Expression and roles of sympathetic and sensory nerves in perivascular adipose tissue

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Abstract

There is a call to elucidate the poorly understood link between perivascular adipose tissue (PVAT) and nervous systems controlling the vasculature. The principle aims of this study were to investigate the expression of sympathetic and sensory nerves in PVAT of rat mesenteric arteries, to characterize their role(s) in vascular tone and to investigate the interaction between these nervous systems and PVAT-derived vasoactive compounds. Immunofluorescence staining was employed to investigate the localisation of sympathetic and sensory nerves in mesenteric arteries. Rat mesenteric arterial beds (MABs; with and without PVAT, removed by careful dissection) were perfused with Krebs'-Henseleit buffer and changes in pressure recorded, or different sized rat mesenteric arteries (MA), rat abdominal aortas, porcine splenic arteries (PSA) and porcine coronary arteries (PCA) (with and without PVAT, removed by careful dissection) were isolated and set up for isometric recording. Responses to electrical field stimulation (EFS) were obtained under basal and raised tone conditions induced by methoxamine, an α1-adrenoceptor agonist, in the presence and absence of agonists/antagonists. Enzyme immunoassay (EIA) was conducted to quantify capsaicin-evoked calcitonin gene-related peptide (CGRP) release of different mesenteric arterial segments and the dissected PVAT. A multiplex assay was carried out to investigate the link between sympathetic and sensory nerves and PVAT-derived vasoactive compounds.

Immunofluorescence showed the presence of both tyrosine hydroxylase (TH; the rate-limiting enzyme of catecholamine biosynthesis)-immunoreactive and CGRP (the principal neurotransmitter for sensory nerves)-immunoreactive nerves at the adventitia and within PVAT. EFS elicited frequency-dependent vasoconstriction of the mesenteric beds. These responses were abolished by guanethidine, a sympathetic nerve

blocker, indicating an involvement of sympathetic nerves. In the absence of PVAT, neurogenic contractile responses were markedly attenuated. There was no significant difference in concentration-dependent contractions to methoxamine in preparations with and without PVAT, thus suggesting that vascular smooth muscle remains intact in PVAT-denuded preparations. Contractile responses to EFS were significantly decreased after Krebs'-Henseleit solution containing PVAT was transferred to preparation without PVAT, indicating PVAT contains transferrable factor/s. Incubation with candesartan, but not losartan, angiotensin II receptor inhibitors, in the presence of PVAT significantly attenuated EFS-evoked contraction, indicating a potential involvement of PVAT-derived angiotensin II in PVAT-enhanced neurogenic contractile response. In PSA, removal of PVAT significantly attenuated EFS-evoked contractile responses. Exogenous methyl palmitate had no effect on sympathetic neurogenic contractions while exogenous apelin-13 reduced sympathetic responses in both PVAT-intact and PVAT-denuded PSAs.

EFS of PVAT-intact MABs in the presence of guanethidine and methoxamine elicited frequency-dependent vasodilatation due to stimulation of sensory nerves. Neurogenic vasodilatation was abolished in preparations with PVAT removed. In contrast, dose-dependent vasodilator responses to capsaicin, an agonist at vanilloid receptor subtype 1 (TRPV1), and exogenous CGRP, were comparable between PVAT-intact and PVAT-denuded preparations. This suggests that the PVAT removal procedures did not damage vascular smooth muscle relaxation in PVAT-denuded preparations. Myography experiments revealed that EFS-evoked vasodilator responses were greater in PVAT-intact preparations than in PVAT-denuded preparations in both MA and second order MA (2OMA) segments. EIA indicated that CGRP release was greater in dissected PVAT with capsaicin compared to dissected PVAT without capsaicin in 2OMA

preparations, which further supports the concept of the presence of sensory nerves in PVAT of MABs.

Sodium sulfide (Na_2S), a hydrogen sulphide (H_2S) donor, caused concentration-dependent vasodilation and this effect was attenuated by incubation with HC030031, a TRPA1 antagonist, and pre-treatment with capsaicin. The vasodilator response was greatly attenuated in the second response curve, indicating the involvement of desensitization mechanism. EFS elicited frequency-dependent vasodilatation due to stimulation of sensory nerves but these responses were attenuated in the presence of Na_2S. Incubation with H_2S-synthesizing enzyme inhibitors, DL-propargylglycine, aminooxyacetic-acid and aspartate, had no significant effect on EFS-evoked neurogenic vasodilatation. The presence of PVAT enhanced leptin release under normal oxygenation (95 % O_2 and 5 % CO_2), while gassing with 95 % N_2 and 5 % CO_2 enhanced interleukin-6. Leptin release was enhanced during EFS of sympathetic nerves under low oxygen level and EFS of sensory nerves under normal oxygen level in the presence of PVAT.

In conclusion, the present study provides clear evidence for the expression of sympathetic and sensory nerves within PVAT of mesenteric arteries and shows these nerves contribute to the regulation of vascular tone. H_2S causes vasodilatation of MABs by activating sensory nerves through the TRPA1 signalling pathway, and subsequently impairs sensory nerve function, demonstrating a capsaicin-like action. H_2S-producing enzymes and endogenous H_2S are not involved in EFS-evoked neurogenic vasodilator responses under the conditions of the present study. Activation of sympathetic and sensory nerves in PVAT can modify PVAT-derived mediator(s) release. Collectively, these data show that sympathetic and sensory nerves are expressed in PVAT and have functional roles in modulation of vascular contractility and PVAT-derived mediator release.

Conference presentations

1. Bakar H. A., Dunn, W.R. and Ralevic, V. *Influence of perivascular adipose tissue on sympathetic and sensory perivascular neurotransmission.* YLS2013 Cardiovascular Medicine: Bridging Basic and Clinical Researchers, Barts and the London Charterhouse Square Campus, United Kingdom.
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Abbreviations

AA	Abdominal aorta
ACE	Angiotensin-converting enzyme
ACh	Acetylcholine
AChE	Acetylcholinesterase
ADP	Adenosine diphosphate
ADRF	Adipocyte-derived relaxing factor
AITC	Allyisothiocyanate
AMPK	5'-adenosine monophosphate-activated protein kinase
AOAA	Amino oxyacetic acid
AT	Adipose tissue
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BK_{CA}	Large conductance calcium-activated potassium channels
BP	Blood pressure
cAMP	Cyclic adenosine monophosphate
CAT	Cysteine aminotransferase
CBS	Cystathionine-ß-synthase
CGRP	Calcitonin gene-related peptide
CLR	Calcitonin receptor-like receptor
CO	Carbon monoxide
CO_2	Carbon dioxide
CRP	C-reactive protein
CSE	Cystathionine-γ-lyase
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
EDHF	Endothelium-derived hyperpolarizing factor

EDRF	Endothelium-derived relaxing factor
EFS	Electrical field stimulation
EIA	Enzyme immunoassay
FRC	Frequency response curve
GPCR	G protein-coupled receptor
H_2S	Hydrogen sulfide
HNO	Nitroxyl
Hz	Hertz
IK_{Ca}	Calcium conductance-activated intermediate potassium channel
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
IP_3	Inositol trisphosphate
JAK/STAT	Janus kinase/signal transducers and activators of transcription
K_{ATP}	ATP-sensitive potassium channel
KCl	Potassium chloride
L-NAME	L-NG-nitroarginine methyl ester
MA	Mesenteric arteries
MAB	Mesenteric arterial bed
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
min	Minutes
ml	Millilitre
mM	Millimolar
mmHg	Millimetre of mercury
MP	Methyl palmitate

ms	Milliseconds
N	Nitrogen
NA	Noradrenaline
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NaHS	Sodium hydrogen sulphide
NANC	Non-adrenergic non-cholinergic
nM	Nanomolar
NO	Nitric oxide
NPY	Neuropeptide Y
O_2	Oxygen
PAI-1	Plasminogen activator inhibitor-1
PCA	Porcine coronary arteries
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PI3–Akt	Phosphatidylinositol 3' -kinase
PKA	Protein kinase A
PLC	Phospholipase C
PNS	Parasympathetic nervous system
PaO_2	Partial oxygen level
PPADS	Pyridoxalphosphate-6-azophenyl-2’-5’-disulfonate
PPAG	DL-propargylglycine
PSA	Porcine splenic arteries
PVAT	Perivascular adipose tissue
PVATRF	Perivascular adipose tissue relaxing factor
PVCF	Perivascular contractile factor
PVN	Perivascular nerves
RAAS	Renin-angiotensin-aldosterone system
RAMP	Receptor activity modifying protein
RC	Response curve
s	Second

SAT	Subcutaneous adipose tissue
SEM	Standard error mean
SK_{Ca}	Calcium conductance-activated small potassium channel
SMA	Superior mesenteric artery
SNA	Sympathetic nerve activity
SNS	Sympathetic nervous system
SP	Substance P
SR	Sarcoplasmic reticulum
TH	Tyrosine hydroxylase
TNF-α	Tumor necrosis factor α
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin 1
TRPV1	Transient receptor potential vanilloid 1
TTX	Tetrodotoxin
UCP1	Uncoupling protein 1
V	Volt
VAT	Visceral adipose tissue
VSMC	Vascular smooth muscle cell
WAT	White adipose tissue
αAR	α-adrenoceptor
βAR	β-adrenoceptor
$[Ca^{2+}]_i$	Intracellular calcium concentration
μM	Micromolar
1OMA	First order mesenteric artery
2OMA	Second order mesenteric artery
3-MST	3-mercaptopyruvate sulfurtransferase
3OMA	Third order mesenteric artery

Chapter 1

General introduction

1.1 The regulation of systemic blood pressure

Systemic blood pressure (BP) is determined by cardiac output and peripheral vascular resistance. Vascular tone is controlled by a combination of intrinsic and extrinsic mechanisms as well as by neuronal reflexes and central nervous system influences. Intrinsic mechanisms include the myogenic response, endothelial regulation, regulation by metabolic vasoactive factors, autacoids and autoregulation, while extrinsic mechanisms involve perivascular nerves and hormones (Levick, 2010). The baroreflex and arterial BP regulation by the kidney also play an integral role in cardiovascular homeostasis.

Blood vessels are composed of three functional layers (see Figure 1) that regulate vascular tone. The innermost layer is called the endothelium and it is a metabolically active organ lining the luminal side of all blood vessels; it has a role in the maintenance of vascular homeostasis, mediated by a number of endothelium-derived factors (Villar et al., 2006). Both constricting and vasodilating substances can be produced by endothelial cells when stimulated by blood-borne substances or by shear stress associated with the flow of blood (Aaronson and Ward, 2007). Nitric oxide (NO), prostanoids (such as prostacyclin (PGI_2)) and endothelium-derived hyperpolarizing factor (EDHF) are the most significant vasodilators released by endothelial cells. Important endothelial vasoconstrictors are endothelin-1, thromboxane A_2, and prostaglandin H_2 (Aaronson and Ward, 2007).

The adventitia, the outermost layer of blood vessels, is densely innervated in many blood vessels (Figure 1). Vasoactive signals from perivascular

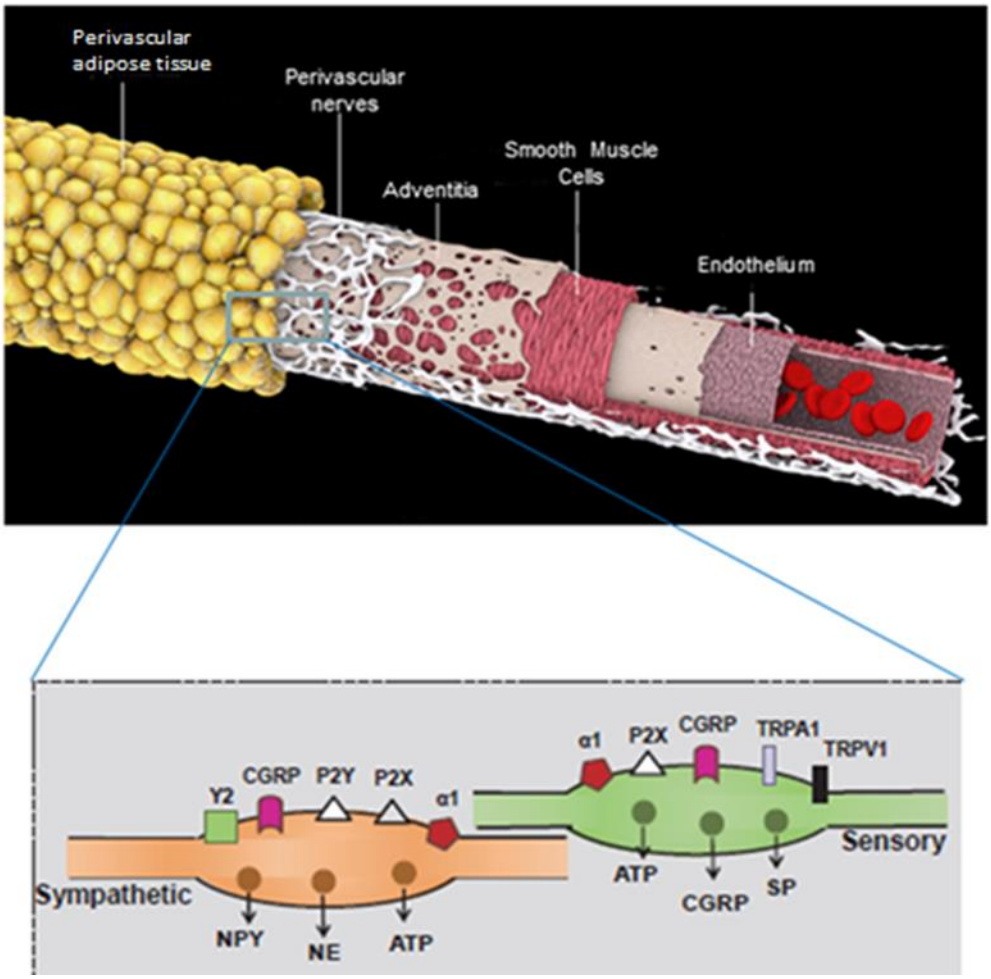

Figure 1: A schematic representation of vascular anatomy. Sympathetic and non-adrenergic non-cholinergic (NANC) nerves lie extensively at the adventitia-medial border. Receptors on sympathetic and sensory nerves: Y2 (neuropeptide Y2), CGRP (calcitonin gene-related peptide), P2Y (purinergic metabotropic receptors), P2X (purinergic ionotropic receptors), α1 (alpha-1 adrenoceptor), TRPA1 (transient receptor potential ankyrin 1), TRPV1 (transient receptor potential vanilloid 1). Neurotransmitter released from sympathetic and sensory nerves: NPY (neuropeptide Y), NE (norepinephrine/noradrenaline), ATP (adenosine 5'-triphosphate), CGRP and SP (substance P). Adapted from Westcott and Segal (2013b).

sympathetic and non-adrenergic non-cholinergic (NANC) nerves are integrated with endothelium-derived signals to generate vasoconstriction or vasodilatation (Westcott and Segal, 2013b). Perivascular nerves (PVN) exert their functions via the release of neurotransmitters, which then act principally on the vascular smooth muscle cells (VSMC). VSMC lie in a layer between the endothelial and adventitial layers. On VSMC, there are numerous receptors that respond to the different signals released from the endothelium or PVN in the adventitia, and the activation of these receptors changes the diameter of the blood vessel, and thus maintains or alters BP.

