDTA, disodium	0.5 mM	Metalloproteases
Leupeptin, hemisulphate	1 µM	Cysteine proteases and trypsin-like proteases

Table 2 Composition of protease inhibitor cocktail. AEBSF: 4(2-amino ethyl) benzene sulfonyl fluoride; EDTA: ethylene diamine tetra acetic acid.

C. Solubilisation buffer (6X) (pH 6.8)

Contents	Concentration
SDS	24% w/v
Glycerol	30% v/v
Beta mercaptoethanol	5% v/v
Bromophenol blue	2.5% v/v
Tris hydrochloride	1.5 M

Table 3 Materials in solubilisation buffer (6X). SDS: sodium dodecyl sulphate.

D. Electrophoresis buffer 10X

Contents	Concentration
Tris	0.19 M
Glycine	1.9 M
SDS	35 mM

Table 4 Composition of electrophoresis buffer (10X).

E. Transfer buffer (1X)

Contents	Amount
Tris	30.3 g
Glycine	144 g
Dissolved in distilled water	8 L
Added Methanol	2L

Table 5 Materials used in transfer buffer (1X).

F. Tris-buffered saline with Tween 20 (TBS-T) (pH 7.6)

Contents	Concentration
Tris	25 mM
Sodium chloride	125 mM
Tween 20	0.1% v/v

Table 6 Composition of TBS-T.

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