Autoregulation enables blood flow to remain nearly constant over a wide range of pressures and is crucial for certain organs like the brain, heart and kidney. Autoregulation involves two homeostatic negative feedback mechanisms, namely the myogenic response and the involvement of vasodilator metabolites (Aaronson and Ward, 2007). The myogenic response is stimulated by stress or increases in pressure, with opening of ion channels leading to Na^+ and Ca^{2+} influx and cell depolarization, providing an additional stimulus for the opening of L-type Ca^{2+} channels and subsequent constriction. This phenomenon occurs in resistance arteries with the aim of maintaining a constant blood supply (Aaronson and Ward, 2007). On the other hand, vasodilator metabolites may be 'washed out' of the tissue when the BP increases, resulting in inhibition of vasodilation thus counteracting the rise of blood flow (Aaronson and Ward, 2007). Other local mechanisms involve the release of autacoids (local hormones), such as histamine, prostaglandin E2, bradykinin and platelet activating factor during certain circumstances, including inflammatory reactions, local infection or trauma (Aaronson and Ward, 2007).

Sympathetic vasoconstrictor nerves are the main extrinsic controllers of BP and the regulation of this system is discussed further in Section 1.2.1. Non-adrenergic non-cholinergic (NANC) nerves, which include nitrergic and sensory nerves, are involved in vasodilatation (as discussed in Section 1.2.2-1.2.4). Most blood vessels receive little or no parasympathetic innervation. Many hormones can influence vascular tone. In general, hormones including adrenaline, vasopressin, insulin and angiotensin II are less important compared to the autonomic nervous system for physiological or acute cardiovascular regulation (Levick, 2010). The notable organs in regulating blood volume are the kidneys which work together with the renin-angiotensin-aldosterone system (Raasch et al.) and baroreceptors in the brain to control extracellular fluid volume. The RAAS system is activated when BP is low and this system may or may not involve central reflexes and the sympathetic nervous system. The sympathetic nerves and RAAS work together in accomplishing their regulatory functions (Abdulla et al., 2009; Dunn et al., 1991; Rathore et al., 2009). Noradrenaline (NA) and angiotensin II exert synergistic actions on vascular tone (Marano and Argiolas, 1994) while vascular reactivity to exogenous NA was found to be altered with a blockade of endogenous angiotensin II (Abdulla et al., 2009; Raasch et al., 2004). It is clear that there are many mechanisms to sustain normal BP as this is essential to homeostasis of tissues and organ systems throughout the body.

1.2 Perivascular nerves

1.2.1 Sympathetic nerves

Sympathetic preganglionic neurons originate in the intermediolateral columns of the spinal cord and exit the spinal cord through ventral roots and synapse with the postganglionic fibres (Aaronson and Ward, 2007) which have their cell bodies located in the paravertebral ganglia (McLachlan, 2003). The postganglionic fibres terminate in the effector organs and adrenal medulla, and release NA and/or adrenaline. These catecholamines activate adrenoceptors which are G-protein coupled receptors (GPCR) in the effector organs. These amines exert their effects by acting on adrenoceptors which are: i) α_1-adrenoceptors, linked to G_q, with subtypes α_{1A}, α_{1B} and α_{1D}, ii) α_{2}-adrenoceptors, linked to $G_{i/0,}$ with subtypes α_{2A}, α_{2B} and α_{2C}, and iii) β-adrenoceptors, linked to G_s, with subtypes $\beta_{1-,}$ β_{2-} and β_{3-}adrenoceptors (Levick, 2010).

In nearly every vascular bed studied across different animal species, the expression of sympathetic nerves has been shown and it accounts for the largest proportion of innervation in the resistance vasculature (Westcott and Segal, 2013b). Sympathetic nerves mainly regulate medium and small arteries, and innervation is sparser in large conduit arteries (Ralevic and Dunn, 2015). It has been reported that vascular responses to sympathetic nerve activity (SNA) can vary, depending on the content and composition of vesicles released from specific axon varicosities (Blair et al., 2003; Stjarne, 2001), the size and location of vessel branches within resistance networks (Marshall, 1982; Ruocco et al., 2002; Zang et al., 2006), and the frequency and firing pattern of action potentials (Bradley et al., 2003).

NA is the principal neurotransmitter in most sympathetic nerves (Biaggioni, 2012). This neurotransmitter is synthesized from its precursor, tyrosine, via tyrosine hydroxylase (TH) enzyme in nerve fibers, and stored with its co-transmitters, adenosine triphosphate (ATP) and neuropeptide Y (NPY) (Hirst and Edwards, 1989). NA binds to postsynaptic α-adrenoceptors (αARs) on smooth muscle cells and this results in vasoconstriction, while activation of β-adrenoceptors (βARs) causes vasodilation in limited vascular beds (Burnstock, 2009a; Guimaraes and Moura, 2001; Moore et al., 2010).

Activation of α_1-adrenoceptors (α_1ARs), a subtype of αAR on the post-junctional membrane of VSMC, stimulates phospholipase C through G_q and evokes production of inositol trisphosphate (IP_3) leading to the intracellular release of Ca^{2+} following activation of IP_3 receptors in the sarcoplasmic reticulum (SR) (Minneman, 1988). α_2-adrenoceptors (α_2ARs) are expressed in both pre- and post-junctional membranes, with α_2ARs stimulation on pre-junctional nerve fibers providing negative feedback by inhibiting NA released during sympathetic neurotransmission and leading to a reduction in transmitter release (Guimaraes and Moura, 2001; Levick, 2010; Vanhoutte et al., 1981). The binding of NA to post-junctional α_2ARs on smooth muscle cells activates G_i protein-mediated signalling leading to diminished adenylyl cyclase activity and thus reduces the cyclic adenosine monophosphate (cAMP) concentration. Low cAMP concentration leads to an increased concentration of calcium (Ca^{2+}) via a reduction in protein kinase A (PKA)-mediated phosphorylation of Ca^{2+} channels in SR and L-type Ca^{2+} channels in the plasma membrane, and this causes contraction in vascular smooth muscle cells (Straub et al., 2004; Xiong and Sperelakis, 1995).

In addition to αARs as the predominant effectors of sympathetic nerves, βARs also contribute to vascular regulation (Guimaraes and Moura, 2001;

Queen and Ferro, 2006). Activation of βARs leads to vasodilation (Fronek and Zweifach, 1975; Garland et al., 2011a) through the activation of adenylyl cyclase via G_s, and an increase in cAMP hence generating VSMC relaxation through a reduction of the intracellular calcium concentration $[Ca^{2+}]_i$ (Kornfeld et al., 2000; Pelligrino and Wang, 1998). βAR activation also elicits vasodilatation through VSMC hyperpolarization, potentially via activation of ATP-sensitive potassium channel (K_{ATP}) channels (Fujii et al., 1999; Garland et al., 2011b). Although it is still debatable, the expression of β_2ARs has been reported to evoke vasodilatation via a nitric oxide (NO)-dependent mechanism (Queen and Ferro, 2006). Removal of the endothelium has been shown to lessen relaxation to βAR agonists (Gray and Marshall, 1992; Molenaar et al., 1988; Toyoshima et al., 1998), however, others have found that endothelial βARs have no role in vasorelaxation (Cohen et al., 1984; Eckly et al., 1994; Satake et al., 1996). The diversity of experimental conditions, species and vessels may account for this discrepancy.

The concept of cotransmission in neuronal function was proposed in 1976 (Burnstock, 1976) although it was not well accepted until the 1990s. To date, it is firmly established that cotransmission has a key role in neurogenic regulation of the vasculature as well as other systems. NA is co-released with adenosine 5'-triphosphate (ATP) as a main sympathetic cotransmitter and neuropeptide Y (NPY) as a second major sympathetic neurotransmitter during activation of vascular adrenergic nerves (Burnstock, 2009a; Hobara et al., 2006; Lundberg, 1996; Westcott and Segal, 2013b).

Purinergic signalling was coined as a system where the purine nucleotides, ATP and adenosine diphosphate (ADP), and the nucleoside, adenosine, act

as extracellular messengers (Burnstock, 2008c). To date there are 19 different receptor proteins for adenosine, adenine and uridine nucleotides, and nucleotide sugars, belonging to three families of receptors: G protein-coupled adenosine and P2Y receptors, and ionotropic P2X receptors (Ralevic and Dunn, 2015). Purinergic mechanisms and specific receptor subtypes have been shown to be involved in physiological and pathological conditions of most studied systems across species (Burnstock, 2007; 2008b). In the vasculature, ATP can be released from both nerves and the endothelium (Burnstock, 1990a; 2010). Sympathetic purinergic cotransmission has also been demonstrated in many different blood vessels (Burnstock, 1988; 1990b; 2007; Burnstock and Ralevic, 2014; Sneddon and Burnstock, 1984)). On VSMC, purinoceptor subtypes P2X1, P2X2 and P2X4, $P2Y_1$ and $P2Y_2$ have been identified, while in endothelial cells, P2X1, P2X2, P2X3, P2X4 and $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{11}$ receptors have been recognized (Burnstock, 2008a).

ATP has been shown to be the predominant sympathetic neurotransmitter in rat mesenteric small arteries (Rummery et al., 2007). Furthermore, it has been demonstrated that in pre-constricted rat and porcine mesenteric arteries, a condition that is more relevant to the normal condition of vascular smooth muscle, the purinergic component of sympathetic neurotransmission became more apparent compared to basal conditions (Pakdeechote et al., 2007; Shatarat et al., 2014). ATP released from sympathetic nerves produces contraction of vascular smooth muscle via P2X (ion channel) receptors. These receptors on VSMCs are intrinsic cation channels, that when activated, cause Ca^{2+} and/or Na^{2+} influx, subsequently leading to transient and rapid depolarization (Burnstock, 2012b; Hirst and Edwards, 1989). As a result, L-type Ca^{2+} channels are activated and the concentration of intracellular calcium concentration ($[Ca^{2+}]_i$) is increased

(Lagaud et al., 1996). Irrefutable evidence for an involvement of P2X1 receptors in sympathetic perivascular neurotransmission comes from studies in mesenteric arteries from P2X1 receptor knockout mice (Vial and Evans, 2002). Neurogenic vasoconstriction was reduced in P2X1 receptor-deficient arteries, and this reduced response was abolished by α_1-adrenoceptor blockade (with prazosin) but was unaffected by a P2 receptor antagonist (pyridoxalphosphate-6-azophenyl-2'-5'-disulfonate, PPADS), whilst the neurogenic response of the wild type arteries was inhibited by both antagonists, consistent with cotransmission involving NA and ATP/P2X1 receptors (Vial and Evans, 2002). The P2X receptor subfamily encompasses 7 purinoceptor subtypes (P2X1-7), with P2X1 being the main purinoceptor in vascular VSMCs (Burnstock, 2009b; Lamont et al., 2006).

As metabotropic receptors, activation of P2Y (G protein-coupled) receptors by ATP activates phospholipase C (PLC), and consequently leads to an increase in IP_3 and Ca^{2+}; in endothelial cells, this then promotes NO production via endothelial nitric oxide synthase (eNOS), and results in vasodilatation (Burnstock, 2012a). P2Y receptor activation by ATP released from endothelial cells in response to changes in blood flow (shear stress) and hypoxia, causes endothelial cells to release NO resulting in vasodilation (Burnstock, 1990a). Although the link between P2Y receptors and the endothelium has been defined, it is still unclear whether the ATP that is released from PVNs can reach endothelial cells and activate P2Y receptors (Westcott and Segal, 2013b).

NPY is synthesized in sympathetic neurons and transported along the axons (Fried et al., 1985). NPY immunoreactivity has been reported to be co-localized with that of TH throughout the arteriolar network in rat cremaster muscle (Fleming et al., 1989). Furthermore, expression of NPY

has also been demonstrated in subpopulations of sympathetic (TH-immunoreactive) neurons in guinea pig cutaneous vessels of ears (Morris et al., 2001). Under experimental conditions, the release of NPY is only apparent at higher stimulation frequencies (Lundberg and Stjarne, 1984) and it is still unclear about the contribution of NPY to vasomotor control under resting physiological conditions (Hodges et al., 2009). NPY exerts its effects through the activation of six receptor subtypes (Y1-Y6) with Y1 being the primary post-junctional receptor expressed on vascular VSMCs (Larhammar and Salaneck, 2004). The activation of NPY receptors enhances PLC activity and subsequently increases IP_3 production and $[Ca^{2+}]_i$ (Brain and Cox, 2006; Heredia et al., 2002). Although NPY alone can evoke vasoconstriction, the essential role of this neuropeptide is predominantly neuromodulation, augmenting post-junctional responses and inhibiting pre-junctional release of neurotransmitter (Edvinsson et al., 1984; Lundberg, 1996)

1.2.2 Parasympathetic nerves

The parasympathetic nervous system has almost no impact on the vasculature. It has no effect on total peripheral resistance, because it innervates only a limited number of vascular beds. However, it can cause vasodilatation in the pancreas, salivary glands, cerebral vessels and erectile tissue of the genitalia (Aaronson and Ward, 2007; Talman, 2004). More recent research suggests that rat mesenteric arteries have cholinergic innervation, however, it is not associated with vascular tone regulation (Tangsucharit et al., 2012).

1.2.3 Sensory nerves

The existence of NANC neurotransmission is firmly accepted in the central and peripheral nervous systems (Burnstock, 2007). Evidence has accumulated that NANC nerves play important roles in regulating vascular tone (Kawasaki et al., 2011). In NANC neurotransmission, sensory nerves play a vital role in vasoregulation, and generally cause vasodilatation (Kawasaki et al., 1988; Kawasaki et al., 2011). Sensory nerves demonstrate physiological antagonism of sympathetic nerve-dependent vasoconstriction (Mione et al., 1990), and also possesses both antidromic and orthodromic conduction, thus this allows their participation in local axon reflexes independent of efferent signalling from the cell body (Krogh et al., 1922; Yaprak, 2008). As a consequence, noxious stimuli, chemical and mechanical irritation, extreme pH or temperature can stimulate antidromic activity of sensory nerves to cause neurotransmitter release and induce vasodilation or pain sensation (Burnstock and Ralevic, 1994; Krogh et al., 1922).

Early studies revealed that the cell bodies of sensory nerves lie within the dorsal root ganglia (DRG) (Hobara et al., 2004b; Ishikawa et al., 2003). However, Somasundaram et al. (2006), through molecular, reverse transcription polymerase chain reaction (RT-PCR), immunocytochemical and confocal analyses recently challenged this concept and proposed that the neuronal somata are also present in the adventitia of rat mesenteric arteries. Other studies also provide some evidence that sensory cell bodies are expressed in the arterial wall of the heart (Page et al., 1995), and axonal swellings, possibly of sensory origin, have been located in the wall of the carotid sinus or arterial vascular bed (Hohler et al., 1994).

Calcitonin gene-related peptide (CGRP), a potent vasodilator, has been recognized as the primary neurotransmitter of sensory nerves while substance P (SP) is a cotransmitter (Brain and Grant, 2004; Kawasaki et al., 1988). CGRP is a 37-amino acid neuropeptide that is primarily localized to C and Aδ sensory fibers and has two major forms, α and β (Russell et al., 2014). αCGRP is predominantly expressed in the nervous system while βCGRP is primarily expressed in the enteric sensory system (Eftekhari and Edvinsson, 2010). These isoforms are synthesized from two distinct genes at different sites of chromosome 11 in the human (Wimalawansa et al., 1990). The putative receptor for CGRP is a GPCR of the B-subtype (Hay et al., 2008), a complex receptor that consists of a seven transmembrane spanning protein, and calcitonin receptor-like receptor (CLR), a single transmembrane-spanning protein, designated receptor activity modifying protein 1 (RAMP)1 (McLatchie et al., 1998). It has been reported that RAMP1 is essential for CGRP receptor ligand binding and specificity (Sexton et al., 2001).

Sensory nerves express transient receptor potential vanilloid 1 (TRPV1) channels and are commonly called "capsaicin (a TRPV1 channel agonist) sensitive nerves", as the application of capsaicin leads to sensory nerve-induced responses, through the release of CGRP (Kawasaki et al., 1988; Kawasaki et al., 2011). Impaired vasodilatation responses to electrical field stimulation (EFS)-induced perivascular nerve stimulation in mesenteric arteries of TRPV1-null mutant mice has been reported (Wang et al., 2006). The TRPV1 agonist, capsaicin, is known for its pungent property and is responsible for the hot and spicy sensation of chilli peppers, properties which are due to its activation of TRPV1 receptors on sensory nerves (O'Neill et al., 2012). Capsaicin serves a 'dual-sensory efferent function', which can be defined as initiation of afferent signals and neuropeptide

release; both events are coupled at the same nerve endings (Szolcsanyi, 2004). Therefore, this enables capsaicin to be used widely as a tool to desensitize sensory nerves, through the depletion of CGRP, as well as to activate sensory nerves through TRPV1 channel activation.

TRPV1 channels are one of the largest families of ion channels, and are members of transient receptor potential (TRP) superfamily of structurally related, non-selective ligand-gated cation channels (Fernandes et al., 2012). These channels can be activated by physical and chemical stimuli, including capsaicin, temperature, pH less than 5.2, endogenous lipids and some inflammatory mediators (Szallasi and Blumberg, 1999). TRPV1 sensitization is achieved through phosphorylation by certain kinases and these enzymes are activated through second messenger cascades (Jung et al., 2004; Premkumar and Ahern, 2000). TRPV1 channel sensitization leads to lowering of the threshold for activation (Moriyama et al., 2005), and subsequently enhanced CGRP release from sensory nerves. The release of CGRP from sensory nerves activates either one or both CGRP receptor subtypes, CGRP1 and CGRP2. The activation of these GPCRs increases cAMP and protein kinase A (PKA)-activated potassium (K^+) channels such as K_{ATP} and large conductance calcium-activated potassium channels (BK_{CA}) (Brain and Cambridge, 1996; Nelson et al., 1990; Reslerova and Loutzenhiser, 1998; Wellman et al., 1998). As a result, VSMC hyperpolarization takes place and evokes closure of voltage-gated Ca^{2+} channels and reduces the concentration of Ca^{2+} to promote vasodilatation (Westcott and Segal, 2013b).

The presence of sensory PVNs has been described in a variety of vascular beds across several species including humans (Birch et al., 2008; Eguchi et al., 2004; Gulbenkian et al., 1993; Hobara et al., 2010; Morris et al.,

1986; Sequeira et al., 2005). In the mesenteric arterial bed of rat and mouse, transmural complexes contain fibres releasing CGRP and substance P (SP), with vasoactive intestinal peptide (VIP) and NO additionally found in the mouse (Lundberg, 1996), and these sensory nerves act as modulators for the autonomic control of the intestinal tract (Maggi and Meli, 1988; Mione et al., 1990). CGRP was shown to be the main vasodilator neurotransmitter in rat mesenteric arteries as EFS-induced vasorelaxation was abolished in the presence of capsaicin and exogenous capsaicin mimicked EFS-induced relaxation, being slow in both onset and decay (Kawasaki et al., 1988). This group further confirmed their findings using immunohistochemical staining; networks of CGRP-like immunoreactive fibres were observed on mesenteric arteries and these markedly diminished after treatment with capsaicin (Kawasaki et al., 1988).

Substance P (SP) is a neurokinin which is involved in certain physiological and pathological conditions including learning and memory, pain and vascular tone regulation (Abdelrahman and Pang, 2005; Ikeda et al., 2003; Quartara and Maggi, 1998; Whittle et al., 1989). It is synthesized in the DRG, transported along axons and stored in vesicles within sensory nerve terminals (White et al., 1985). Of three receptor isoforms that have been identified (NK1-3), SP primarily binds to the neurokinin 1 (NK1) receptor (Langosch et al., 2005). A few studies have shown that exogenous SP is a potent NO-dependent vasodilator (Abdelrahman and Pang, 2005; Kawasaki et al., 1990; Whittle et al., 1989). The binding of SP to NK1 receptors on endothelial cells increases $[Ca^{2+}]_i$ activates eNOS (Brain and Cambridge, 1996). Furthermore, it has been shown that either endothelial denudation, or scavenging NO, inhibited SP-mediated dilation of mesenteric arteries (Bolton and Clapp, 1986). Nonetheless, the role of SP remains

controversial as its levels in the microcirculation may not be sufficient to affect vessel diameter or permeability (Brain, 1997; Westcott and Segal, 2013b). In addition, exogenous SP has no effect on the tone of hepatic and mesenteric arteries (Kawasaki et al., 1988; Li and Duckles, 1992; Phillips et al., 2000).

The involvement of purinergic neurotransmission from sensory nerves was first demonstrated by detection of ATP release in the perfusate during antidromic stimulation of sensory nerves in the rabbit ear artery (Holton, 1959). A later study showed that sensory neurogenic relaxation was blocked by P2 receptor antagonists, but not by adenosine receptor antagonists, in small coronary arteries of the lamb (Simonsen et al., 1997). The removal of endothelium had no effect on the neurogenic responses, while the responses to ATP were attenuated, indicating that the P2Y receptors might be located on the VSMC (Simonsen et al., 1997). This finding indicates the possible vasorelaxant role of P2Y receptors that are present on vascular VSMCs, as well a role in mediation of NANC vasorelaxation (Ralevic and Dunn, 2015).

1.2.4 Nitrergic innervation

Nitrergic nerves contribute to PVN-mediated vasodilation through the production of NO, produced within nerve terminals that contain nitric oxide synthases (Burnstock, 2009a), including some sensory and parasympathetic PVN (Westcott and Segal, 2013b). NO is also synthesized in endothelial cells (Furchgott and Zawadzki, 1980). NO secreted from PVNs diffuses into smooth muscle cells (VSMCs), leading to guanylate

cyclase activation which then generates cGMP and causes vasodilatation (Denninger and Marletta, 1999). In rat mesenteric arteries (MA), it has been shown that NO pre-junctionally inhibits the effect of NA, thus diminishing adrenergic vasoconstriction (Hatanaka et al., 2006; Koyama et al., 2010). This observation indicates that nitrergic nerves can interact and modulate other PVNs (Westcott and Segal, 2013b). Furthermore, Si and Lee (2002) showed that activation of α7-nicotinic acetylcholine receptors on sympathetic nerves of porcine cerebral arteries causes calcium influx and NA release, and leads to activation of presynaptic α2-adrenoceptors located on the neighbouring nitrergic nerve terminals, resulting in NO release and vasodilation.

1.3 Adipose tissue

It is now has been accepted that adipose tissue (AT) is not just a store of lipid or an inert structure with little functional significance (Szasz and Webb, 2012), as it has been shown to play an integral role in regulating energy balance through its metabolic, cellular and endocrine functions (Lee et al., 2014). The physiological roles of AT will be discussed later in this chapter (Section 1.4) with specific focus on perivascular adipose tissue (PVAT). AT is widely distributed around blood vessels (perivascular), around internal organs (visceral or abdominal) or is present subcutaneously (Maenhaut and Van de Voorde, 2011) and it is classically defined as either white or brown (Szasz and Webb, 2012). White adipose tissue (WAT) constitutes up to 20 to 25 % of total body weight and plays a crucial role as an energy store (Maenhaut and Van de Voorde, 2011). WAT also acts as a thermal insulator and supports the body against mechanical shocks (Mariman and Wang, 2010). Brown adipose tissue (BAT) regulates

body temperature by lipid metabolism in newborn mammals and some hibernating animals (Cannon and Nedergaard, 2004). BAT contains many mitochondria which have a unique protein called uncoupling protein 1 (UCP1) (Seale and Lazar, 2009). This protein acts by eliminating the proton motive force that is normally used to stimulate the synthesis of cellular ATP (Cannon and Nedergaard, 2004). Consequently, it leads to the production of energy in the mitochondrial gradient which is released in the form of heat (Seale and Lazar, 2009).

There is evidence that the BAT level is inversely correlated with body mass index (BMI) (Cypess et al., 2009; Seale and Lazar, 2009). BAT and WAT adipocytes derive from different precursors, in which BAT adipocytes share origins with skeletal muscle while WAT adipocytes have a different and not fully characterized origin (Lazar, 2008; Richard et al., 2010). WAT is less well vascularised, less innervated and less metabolically active compared to BAT (Cinti, 2005). Both types of adipocytes receive innervation by the sympathetic nervous system (Berthoud et al., 2006) as discussed below.

Recently, the concept of beige adipose tissue has been introduced, and is distinct from both BAT and WAT (Petrovic et al., 2010; Wu et al., 2012) although it has been reported that the formation of beige/brite adipose tissue is due to "browning" of WAT (Park et al., 2014). Interestingly, the beige/brite cells in WAT are derived from precursor cells that are different from classical brown adipocytes and are closer to the white adipocyte cell lineage (Petrovic et al., 2010). These beige/brite cells resemble the white adipocyte-like phenotype, including large lipid droplets and the lack of UCP1 expression under basal conditions, but when they respond to certain stimuli such as cold exposure or β_3-adrenoceptor activators, beige/ brite cells transform into cells having BAT-like characteristics, such as

multilocular/small lipid droplets and UCP1 expression (Himms-Hagen et al., 2000; Park et al., 2014).

1.3.1 Sympathetic nervous system (SNS) innervation of adipose tissue

The innervation of BAT by the sympathetic nervous system (SNS) has long been recognized both at the level of blood vessels and also directly on adipocytes (Norman et al., 1988). In fact, its physiological function, involvement in nonshivering thermogenesis in rodents is directly controlled by SNS innervation (Bartness et al., 2010; Richard and Picard, 2011). The release of NA from sympathetic nerve terminals stimulates β_3-adrenoceptors that turns on a cascade of intracellular events ending in activation of UCP1 and triggering BAT thermogenesis (Bartness et al., 2010; Cannon and Nedergaard, 2004).

In 1964, Wirsen (1964) (cited in Bartness et al. (2010)) proposed that there was no direct sympathetic innervation of brown adipocytes as evidenced by the absence of catecholaminergic nerve fibers in the parenchymal space using histofluorescence staining. However, a year later, the same author found catecholaminergic varicosities in the parenchymal space and among blood vessels using the same methods ((Wirsen, 1965); cited in Bartness et al., 2010). Further investigation using electron microscopy revealed the existence of sympathetic innervation in BAT ((Bargmann et al., 1968); cited in Bartness et al., 2010). An elegant approach by Bartness and co-workers, using a unilateral denervation model (Bartness et al., 2010; Bartness, 2001), verified the sympathetic innervation of BAT. Furthermore, the retrograde viral transneuronal tract

tracer, pseudorabies virus, injected into the central sympathetic outflow, revealed that circuits ultimately terminate in BAT, indicating that the SNS outflow directly originates from the brain (Bartness et al., 2010).

Various experimental approaches, including neurochemical (NA turnover), neuroanatomical (viral tract tracing) and functional (sympathetic denervation-induced blockade of lipolysis) studies have revealed SNS innervation in WAT (Bartness and Bamshad, 1998; Bartness et al., 2014; Bartness and Song, 2007; Foster and Bartness, 2006; Ryu and Bartness, 2014). In WAT, the SNS is the principal initiator of lipolysis in mammals (Nguyen et al., 2014). Previous studies have shown that four adrenoceptor subtypes, α_2-, β_1-, β_2- and β_3-adrenoceptors exist in WAT (Collins et al., 2004; Collins and Surwit, 2001; Lafontan, 1994; Lafontan and Berlan, 1995). Of these adrenoceptors, all beta (β_1-, β_2- and β_3-)adrenoceptors stimulate lipolysis while α_2-adrenoceptors inhibit the process (Lafontan and Berlan, 1995). The SNS innervation in WAT also plays a role in the control of lipid proliferation (Bartness and Bamshad, 1998; Jones et al., 1992). It has been shown that sympathetic denervation of WAT *in vivo* stimulates fat cell proliferation (Bartness and Song, 2007; Cousin et al., 1993).

1.3.2 Parasympathetic nervous system (PNS) innervation of adipose tissue

In BAT, the parasympathetic nervous system (PNS) innervation only appears in two minor BAT depots, pericardial, normal structures that lie in the cardiophrenic (Schafer et al., 1998) and mediastinal, the central compartment of the thoracic cavity surrounded by loose connective tissue (Giordano et al., 2004), but not in the major interscapular BAT (IBAT), a

region between shoulder blade depot that is found in rodent and in human infant (Bartness et al., 2010). In contrast, a few studies have shown that WAT is not innervated by the PNS (Bartness et al., 2014; Bartness and Song, 2007; Berthoud et al., 2006). The evidence to date indicates a limited involvement of the PNS in adipose tissue innervation, with no clear physiological role has been described thus far.

1.3.3 Sensory innervation in adipose tissue

There is accumulating evidence that BAT has marked sensory innervation, as shown by immunohistochemical markers of sensory neuropeptides such as CGRP and substance P (Bartness et al., 2010). Denervation of sensory nerves in BAT results in general decreases in BAT growth and protein content, mitochondrial content such as cytochrome oxidase activity, and thermogenic capacity, such as UCP1 content (Cui and Himms-Hagen, 1992; Himms-Hagen et al., 1990).

Collective data obtained from different experimental protocols including anterograde viral tract tracer (Fishman and Dark, 1987), co-culture of DRG cell and adipocytes (Kosacka et al., 2006) and immunohistochemistry (Fredholm, 1985) strongly indicate that WAT is innervated by sensory nerves. Recently, Murphy et al. (2013) showed endogenous leptin secreted from WAT activates spinal sensory nerves innervating Siberian hamster brain in a paracrine manner. Despite the innervation and feedback loops (Ryu and Bartness, 2014) of sensory nerves that have been recognized in WAT, the significance of the innervation is still poorly understood and warrants further exploration.

1.4 Perivascular adipose tissue

Perivascular adipose tissue (PVAT) surrounds most systemic blood vessels, except the cerebral circulation (Gao, 2007). It is often absent in the microcirculation, but is normally present within most arteries and veins with a diameter of more than 50 μm (Houben et al., 2012), and differs from adipose tissue in general due to its specific location (surrounding blood vessels) (Gil-Ortega et al., 2015; Szasz and Webb, 2012). PVAT is located on the outside of the adventitia, although no clear barrier exists between them (Gil-Ortega et al., 2015; Szasz et al., 2013) as shown in Figure 2. However, it has been reported that PVAT of large vessels is separated by an anatomical barrier compared to small vessels and this may contribute to heterogeneity in the mechanisms by which PVAT-derived factors reach the muscular or intimal layers of the vascular wall (Ahmed et al., 2004; Gil-Ortega et al., 2015).

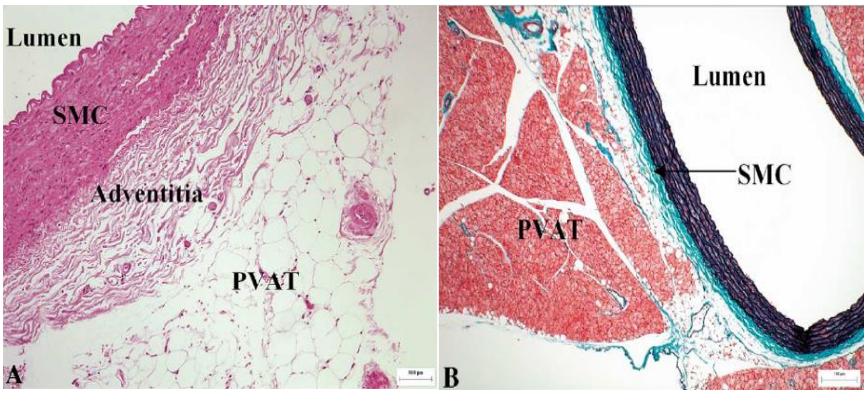

Figure 2: (A) Transection of a human internal thoracic artery and (B) longitudinal section of a rat thoracic aorta showing the perivascular adipose tissue (PVAT) is located at the outside of adventitial layer, which separated SMC (smooth muscle cell) and PVAT. PVAT in human subjects is composed of white adipose tissue compared with brown adipose tissue in rat aortae. Magnification bar represents 100 μm. This figure is taken from Gao et al. (2005b).

There is increasing evidence for regional phenotypic and functional differences among PVAT depots, depending on the specific vascular bed or different regions in the vascular bed where the PVAT is located (Amis and Cronan, 1988; Gil-Ortega et al., 2015; Hill et al., 2001; Iacobellis, 2014). The regional differences in the vascular networks are due to adaptations to meet the hemodynamic and metabolic demands of a specific organ (Gil-Ortega et al., 2015; Hill et al., 2001). Vascularization, innervation and distribution of the different types of PVAT (BAT and WAT) vary enormously with location (Gil-Ortega et al., 2015; Rittig et al., 2012; Szasz and Webb, 2012). For instance, aortic PVAT consists of mixed BAT and WAT, whilst WAT is the dominant tissue for mesenteric PVAT (Gao, 2007). Furthermore, it has been shown that in humans, perivascular coronary adipocytes exhibit a reduced state of differentiation, are more irregular in shape, and are smaller in size than subcutaneous and perirenal adipocytes, with a smaller lipid droplet accumulation (Chatterjee et al., 2009).

A large body of evidence suggests that PVAT has distinct secretory profiles, depending on the vasculature (Galvez-Prieto et al., 2008; Gil-Ortega et al., 2015; Rittig et al., 2012) and it is hypothesized to be a functionally specialized type of adipose tissue with different developmental and secretory properties (Chatterjee et al., 2009). In general, the secretion pattern of PVAT cells differed from visceral and subcutaneous PVAT cells. It can be exemplified by mouse aortic PVAT which secretes less adiponectin, leptin, and resistin, and expresses lower levels of lipid oxidation genes, compared with subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) (Chatterjee et al., 2009; Fitzgibbons et al., 2011). Similarly, levels of leptin, adiponectin, tumor necrosis factor alpha (TNF-α), and monocyte chemoattractant protein-1 (MCP-1) were found to be lower in human peritibial and peripopliteal PVAT compared with subcutaneous AT

(Mauro et al., 2013). Furthermore, Rittig et al. (2012) also showed that perivascular preadipocytes secreted higher levels of MCP-1 and vascular endothelial growth factor (VEGF) than visceral and subcutaneous preadipocytes. Collectively, these data suggest that there is structural and functional regional heterogeneity among different PVAT depending on the type of blood vessel.

PVAT has been largely ignored in the past but is currently receiving increased attention due to its role in physiological and pathological conditions. Previously, most *in vitro* experiments which studied blood vessels began with 'cleaning' the vessels, which directly removed PVAT as it was believed to provide only mechanical support without any physiological function (reviewed in Szasz and Webb, 2012). However, the scenario changed after Soltis and Cassis (1991) discovered that PVAT significantly attenuated contractile responses to NA in rat aorta. In their series of experiments, they also demonstrated that there was no difference between cleaned and PVAT-intact tissues in contractile responses to KCl and phenylephrine (selective α_1-adrenoceptor agonist) as well as relaxation responses to acetylcholine, isoproterenol (non-selective β-adrenoceptor agonist) and sodium nitroprusside (NO releaser). In contrast, PVAT-intact tissues exhibited greater contractile responses to tyramine (indirect sympathomimetic agonist) compared to cleaned tissue. In EFS experiments, intact tissues elicited a frequency-dependent contraction while no response was observed with cleaned tissues (Soltis and Cassis, 1991). These findings indicated that PVAT significantly influences vascular responsiveness in the *in vitro* setting and thus prompted the initiation of extensive research.

1.4.1 Perivascular adipose tissue innervation

Perivascular adipose tissue (PVAT) can release several vasoactive compounds including adipokines, cytokines, angiotensin II and reactive oxygen species (as discussed in Section 1.4.2-1.4.7). It is established that these substances can cause either vasoconstriction or vasodilatation by acting on targets located on either smooth muscle cells or endothelial cells. However, little is known about whether PVAT-derived substances can influence neuronal responses in blood vessels. A recent review by Bulloch and Daly (2014) highlighted our poor understanding of the link between PVAT and the autonomic nervous system, or other nerves, that are involved in controlling the vasculature.

Despite the limited literature on the innervation of PVAT and its impact, there exist a few studies that suggest the innervation of PVAT. In fact, the innervation of PVAT was first demonstrated in 1970, when Diculescu and Stoica (1970) produced a set of Falk fluorescence plates demonstrating the clear innervation of perivascular fat in the rat mesenteric artery. However, it is only recently that the interest to investigate neurotransmission in PVAT has been revived. Recently, Dashwood and Loesch (2011) showed that PVAT of human saphenous vein receives direct sympathetic innervation. Bulloch and Daly (2014) also provided evidence of sympathetic innervation in PVAT, of mouse mesenteric arteries, using immunofluorescence techniques.

Interactions between nerves and PVAT-derived adipokines have been demonstrated, in which the sympathetic nervous system suppresses serum adiponectin levels and adiponectin synthesis in WAT (Imai et al., 2006). It has been postulated that sympathetic innervation in small arteries is

responsible for maintaining BP, and may be influenced indirectly via factors including perivascular contractile factors (PVCFs), perivascular relaxing factors (PVATRFs) and NO released as a result of nerve mediated WAT activation, as well as via direct actions of sympathetic (NA and ATP) and sensory neurotransmitters (Bulloch and Daly, 2014). A recent publication illustrated a single branching nerve fibre innervating both PVAT and adventitial fibroblasts (Miao and Li, 2012). Other studies indicate that sympathetic nerve tracts lie within adipocytes and regulate lipolysis (Cinti, 1999; Rebuffe-Scrive, 1991). To our knowledge, there is no existing literature on the innervation of sensory nerves within PVAT. The interaction between nerves and PVAT is very unclear, and thus warrants exploration.

1.4.2 Bioactive molecules from PVAT

Perivascular adipose tissue (PVAT) is now established to play a role in regulating vascular tone. Adipocytes and stromal cells lying within PVAT are sources of an ever-growing list of molecules including adipokines, cytokines, reactive oxygen species and gaseous compounds (Szasz et al., 2013) as shown in Table 1. PVAT mainly exerts its effect by a paracrine manner while a few PVAT-derived compounds have an endocrine role (Szasz and Webb, 2012). Data obtained from a number of studies have shown that PVAT secretes several important vasoactive molecules with pharmacological promise that will be discussed in this chapter.

Table 1: PVAT-derived vasoactive compounds and receptors

	Vasoactive compounds
Adipokines	Leptin, adiponectin, methyl palmitate, apelin, resistin, visfatin, adrenomedullin, omentin, nesfatin, vasfin, chemerin, methyl palmitate
Cytokines	interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), plasminogen-activator inhibitor-1 (PAI-1) and cytokines receptors
Gaseous molecules	Superoxide, hydrogen peroxide, nitric oxide and hydrogen sulfide
Renin-angiotensin-aldosterone-system (Raasch et al.) molecules	Angiotensinogen, angiotensin I, angiotensin II, angiotensin (1-7), angiotensin-converting enzyme 1 and 2, pro(renin) receptor and angiotensin II receptors
reactive oxygen species (ROS)	Nicotinamide adenine dinucleotide phosphate-oxidase, superoxide dismutases, and lipoxygenases

Sources include Adeghate (2004); Aghamohammadzadeh et al. (2012); Fang et al. (2009); Fukai et al. (2005); Fukuhara et al. (2005); Galvez-Prieto et al. (2008); Lee et al. (2011); Maenhaut and Van de Voorde (2011); Ohkawa et al. (1994); Szasz and Webb (2012); Thalmann and Meier (2007); Trayhurn and Beattie (2001); Viengchareun et al. (2002); Yamawaki et al. (2010); Zhang et al. (1994).

1.4.3 PVAT-derived adipokines

Leptin, a 16 kD peptide hormone encoded by the *ob* gene, was the first adipokine to be identified (Zhang et al., 1994). It is an adipocyte-specific hormone, mainly secreted by WAT adipocytes, with a key role in energy homeostasis (Brennan and Mantzoros, 2006; Szasz et al., 2013). Leptin regulates appetite, insulin sensitivity, glucose uptake, bone metabolism and the immune system, as well as modulating the endocrine hypothalamic-pituitary system (Barr et al., 1997; Cammisotto et al., 2005; Mueller et al., 1998; Szasz et al., 2013). Leptin and its receptors have been shown to be expressed in the cardiovascular system (Beltowski, 2006). Leptin acts on its receptor which includes at least six isoforms, with Ob-Ra as the predominant receptor (Mattu and Randeva, 2013). The actions of leptin are mediated via Ob-Ra modulation of the janus kinase/signal transducers and activators of transcription (JAK/STAT) (Zabeau et al., 2003) and 5'-adenosine monophosphate-activated protein kinase (AMPK) pathways (Minokoshi et al., 2002), or via phosphatidylinositol 3'-kinase (PI3)–Akt and mitogen-activated protein kinases (MAPK) pathways (Fruhbeck, 2006; Ren, 2004).

In the cardiovascular system, under normal conditions, leptin demonstrates vasorelaxing effect (Mohammed et al., 2007; Nakagawa et al., 2002; Sahin and Bariskaner, 2007). The anti-contractile effect of leptin is achieved via both endothelial dependent (through involvement of NO) and endothelial independent mechanisms (Kimura et al., 2000; Lembo et al., 2000; Sahin and Bariskaner, 2007). It has been shown that leptin dilates canine small mesenteric arteries and veins by a mechanism involving endothelial release of nitric oxide (Mohammed et al., 2007). Leptin directly antagonizes angiotensin II-induced vasoconstriction in rat aorta (Fortuno et al., 2002)

through inducible NOS (iNOS) activity in vascular VSMC (Rodriguez et al., 2007). In measurements of forearm vascular resistance of healthy men, leptin was shown to directly cause vasodilation via a NO-independent mechanism (Nakagawa et al., 2002). Conversely, an indirect vasoconstrictor effect of leptin has been reported in which leptin can excite the sympathetic nervous system, consequently leading to a rise in BP (Fruhbeck et al., 1999). Furthermore, it has also been shown that chronic leptin infusion results in endothelial dysfunction (Knudson et al., 2005) and hypertension (Shek et al., 1998). Leptin is being studied extensively as it holds promise in obesity treatment. Obesity is closely related to hyperleptinemia and most obese humans are found to be resistant or tolerant to leptin (Dardeno et al., 2010).

Adiponectin is regarded as the most abundant adipokine and is released by both brown (Viengchareun et al., 2002) and white (Trayhurn and Beattie, 2001) adipocytes. There are two forms of adiponectin, full length or a smaller globular fragment (Kadowaki and Yamauchi, 2005). The full length adiponectin acts on the R2 receptors while the globular form acts via R1 receptors (Yamauchi et al., 2003). Adiponectin is an insulin sensitizing hormone and acts through the receptors AdipoR1, AdipoR2, and T-cadherin (Shehzad et al., 2012). Interestingly, it has been shown that adiponectin levels are decreased in obesity (Arita et al., 1999) possibly identifying it as negative marker for obesity related diseases (Shehzad et al., 2012). Adiponectin possesses antiinflammatory and antioxidative properties (Antoniades et al., 2009) and prevents macrophage activation and foam cell accumulation, which enables adiponectin to act as an anti-atherosclerotic agent (Shehzad et al., 2012). Adiponectin exhibits a vasorelaxing role in humans via an endothelium-dependent mechanism and the potential mechanism for the vasorelaxation is activation of

phosphorylation and stimulation of endothelial NO synthase via phosphatidylinositol 3-kinase-dependent pathways. As a result, NO production increases in vascular endothelial cells in response to adiponectin (Tan et al., 2004). In rat aorta, vasorelaxation is produced through NO and activation of potassium channels (Greenstein et al., 2009; Xi et al., 2005). The rise of NO concentration inhibits platelet aggregation, leukocyte adhesion to endothelial cells and vascular smooth muscle proliferation (Maenhaut and Van de Voorde, 2011). In addition, it has been shown that the endothelium-dependent vasorelaxation response to acetylcholine is significantly attenuated in adiponectin receptor knockout mice (Ouchi et al., 2003). Incubation with a blocking peptide for the adiponectin R1 receptor almost completely abolished the vasodilator effect of PVAT (Greenstein et al., 2009). Moreover, adiponectin has been found to reduce oxidative stress by suppressing reactive oxygen species (ROS) production in endothelial cells (Maenhaut and Van de Voorde, 2011).

Adrenomedullin is a 52 amino acid peptide which is produced by the adrenal glands, heart, vascular endothelial, smooth muscle cells and white adipocytes in both humans and rodents (Fukai et al., 2005). Adrenomedullin exerts a direct and potent vasodilatory action on blood vessels (Aghamohammadzadeh et al., 2012). It also possesses antioxidant properties (Aghamohammadzadeh et al., 2012). Nuki et al. (1993) reported that adrenomedullin causes vasodilatation in mesenteric vessels from isolated rats. It has been shown that adrenomedullin induced vasodilatation via NO dependent pathways in coronary (Terata et al., 2000) and skeletal muscle arteries of humans (Nakamura et al., 1997). Furthermore, intravenous infusion of adrenomedullin results in significant vasodilation of pulmonary vessels, suggesting a potential therapeutic strategy for pulmonary hypertension (Nagaya et al., 2003; Nagaya et al.,

2000). Adrenomedullin has been demonstrated to reduce levels of ROS in vascular smooth muscle (Yoshimoto et al., 2005), while adrenomedullin knockout mice express higher levels of ROS (Shimosawa et al., 2003). In patients with coronary artery disease (Gryglewski et al.), the level of adrenomedullin is higher than in healthy people (Aghamohammadzadeh et al., 2012). Theoretically, adrenomedullin serves as a protective mechanism against CAD (Shibasaki et al., 2010). However, there is a possibility that the elevation of adrenomedullin level can contribute to vascular dysfunction (Aghamohammadzadeh and Heagerty, 2012).

Omentin is a PVAT-derived cytokine which consists of 313 amino acids and is chiefly expressed in visceral rather than in subcutaneous adipose tissue (Yamawaki et al., 2010). Of the two isoforms, omentin-1 appears to be the main isoform in human plasma (de Souza Batista et al., 2007). It has been shown to induce both endothelium-dependent and endothelium-independent vasorelaxation (Maenhaut and Van de Voorde, 2011) in isolated rat aorta and small mesenteric arteries (Yamawaki et al., 2010). Plasma levels of this protein and its expression have been observed to be reduced in obesity (de Souza Batista et al., 2007) and type II diabetes mellitus (Cai et al., 2009). Moreover, decreased omentin-1 levels are linked with low plasma adiponectin and high-density lipoprotein levels (Maenhaut and Van de Voorde, 2011). Furthermore, omentin-1 levels are negatively associated with leptin levels, waist circumference, body mass index and insulin resistance (de Souza Batista et al., 2007). In parallel to adiponectin, omentin-1 concentration increases after weight loss and leads to improvement of insulin sensitivity (Moreno-Navarrete et al., 2010). Omentin has recently been found to reduce BP in rats by inhibiting NA-induced increases in systolic BP and mean BP, and dimorpholamin and

angiotensin II-induced increases in diastolic BP *in vivo* in the rat (Kazama et al., 2013).

Visfatin, also known as pre-B-cell colony-enhancing factor (PBEF) is a protein with a molecular weight of 52 kDa. It is secreted by PVAT and VAT (Fukuhara et al., 2005). It has strong insulin-mimetic effects as well as having been proven to play important roles in intracellular signalling as it is homologous with nicotinamide phosphoribosyl transferase (NAMPT) (Stastny et al., 2012). This protein has been confirmed as NAMPT and involved in nicotinamide adenine dinucleotide (NAD) biosynthesis (Rongvaux et al., 2002). Visfatin enhances growth of vascular smooth muscle cells (Wang et al., 2009) and endothelial angiogenesis through upregulating VEGF and matrix metalloproteinases (Adya et al., 2008). In rat isolated aorta, visfatin has been shown to cause endothelium-dependent vasorelaxation through NO production (Maenhaut and Van de Voorde, 2011). In small MA of rats, visfatin also causes vasorelaxation through a mechanism involving activation of phosphoinositide 3-kinase (PI3K)/Akt/eNOS pathways but not insulin receptors (Yamawaki et al., 2009).

Visfatin showed direct cardioprotective effects in an *in vivo* ischaemia/reperfusion model with a 50% reduction in the size of the induced infarct following a single intravenous bolus dose of visfatin (Lim et al., 2008) and the effects were found to be dependent on PI3K and mitogen-activated protein kinase 1/2 activation (Hausenloy, 2009; Hausenloy and Yellon, 2009). A number of studies have shown that the visfatin levels have a positive correlation with obesity (Pagano et al., 2006; Saddi-Rosa et al., 2010) or specifically with body mass index and percentage of body fat, but not with abdominal circumference or visceral

fat, estimated by computed tomography (Saddi-Rosa et al., 2010). Increased levels of visfatin are linked with endothelial inflammation and plaque destabilisation, acceleration of oxidative stress and pro-inflammatory cytokine levels (Mattu and Randeva, 2013). These findings indicate that visfatin appears to have mixed effects on the cardiovascular system.

Apelin is the endogenous ligand of the G protein-coupled receptor, APJ (Kunduzova et al., 2008; Lee et al., 2006a). This peptide is produced and secreted by adipocytes, stromal vascular fraction and cardiovascular tissues (Lee et al., 2006a). Apelin receptor (APJ) was first found as an orphan GPCR, and has close identity to the angiotensin II receptor, type AT_{1a} (O'Dowd et al., 1993). The gene encoding the APJ shares the greatest sequence identity with the angiotensin AT1 receptor (O'Dowd et al., 1993). After its cognate ligand, apelin, was discovered from bovine stomach extracts, the receptor was deorphanised (Tatemoto et al., 2001). Recently, the apelinergic system has been shown to be critically involved in multiple homeostatic processes. Apelin and APJ receptors are ubiquitous, and have been found in many tissues where they play roles in satiety, immune function, cardiovascular system and fluid balance (Habata et al., 1999; Katugampola et al., 2001; Taheri et al., 2002). Apelin and APJ have been detected in endothelial cells of blood vessels (Katugampola et al., 2001). Apelin (apelin-12, -13 and -36) exerts endothelium-dependent vasodilatation *in vivo* via eNOS phosphorylation and results in increased NO production in the femoral arteries of Wistar rats (Katugampola et al., 2001). Administration of apelin reduced body adiposity and serum levels of insulin and triglycerides in obese mice fed a high-fat diet (Heinonen et al., 2005). Apelin plays a role in regulating insulin resistance by influencing serum adiponectin levels, energy expenditure and expression of uncoupling

proteins in brown adipose tissue in obese mice fed a high-fat diet (Higuchi et al., 2007). The same report also showed that apelin increased the serum adiponectin but decreased the leptin level (Higuchi et al., 2007). In addition, apelin and its receptors are expressed in the heart; apelin is found in endocardial cells whereas APJs are located in cardiomyocytes (Kleinz and Davenport, 2004; Szokodi et al., 2002). Apelin is well known as a potent inotrope and chronotrope (Kleinz and Davenport, 2004; Szokodi et al., 2002).

Resistin is a family member of cysteine-rich proteins called resistin-like molecules (Viengchareun et al., 2002). It is a 12.5 kDa peptide which is normally found in inflammatory zone 3 and adipocyte-secreted factor (Adeghate, 2004; Steppan et al., 2001) while its receptors are still unknown (Mattu and Randeva, 2013). In humans, circulating monocytes and macrophages have been identified to produce resistin (Bastard et al., 2006). Although resistin does not directly affect the contractility of isolated coronary blood flow, mean arterial pressure or heart rate (Dick et al., 2006; Gentile et al., 2008), it has been shown to have associations with endothelial dysfunction and coronary artery disease (Reilly et al., 2005). Resistin expression is triggered by TNF-α and IL-6, both of which are increased in obesity (Rabe et al., 2008) and thus explains an increased level of resistin in obesity. Resistin impairs endothelial function through augmenting superoxide production (Kougias et al., 2005) or vice versa (Dick et al., 2006), therefore resulting in decreased expression of endothelial NO synthase and NO levels (Chen et al., 2010).

1.4.4 PVAT-derived cytokines

PVAT has recently been identified as a source for the production of tumor necrosis factor α (TNF-α) (Thalmann and Meier, 2007). TNF-α can act both as a potent vasoconstrictor (Wagner, 2000; Zhang et al., 2002) as well as vasodilator (Baudry and Vicaut, 1993; Shibata et al., 1996). Vasoregulatory activities of TNF-α are divergent in different vascular beds and various experimental protocols used may explain the diversity of observations reported (Maenhaut and Van de Voorde, 2011). It has been suggested that vasorelaxant actions of TNF-α are achieved through both endothelium-dependent (Brian and Faraci, 1998) and endothelium-independent mechanisms (Johns and Webb, 1998). Several studies have proposed that TNF-α mediates vasorelaxation through an increase in NO and prostaglandins production (Brian and Faraci, 1998; Shibata et al., 1996) and possibly with an involvement of hydrogen peroxide (Cheranov and Jaggar, 2006). Conversely, TNF-α induces vasoconstriction by increasing endothelin-1 (Wort et al., 2009) and angiotensinogen production (Brasier et al., 1996). TNF-α has been reported to impair endothelium-dependent vasorelaxation in many vascular beds and leads to a reduction in endothelial NO release or an increase in NO scavangers such as reactive oxygen species (ROS) (Zhang et al., 2002). In addition, a recent study showed that a decreased vasorelaxing effect was a result of ROS up regulation by TNF-α and IL-6 activities (Greenstein et al., 2009).

In clinical studies involving obese patients as well as different rodent models of obesity, expression of TNF-α mRNA increased in parallel with increased adipose tissue and this phenomenon is believed to contribute to inflammation (Bastard et al., 2006). Furthermore, TNF-α levels also increase as the infiltration of macrophages accelerates in adipose tissue

during obesity (Clement et al., 2004). Consequently, ROS production increases and results in endothelial dysfunction in obesity and obesity-related disorders such as hypertension, atherosclerosis and type 2 diabetes (Zhang et al., 2009). Moreover, TNF-α can reduce adiponectin expression (Hector et al., 2007) and enhances secretion of proinflammatory proteins which exacerbates the chronic inflammatory state of adipose tissue in obesity (Bullo et al., 2003).

Acute exposure to the proinflammatory cytokine IL-6*,* another PVAT-derived cytokine, has been shown to relax aorta of Sprague-Dawley rats (Ohkawa et al., 1994) and this effect is possibly regulated by an endothelium-independent pathway involving an increase in prostacyclin in vascular smooth muscle cells (Maenhaut and Van de Voorde, 2011). It has also been demonstrated to relax skeletal muscle resistance vessels of rat *in vivo* (Minghini et al., 1998). However, IL-6 is ineffective in causing relaxation of similar arterioles under isolated *in vitro* conditions, suggesting that IL-6 interacts with parenchymal or intravascular factors to elicit arteriolar relaxation (Minghini et al., 1998). By contrast, a prolonged increase in IL-6 plasma levels is associated with high BP (Lee et al., 2006b; Ridker et al., 2000). Continuous elevation of IL-6 alters endothelial function by increasing angiotensin II-stimulated production of ROS and consequently reduces endothelial NO synthase mRNA expression (Schrader et al., 2007). IL-6 was also found to suppress adiponectin gene expression in cultured adipocytes (Chudek and Wiecek, 2006). Additionally, it also can act as a predictor of future myocardial infarction (Ridker et al., 2000) and is closely associated with cardiovascular mortality (Langenberg et al., 2006) through induction of hepatic C-reactive protein (CRP) production, which has been recognized to be an independent major risk factor for cardiovascular complications (Bastard et al., 2006).

Additionally, there are some other inflammatory cytokines/chemokines that are expressed in PVAT such as interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1) and plasminogen-activator inhibitor-1 (PAI-1). Expression of these PVAT-derived cytokines has been shown to increase in pathological states such as obesity, injury and metabolic syndrome (Eiras et al., 2010; Hug and Lodish, 2005; Rajsheker et al., 2010; Szasz and Webb, 2012; Thalmann and Meier, 2007).

1.4.5 PVAT-derived gaseous molecules

PVAT also releases reactive oxygen species (ROS), a class of oxygen derived molecules, which have also been identified as vascular modulators which possess both contractile and vasorelaxation properties (Maenhaut and Van de Voorde, 2011). Vascular smooth muscle, endothelium and PVAT are recognized to produce ROS (Gao et al., 2006). Superoxide anions can cause vasoconstriction through Ca^{2+} sensitization although the mechanism is not fully characterized, and it is unclear whether they act directly or via conversion to hydrogen peroxide (Knock et al., 2009). Gao et al. (2006) suggested that the contraction in response to perivascular nerve activation by electrical stimulation is enhanced by superoxide anions produced by PVAT. In obese mice, superoxide and hydrogen peroxide production within adipose tissue is elevated which leads to endothelial dysfunction (Maenhaut and Van de Voorde, 2011). Endothelium-dependent vasorelaxation effects *in vitro* are impaired by superoxide anions through reduction of NO bioavaibility via the formation of peroxynitrite, which is in turn another ROS (Gryglewski et al., 1986). Moreover, ROS promotes endothelial dysfunction by up regulating the expression of adhesion and chemotactic molecules in endothelial cells, which stimulate monocyte

adhesion and migration to the vessel wall and thus leads to the development of vascular diseases such as atherosclerosis (Cooper et al., 2002).

Hydrogen peroxide is more likely a paracrine ROS as it is not a free radical, and hence is more stable and less reactive with other tissue radicals (Ardanaz and Pagano, 2006). Hydrogen peroxide is capable of inducing both vasorelaxation and vasoconstriction and the effects may vary, depending on species, vascular bed and concentration (Ardanaz and Pagano, 2006; Ketonen et al., 2010). It induces vasorelaxation possibly through endothelium-dependent mechanisms involving the release of vasodilating cyclooxygenase metabolites (Thengchaisri and Kuo, 2003), NO (Gil-Longo and Gonzalez-Vazquez, 2005) and endothelium independent mechanisms (Lucchesi et al., 2005). Conversely, vasoconstriction is mainly generated in a Ca^{2+}-dependent pathway, although Ca^{2+}-independent pathways have also been reported (Ardanaz and Pagano, 2006; Gil-Longo and Gonzalez-Vazquez, 2005; Moreno et al., 2010). In addition, hydroxyl radicals, cyclooxygenase metabolites, protein kinase C, phospholipase $A_{2,}$ phospholipase C and tyrosine kinase also contribute to hydrogen peroxide-induced contractions (Moreno et al., 2010).

Recently hydrogen sulphide (H_2S) has been regarded as third gaseous signaling molecule after NO and carbon monoxide (CO) (Dunn et al., 2016; Moore et al., 2003; Wang, 2002). H_2S has emerged as a critical cardiovascular signalling molecule with a profound effect on the heart and circulation (Polhemus and Lefer, 2014). Similar to NO and CO, H_2S is synthesized in many different cell types and can easily diffuse without involvement of any transporters (Yu et al., 2014). Adipose tissue has been reported as a critical organ for sulfur-containing amino acid metabolism

(Feller and Feist, 1963) and endogenous H_2S also results from a metabolic production of sulfur-containing amino acids (Kamoun, 2004). Fang et al. (2009) was the first group to show that H_2S is present in the PVAT of rat aorta. The authors demonstrated an endogenous cystathionine γ-lyase (Bailey-Downs et al.)-H_2S pathway in PVAT and observed the vascular tone regulation of H_2S from PVAT *in vitro* and *in vivo*, and its pathophysiological significance in pressure overloaded hypertension to explore the role of H_2S as an ADRF in the pathogenesis of hypertension (Fang et al., 2009). CSE, an endogenous H_2S-producing enzyme, generates H_2S production mainly in vascular smooth muscle and heart (Zhao et al., 2001). Fang et al. (2009) provided evidence for CSE protein expression in both the aorta and PVAT of aorta using immunohistochemical and western blotting approaches. Although the mechanism involved in the endogenous H_2S-induced vasorelaxing effect in PVAT is still unclear, Fang et al. (2009) postulated that PVAT-derived H_2S relaxed the aorta by the involvement of K_{ATP} channels but that this was endothelium and Ca^{2+}-channel independent.

1.4.6 PVAT-derived renin-angiotensin-aldosterone system metabolites

The renin-angiotensin-aldosterone system (Raasch et al.) plays an integral role in regulation of BP and renal function. The substrate of the system, angiotensinogen, is cleaved by renin, producing angiotensin I, which is then converted to angiotensin II by either angiotensin-converting enzyme (ACE) and/or chymase (Paul et al., 2006). Angiotensin II is the major effector peptide of the RAAS and acts by binding to two different receptors, AT_1 and AT_2. The AT_1 receptors are widely distributed and mediate most of the biological responses that contribute to the known pressor, trophic, and

pro-inflammatory effects of angiotensin II, whereas the AT_2 receptors antagonize several of the AT_1 receptor-mediated responses (Paul et al., 2006). The presence of angiotensinogen mRNA in rat perirenal and other adipocytes was discovered in the late 1980s (Campbell and Habener, 1987). Both BAT and WAT are rich sources of angiotensinogen (Zhang et al., 2003). Later studies showed that adipocytes contain all necessary peptide and enzymes (renin, angiotensinogen, angiotensin and ACE) to produce angiotensin II and thus indicate the existence of a local renin-angiotensin system in adipose tissue (Cassis et al., 1988; Engeli et al., 1999; Engeli et al., 2003; Giacchetti et al., 2002). In contrast, Galvez-Prieto et al. (2008) showed the absence of renin mRNA expression in both PVAT of rat periaortic and rat mesenteric arteries. However, the authors also suggested that the functional role of RAS in PVAT does not depend on local renin or prorenin uptake from the circulation (Galvez-Prieto et al., 2008), which thus makes RAAS a functional system in PVAT. The authors also discovered the expression of the (Zhang et al.)renin receptor, ACE_2 and of three AT_{1a} receptor isoforms in perivascular adipose tissue (Galvez-Prieto et al., 2008).

There are similarities and differences between the expression of RAAS components in PVAT of rat periaortic and rat mesenteric arteries. ACE_1 and ACE_2 expression was similar between PVAT of rat periaortic arteries and rat mesenteric arteries. Renin receptor expression was five times higher, whereas expression of chymase, AT_{1a}, and AT_2 receptors were significantly lower in PVAT of rat periaortic arteries compared with PVAT of mesenteric arteries respectively (Galvez-Prieto et al., 2008). Notably, the expression of angiotensin II was higher in PVAT of mesenteric arteries although there was no difference in angiotensin I levels between periaortic and mesenteric tissues (Galvez-Prieto et al., 2008). Furthermore, the direct involvement of

PVAT-derived angiotensin II in promoting sympathetic vasoconstriction responses has been demonstrated by Lu et al. (2010a). The study further corroborated the previous findings on the existence of functional angiotensin II in PVAT and showed that neurogenic vasoconstriction was markedly reduced in the presence of candesartan, an angiotensin II inhibitor (Lu et al., 2010a).

The RAAS is also relevant in pathophysiology of the cardiovascular system. Angiotensin II has been reported to stimulate the metabolism of NO into oxygen free radicals which leads to vascular tissue damage (Cai et al., 2002). As a result, levels of angiotensin II and NO become imbalanced leading to endothelial dysfunction and result in a loss of vasodilator capacity (Maenhaut and Van de Voorde, 2011). Consequently, this causes an increased expression of adhesion molecules and proinflammatory cytokines in endothelial cells (Verdecchia et al., 2008) and thus promotes monocyte and leukocyte adhesion and migration to the vessel wall (Verdecchia et al., 2008). Moreover, angiotensin II also enhances the progression and destabilization of atherosclerotic plaques as it increases the release of plasminogen activator inhibitor-1 (PAI-1). The PAI-1 stimulates thrombosis formation and expression of growth factors, which leads to smooth muscle cell proliferation and migration (Verdecchia et al., 2008). There is a consensus that angiotensinogen mRNA expression in adipose tissue is elevated in obesity (Thatcher et al., 2009). Several studies have emphasized a contribution of adipose tissue-derived angiotensinogen and/or angiotensin II to obesity-related hypertension (Thatcher et al., 2009). Increased levels of angiotensin II may exacerbate obesity-related hypertension as it stimulates the secretion of proinflammatory cytokines such as IL-6 and TNF-α (Das, 2005), decreases

adiponectin (Ran et al., 2006), and increases leptin concentration in adipocytes (Skurk et al., 2005).

1.4.7 PVAT-derived relaxing factor (PVATRF)

PVAT-derived relaxing factor (PVATRF) appears as an interesting vasorelaxing metabolite released from PVAT, although little is known about it. The idea was first described by Soltis and Cassis (1991) and then was supported by Lohn et al. (2002). Their consensus was that adipose tissues possess a modulatory effect which was ascribed to a locally produced PVATRF as an important arterial tone regulator by actively antagonizing contraction (Lohn et al., 2002). PVATRF markedly attenuates the contractile response to angiotensin II and other vasoactive compounds in aortic ring preparations (Lohn et al., 2002). Moreover, the vasorelaxing action of PVATRF has been shown to have a positive correlation with the amount of PVAT (Verlohren et al., 2004).

PVATRF actions have been observed to be independent of cyclooxygenase or P450 pathways, activation of adenosine receptors, or the presence of functional leptin receptors (Lohn et al., 2002). In addition, Dubrovska et al. (2004) affirmed that the anti-contractile effect did not involve neuronal presynaptic N-type Ca^{2+} and Na^{2+} channels or vanilloid, cannabinoid and CGRP receptors. Furthermore, removal of the endothelium did not affect vasorelaxation produced by PVATRF (Lohn et al., 2002). Overall, the mechanism of PVATRF, whether it involves NO formation and the role of endothelium, is still controversial and needs to be scrutinized (Gao et al., 2007; Gao et al., 2005b; Lohn et al., 2002; Verlohren et al., 2004). Several reports indicate that PVATRF-induced vasorelaxation is likely

mediated by the opening of different K^+ channels in vascular smooth muscle and that the types of channels can vary according to tissue and species studied (Gao et al., 2007; Gao et al., 2005b; Lohn et al., 2002; Verlohren et al., 2004). Lohn et al. (2002) showed that K^+ channels are critically involved in the disappearance of anti-contractile effect of PVAT in high (60 mM) external K^+ solutions. In the experiment, raising external K^+ was expected to diminish the effects of any K^+ channel opener by significantly reducing the difference between the K^+ equilibrium potential and the membrane potential. In addition, Gollasch (2012) suggested that KCNQ (K_v7) channels representing the K_v channel family were involved in the effect of PVATRFs. KCNQ channels were found to possess a unique role in vasodilatory control from outside the vessel (Gollasch, 2012). They are neither the target of endothelium-derived relaxing factors (EDRF) nor endothelium-derived hyperpolarizing factors (EDHF) (Gollasch, 2012). His recent study also indicates that K_{ATP} channels have no significant role in vasorelaxation induced by PVATRF (Gollasch, 2012). The variety of the findings suggest the possibility of different distribution of K^+ channels in different vessels and/or species or the existence of different PVATRFs (Maenhaut and Van de Voorde, 2011).

There is increasing evidence to support the existence of different PVATRFs (Maenhaut and Van de Voorde, 2011). PVATRF is postulated to have a Ca^{2+} dependency (Gollasch, 2012) and its release can be inhibited by blockers of tyrosine kinase and protein kinase A (Dubrovska et al., 2004). A number of endogenous metabolites have been proposed to be possible PVATRF candidates. Peptide angiotensin (1-7) was hypothesized to be a possible PVATRF as the PVAT vasorelaxing effect was diminished when peptide angiotensin (1-7) was inhibited by Ang-(1-7) receptor (Mas) antagonist (A779), nitric oxide synthase inhibitor, and NO scavenger (Lee et al.,

2009). In addition, peptide angiotensin (1-7) enhanced NO release from endothelial cells, which resulted in the opening of calcium-activated K^+ (BK_{Ca}) channels (Lee et al., 2009). This theory however, was rejected due to the fact that certain PVATRFs-related potassium channels (K_{ATP} or K_v) were not measured in the observation (Lohn et al., 2002; Schleifenbaum et al., 2010).

Hydrogen peroxide (H_2O_2), a product of NAD(P)H oxidase has been described as being an PVATRF acting via smooth muscle soluble guanylyl cyclase (sGC) without activation of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels (Gao et al., 2007). This contrasts to previous reports stating that BK_{Ca} channels are strongly activated by the sGC/cGMP/PKG pathway (Robertson et al., 1993). Exogenous H_2O_2 has been exhibited to open 4-aminopyridine (4-AP)-sensitive K_v channels in certain vascular beds (Rogers et al., 2007) while endogenously produced H_2O_2 induces EDHF-like responses independent of K_v (KCNQ) channels (Hercule et al., 2009).

Hydrogen sulphide (H_2S) has also been proposed as another novel candidate for PVATRF, or at least to be involved in the PVATRF effect (Schleifenbaum et al., 2010), since neither H_2S nor PVATRF are adsorbed by essentially fatty acid-free serum albumin and both are inactivated by heating (65°C for 10 min), two proposed characteristics for PVATRFs (Lohn et al., 2002). In addition, inhibition of CSE resulted in the disappearance of PVAT anti-contractile effects in mesenteric arteries and aortas of rats and mice (Schleifenbaum et al., 2010).

Previous studies have shown that methyl palmitate (MP) is an endogenous potent vasodilator and has been isolated from rat superior cervical ganglion and the retina (Lee et al., 2010; Lin et al., 2008). MP has recently been

isolated from PVAT of rat aorta and holds a potential as a PVATRF candidate (Lee et al., 2011). MP induces potent vasorelaxation in vascular smooth muscle by opening voltage-activated potassium (K_v) channels on VSMCs (Lee et al., 2011). Lee et al. (2011) proposed MP as a PVATRF candidate based on close to identical concentration-response curves in the presence of 4-aminopyridine (4AP), a specific K_v channel inhibitor. 4AP blocked vasodilation induced by PVATRF from PVAT of rat retina and exogenous MP (Lee et al., 2011). The authors also provide data that support their proposal. First, consistent with Lohn et al. (2002) who indicated that PVATRF is Ca^{2+}-dependent, vasodilation induced by PVATRF and MP decreased markedly in PVAT-intact preparations perfused with Ca^{2+} free Krebs'-Henseleit solution (Lee et al., 2011). Second, the vasorelaxing effect of PVATRF and MP in the aortic PVAT was independent of NOS or the presence of endothelium. This result is in line with other studies (Lohn et al., 2002; Malinowski et al., 2008; Verlohren et al., 2004) in aorta of the rat and in human internal thoracic artery, although this finding contradicts another study in rat aorta (Gao et al., 2007). Third, MP mediates vasorelaxation through K_v channels, which is consistent with other studies in rat aorta and mesenteric arteries (Fesus et al., 2007; Verlohren et al., 2004). Fourth, the aortic relaxations induced by Krebs'-Henseleit solutions containing exogenous MP or PVATRF were decreased after hexane extractions. These results suggest that the PVATRF was lipophilic rather than hydrophilic or a peptide (Lee et al., 2011). In addition, the vasorelaxing effect of MP was not affected with incubation at 70°C for 10 min, suggesting that vasorelaxing activities of PVATRF and MP were heat stable. Finally, the vasorelaxing property of MP was only apparent in wild normotensive rats, but not in hypertensive rats (20-week old spontaneous hypertensive rats) (Lee et al., 2011), and this result is consistent with previous studies (Galvez et al., 2006; Takemori et al., 2007).

1.5 Aims

PVAT has become the subject of intense investigation over the past few years since it was discovered to play a key role in the physiology and pathology of the cardiovascular system. There are numerous vasoactive compounds that have been isolated from PVAT as well as growing evidence for the involvement of PVAT in certain pathophysiological conditions such as obesity, hypertension, insulin resistance and atherosclerosis. Despite increased attention on PVAT, our knowledge on the interaction of PVAT and nervous systems controlling vascular tone is still at its infancy. Therefore, the aims of the present study were:

1) To investigate the influence of PVAT (its absence and presence) on sympathetic neurotransmission using electrical field simulation in different blood vessels, to determine the expression and location of sympathetic nerves in PVAT of different order vessels of mesenteric arterial beds (MABs) and to examine the interaction between PVAT-derived sympathetic nerves with endogenous or exogenous PVAT-derived compounds.

2) To characterize the expression and vasomotor role of sensory nerves in PVAT, and to examine the effect of PVAT on sensory neurotransmission in resistance and conduit arteries.

3) To examine the effect of H_2S on sensory neurotransmission in the absence and presence of PVAT. The involvement of H_2S-producing enzymes and endogenous H_2S in PVAT-induced vasorelaxation in MABs was also investigated.

4) Previous studies have shown the anti-contractile effect of PVAT is diminished in pathophysiological states including hypoxia (Greenstein et al., 2009; Szasz et al., 2013). As an attempt to investigate the interaction between PVAT-derived compounds and PVAT-derived sensory and sympathetic nerves, seven compounds namely adiponectin, leptin, IL-6 and MCP-1, tumor necrosis factor alpha (TNF-α), interleukin beta (IL-β) and total plasminogen activator inhibitor-1 (PAI-1), were investigated for possible changes in their expression during sympathetic and sensory neurogenic activation under normal and low oxygen levels.

Chapter 2:

Materials and methods

2.1 Rat tissue preparation

Male Wistar rats weighing 180-220 g were killed by stunning and exsanguination. These animals were obtained from Charles River Laboratories International, Inc., UK and were housed at the Biomedical Services Unit, University of Nottingham. The animal study is in accordance with The Animal Welfare Act 2006. The rat mesenteric arterial beds (MABs) and rat abdominal aortas (Raasch et al.) were immediately isolated from rats' abdomens, dissected and placed on a petri dish full of Krebs'-Henseleit buffer solution. MABs were used as these vascular beds were densely innervated by both sympathetic and sensory nerves and also encompass both large conduit and small resistant arteries. AAs were chosen for investigation in conduit arteries. The Krebs'-Henseleit buffer was composed of (mM): NaCl, 118; KCl 4.8; $MgSO_4.7H_2O$ 1.1; KH_2PO_4 1.2; $NaHCO_3$ 25; glucose 12; $CaCl.2H_2O$ 1.25; gassed with 95 % O_2 and 5 % CO_2 and maintained at 37º C. MABs and AAs were prepared for two conditions, one with intact PVAT and the other with PVAT removed (see Figure 2.1 for MABs).

In some experiments to determine the role of specific vascular segments, superior mesenteric artery (SMA) and second order mesenteric artery (2OMA) of the MABs were cut into artery rings (approximately 2 mm long). AAs also were cut into small rings (approximately 2 mm long). PVAT removal from MABs was carried out as described by Gao et al. (2005a). PVAT was carefully removed from MABs with fine scissors under a dissecting microscope paying attention in order not to damage the adventitial layer. The SMA was tied off close to its junction with the aorta (and it was also tied off at the distal end) leaving a sufficient length for cannulation with a hypodermic needle (No. 21), which was later attached

to a stimulator (Grass instruments, Quincy, Mass, U.S.A) that acted as the positive electrode. The blood was flushed from the vascular bed with 0.5 ml of Krebs'-Henseleit solution. Finally, the intestine was separated from the mesentery by cutting close to the intestinal border of the mesentery, as described by McGregor (1965).

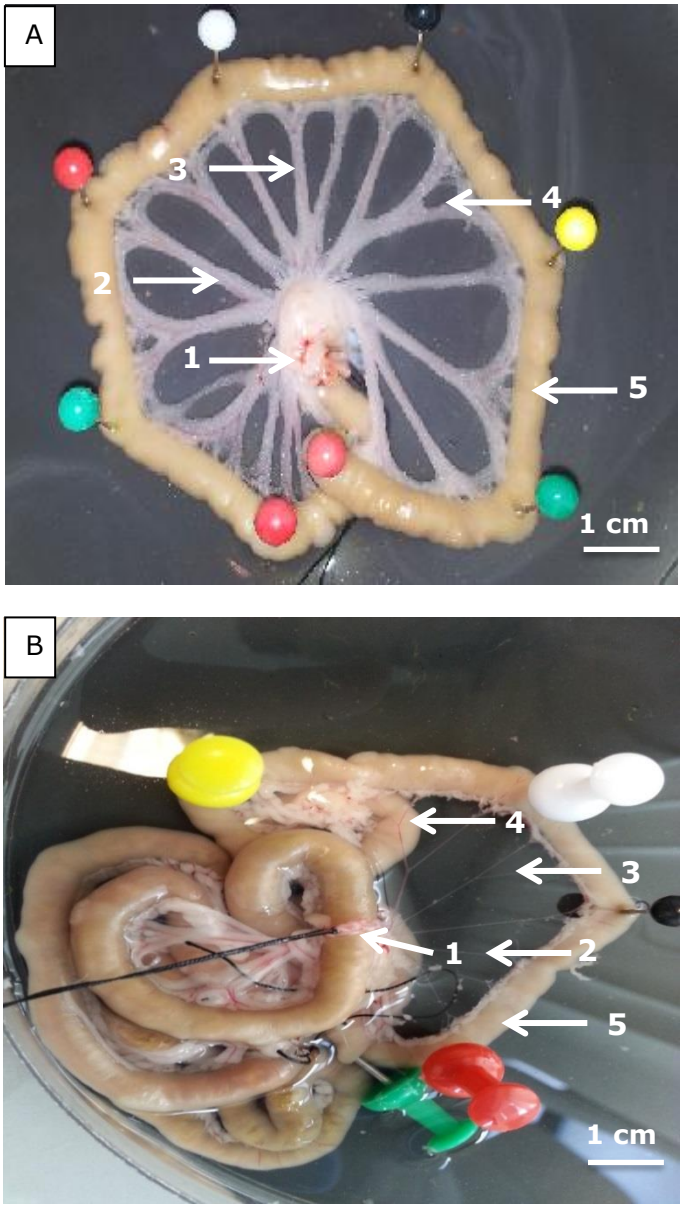

Figure 2.1: MABs with fat (A) and after removal of fat (B). 1- superior mesenteric artery, 2-first order branch artery, 3- second order branch artery, 4-third order branch artery, 5-small intestine. The superior mesenteric artery was cannulated as described in Section 2.1. The mesenteric arcade was then cut from the intestinal wall.

The MAB preparation was mounted on a stainless steel grid (7x5 cm) which was attached to a stimulator and formed the negative electrode. The preparation was contained within a humid chamber and perfused at a constant flow rate of 5 ml min^{-1} using a peristaltic pump (model 7554-30; Cole-Parmer Instrument Co., Chicago, IL, U.S.A) as shown in Figure 2.2. A heating coil was used warm the Krebs'-Henseleit solution, and a circulating heater (model 7553-87; Cole-Parmer Instrument Co., Chicago, IL, U.S.A) was used to maintain the temperature of the heating coil and the humid chamber at approximately 37°C. Preparations were allowed to equilibrate for 30 minutes (min) before experimentation. Changes in perfusion pressure (mmHg) were measured with a pressure transducer (model P23XL; Viggo-Spectramed, Oxnard, CA) and recorded on chart software (ADInstruments, Pty Ltd., Castle Hill, Australia).

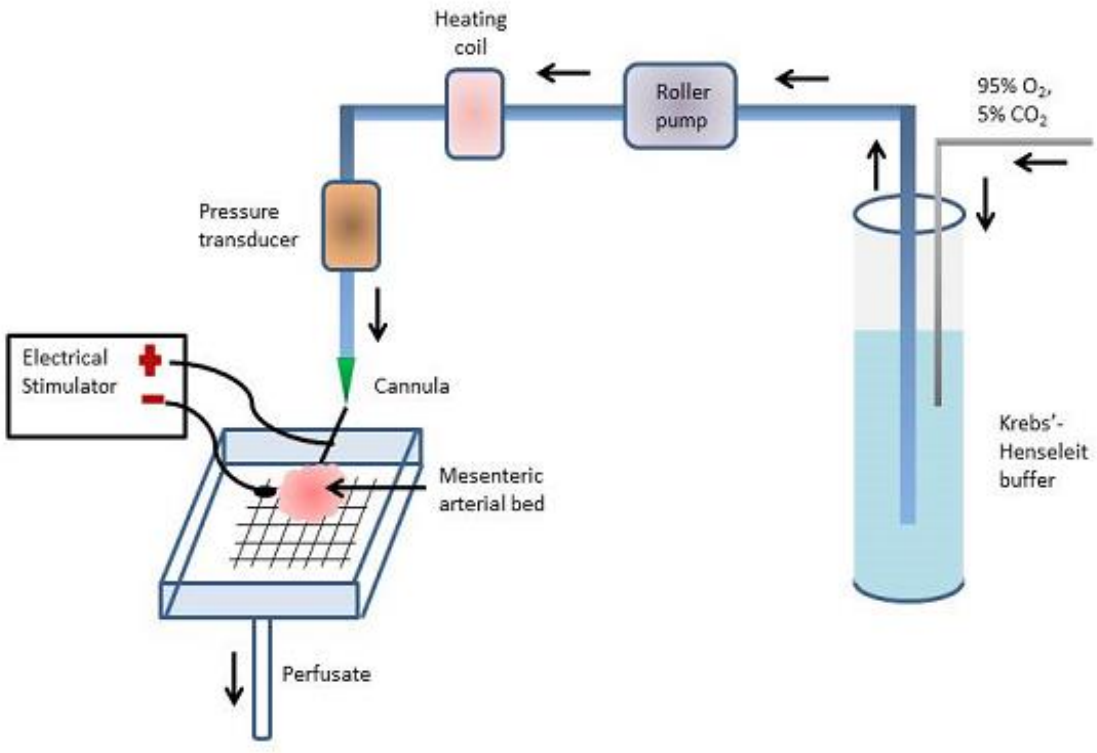

Figure 2.2: Diagrammatic representation of perfusion setup used in perfusing rat isolated mesenteric arterial bed (see text for details).

2.2 Neuronal responses in perfused mesenteric arterial beds

2.2.1 Electrically-evoked vasocontractile responses

A contractile response was elicited by electrical field stimulation (EFS) (Ralevic et al., 1996) in the presence or absence of agonists and antagonists. EFS was conducted at increasing frequencies (4-32 Hz) to obtain frequency-response curves. MABs, with the presence and absence of PVAT, were stimulated at 90 V for 30 s with constant pulse at 1 ms using an electric stimulator (Grass SD9B, Quincy, Mass, U.S.A.). The intervals between stimuli were between 5 to 15 min and the perfusion pressure was allowed to return to baseline before the next stimulation. In order to examine the reproducibility of the responses, a second similar cycle of electrical stimulation (4-32 Hz, 90 V, 1 ms, 30 s) was conducted with a 30 min interval between the two frequency response curves. Vasoconstrictor responses of the preparations were measured as increases in perfusion pressure (mmHg).

2.2.2 Electrically-evoked vasodilatation responses

To investigate the effect of PVAT on sensory neurogenic vasorelaxation, MABs in the presence and absence of PVAT were perfused with Krebs'-Henseleit solution (Section 2.1) containing guanethidine (5 μM), a sympathetic nerve blocker, for 30 min and then methoxamine (0.5-5 μM), an α1-adrenoceptor agonist, was added to pre-constrict the preparations (to 30-70 mmHg above baseline). In both PVAT-intact and PVAT-denuded preparations, EFS at 0.5-12 Hz, 60 V, 0.1 ms and 30 s was applied in the

presence or absence of pharmacological agents. In reproducibility tests, two consecutive frequency response curves were carried out with a 30 min interval. The effect of PVAT on relaxation of mesenteric arteries was also determined using various agonists and antagonists, added either into the perfusate (cumulative concentrations) or as bolus injections (50 µl) (doses). Vasodilator responses of the preparations were measured as decreases in perfusion pressure (mmHg).

2.3 Isometric tension recording (wire myography) of superior and second order rat mesenteric arterial segments

Segments of mesenteric arteries were obtained as described in Section 2.1 Fine dissection was conducted to isolate the segments (superior and second order) using a dissecting microscope. Segments were prepared in two conditions, one with intact PVAT and the other with PVAT removed. Segments were cut into lengths of approximately 2 mm and were mounted on fine tungsten wires, and placed between the plastic jaws of a dual and multi-channel wire myograph (Myograph-Interface Model 410 and Model 620 respectively, Danish Myo Technology, Arhus, Denmark) as shown in Figure 2.3. The isometric tension recordings were carried out as described by Mulvany and Halpern (1977). Segments were equilibrated in Krebs'-Henseleit buffer in bath chambers (5 ml) at 37°C, continuously bubbled with 95% O_2 and 5% CO_2. A tension of 1 g and 0.5 g were applied to SMA and 2OMA, respectively, by stretching these vessels for tension development. Guanethidine (5 µM) was applied for a minimum time of 30 min to block sympathetic activity. Vessels were left to relax to reach a new

level of resting tension over about 30 min. Methoxamine (1-10 μM) was then added to submaximally pre-contract the vessels in order to facilitate investigation of vasorelaxant responses. The vascular relaxation was the decrease in tension against the methoxamine-induced raised tone, expressed as a percentage (the equation is shown below). Tension was measured and recorded on a MacLab recording system (ADInstruments Ltd., Hastings, UK).

$$\text{Relaxation (\%)} = \frac{\text{decrease in tension (g)}}{\text{methoxamine-induced raised tone (g)}} \times 100$$

EFS at 0.5-12 Hz, 60 V, 0.1 ms, 30 s was carried out to investigate nerve-mediated vasorelaxant responses in the presence of guanethidine (5 μM) to block sympathetic neurotransmission. The mesenteric artery segments were stimulated electrically (DS2; Digitimer Ltd, Welwyn Garden City, UK) through two platinum electrodes mounted in the plastic jaws on either side of the blood vessel (Figure 2.3). To investigate the involvement of vanilloid receptor subtype 1 (TRPV1) channels, capsaicin (0.01-10 μM), an agonist at TRPV1 channels, was added as cumulative concentrations after the contraction to methoxamine had reached a stable plateau. The state of the endothelium was assessed by measuring the vasodilator response to acetylcholine (ACh) (1 μM). More than 80 % vasorelaxation induced by ACh was taken as indicating an intact endothelium. At the end of the experiment, tetrodotoxin (1 μM), a neurotoxin which acts by blocking voltage-gated sodium channels in nerve cell membranes, was added to evaluate the neurogenic component of the vasorelaxation responses.

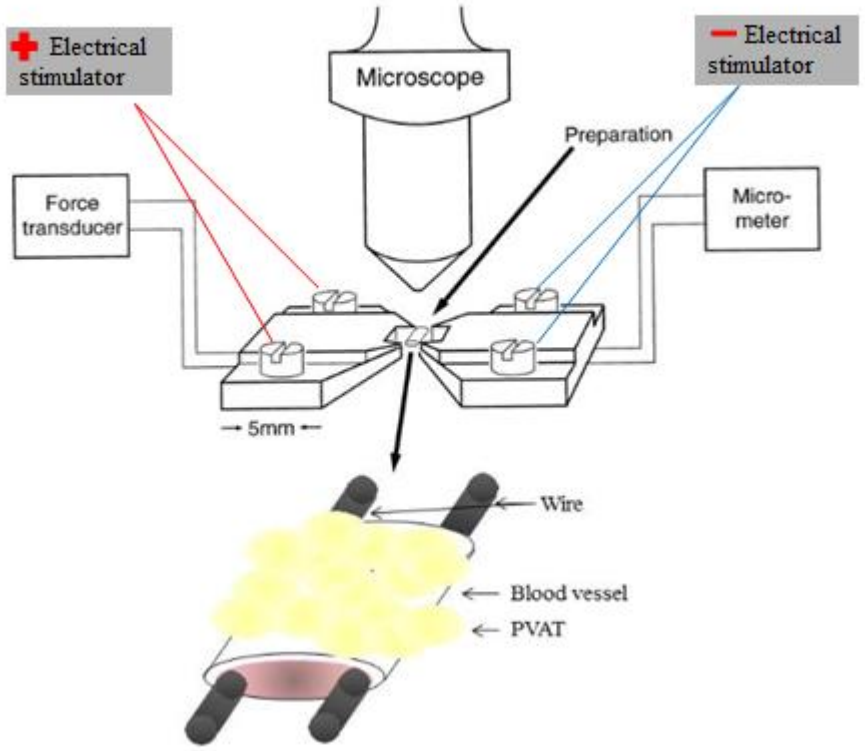

Figure 2.3: Wire myograph setup and dimensions. Figure is a modification of that from Goode et al. (1997).

2.4 Enzyme immunoassay (EIA) for measuring CGRP release

2.4.1 Principle of CGRP assay

Enzyme immunoassay (EIA) utilizes a double-antibody sandwich principle, as described in manufacturer's booklet (SPI-BIO, Bertin Pharma, cited from https://www.caymanchem.com/pdfs/589001.pdf). Each well of the microtiter plate is coated with a monoclonal antibody specific to CGRP. An acetylcholinesterase (AChE)-Fab' conjugate is added to the wells, binds selectively to a different epitope on the CGRP molecule, and then allows

the two antibodies to form a sandwich by binding to different parts of the rat CGRP molecule. The sandwich is immobilised on the plate so the excess reagents can be washed away. The enzymatic activity of the AChE is measured using Ellman's reagent for determining the concentration of the rat CGRP (the AChE tracer acts on the Ellman's reagent to form a yellow compound). The intensity of the colour is proportional to the amount of the rat CGRP (https://www.caymanchem.com/pdfs/589001.pdf) (See Figure 2.4).

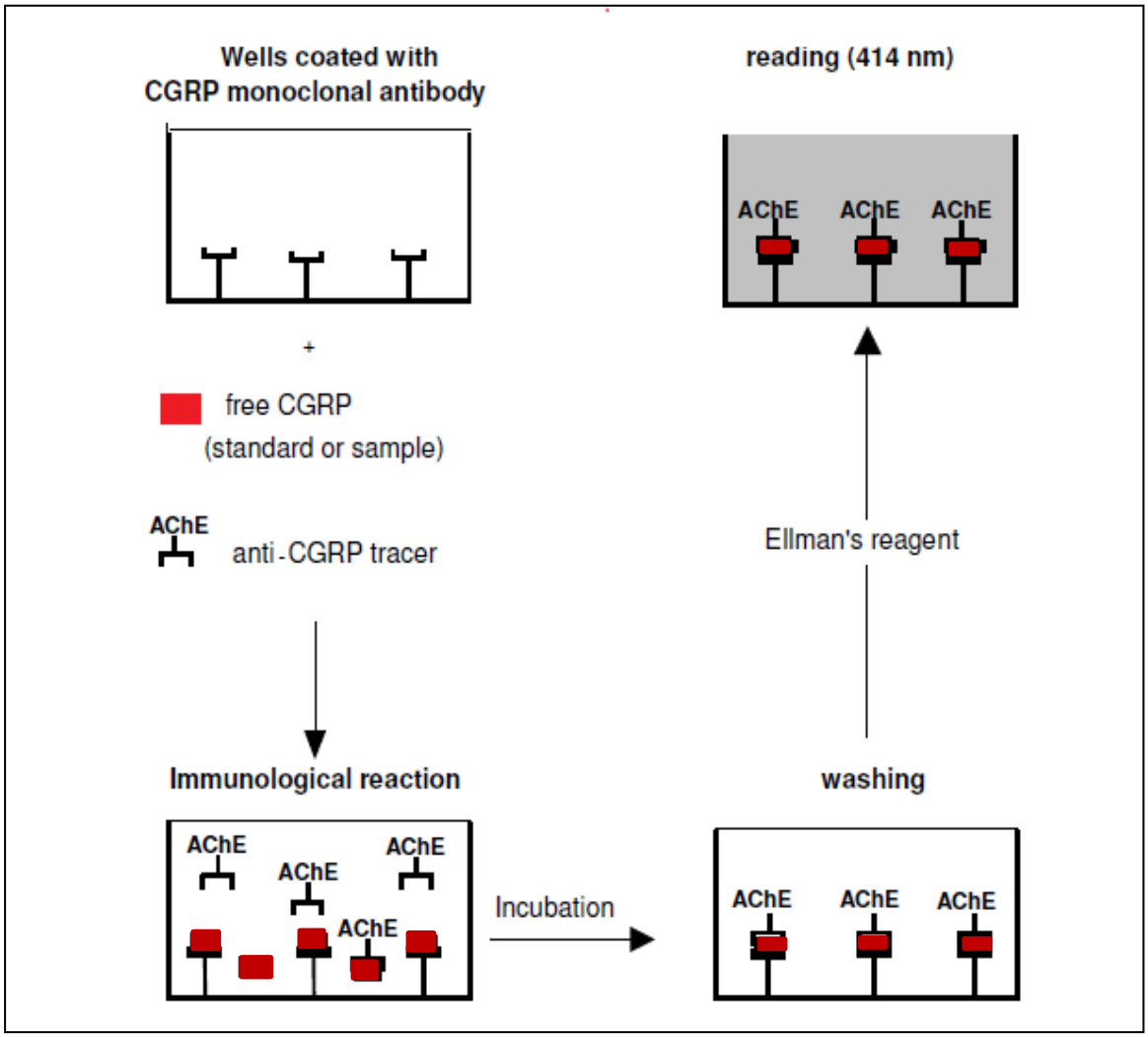

Figure 2.4: Schematic diagram of the CGRP enzyme immunoassay (EIA), taken from https://www.caymanchem.com/pdfs/589001.pdf.

2.4.2 EIA assay procedure

Rats were killed as described in Section 2.1. Segments of mesenteric arteries (superior mesenteric, first order, second order and third order) in the presence and absence of PVAT were collected, and the weights and lengths were measured. All segments were incubated in Krebs'-Henseleit solution with capsaicin (10 μM) in a water bath at 37^0C for 15 min to evoke CGRP release. The segments were then removed and the solutions were assayed for CGRP content using rat CGRP enzyme immunometric assay (EIA) kit purchased from SPI-BIO (Bertin Pharma, Montigny-le-Brentonneux, France). CGRP in each sample was extracted using Oasis® HLB Extraction cartridges (Waters, Massachusetts, USA) in acidic conditions with the presence of 4 % (v/v) acetic acid. One ml of each sample was passed slowly (about 2 ml/min) through the cartridge and then washed with 10 ml 4% (v/v) acetic acid. CGRP content was eluted with 3 ml of methanol:water with 4 % acetic acid (90:10, v/v), at 1 ml per time. It was important to have a pause between each ml of solution as the reproducibility of the recovery depends on this step. The samples were dried by vacuum centrifugation overnight and reconstituted with 250 μl of EIA buffer.

Reagents and samples were then distributed onto a 96 well plate as shown in Figure 2.5; the first column (column 1) of wells was kept empty for Ellman's reagent blanks, the second column was filled with 100 μl of EIA buffer as nonspecific binding wells. The next two columns were filled with 100 μl of 8 standards (in duplicate). 100 μl of quality control, Krebs'-Henseleit and samples were dispensed in duplicate to appropriate wells. Finally, anti-AChE tracer (100 μl) was added to all wells except column 1. The plate was then incubated at 4^0C for 16-20 hours. Following the

incubation period, the plate was washed 6 times with wash buffer, 300 µl of Ellman's Reagent was dispensed into each of the wells and then the last 8/races of buffer were removed by inverting the plate. The plate was incubated in the dark (plate covered with an aluminium sheet) at room temperature on an orbital shaker. Finally, the plate was read between 405 and 414 nm (yellow colour), 30 to 60 min after adding the Ellman's reagent. Standard curve was used to determine the unknown concentrations of CGRP from samples and the data was analysed using Locally Weighted Scatter-plot Smoother (LOWESS), as described in Section 2.10.

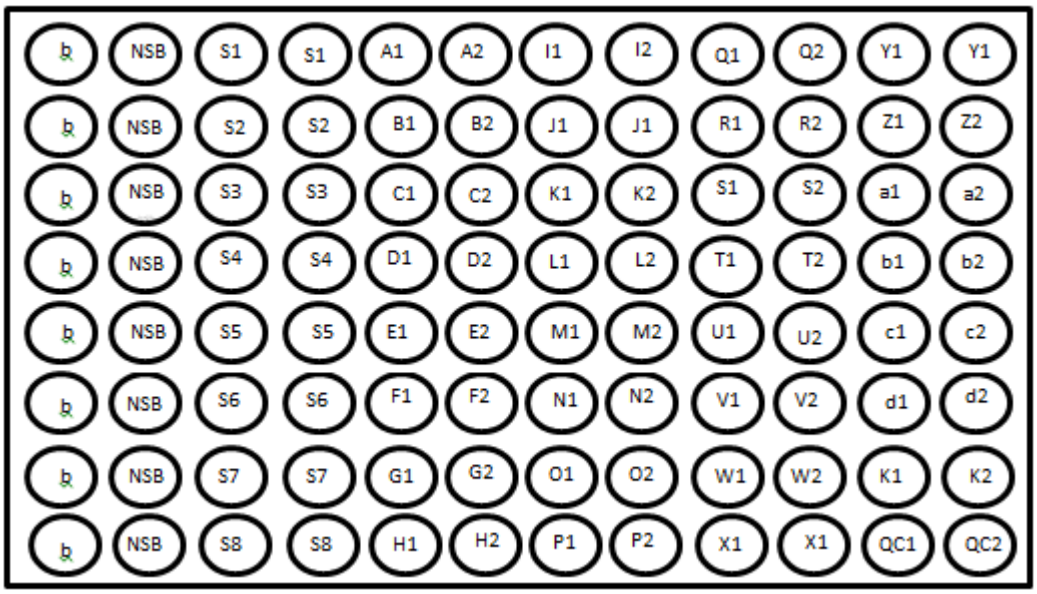

Figure 2.5: A design of an EIA 96-well plate set up. The first column was left empty (blank), the second column was non-specific binding (100 µl of EIA buffer). The next two columns were the duplicate of 8 standards. The remaining wells (A1-d2) were samples collected from mesenteric arterial segments, assayed in duplicate. K1-K2 wells were Krebs'-Henseleit solution and QC1-QC2 wells were quality control in duplicate.

2.5 Immunofluorescence staining

2.5.1 Tissue preparation for immunofluorescence staining

Segments of mesenteric arteries (superior mesenteric, first order, second order and third order) were prepared as PVAT-intact and PVAT-denuded preparations and fixed in 4 % (w/v) paraformaldehyde for 3 hours at room temperature. Specimens were then washed in Dulbecco's phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, U.SA.). Slices of vessels were created by freezing the tissues in optimal cutting temperature (Eberhardt et al.) mounting solution, then cut into either longitudinal (with the aim to examine nerves network at the adventitia and within PVAT) or cross-sections (with the aim to see the distribution of nerves in different layers of vessels, if any and within PVAT) to size (40 µm thick) using a cryostat (Leica model CM1900, Heidelberg, Germany). Longitudinal sections were obtained by positioning vessels horizontally while cross/tranverse-sections were obtained by positioning vessels vertically in the OCT. The slices were then transferred onto gelatinised slides and stored at -80^{0}C. In some preparations, fat from half of the vessel was removed and similar immunofluorescence staining procedures were conducted with omission of the cutting step. This procedure was conducted with the aim to obtain more nerve profile.

2.5.2 Immunofluorescence staining procedures

All segments of mesenteric arteries were stained using the standard indirect immunofluorescence staining technique (Odell and Cook, 2013) and double immunofluorescence staining technique (Sundberg et al.,

2002). The tissues were permeabilised using PBS + 1 % (w/v) bovine serum albumin (BSA) + 0.15 % (v/v) Triton X-100 at room temperature for 30 min. Non-specific binding was blocked with goat serum 10 % (v/v) in PBS at room temperature for 30 min.

The slides were incubated overnight at 4^0C with antibody against calcitonin gene-related peptide (anti-CGRP, rabbit polyclonal, Merck Millipore, UK), the principal neurotransmitter of mesenteric sensory nerves, diluted in 1:500 and/or a general neuronal marker, protein gene product 9.5 (anti PGP 9.5, mouse monoclonal, Abcam, UK) diluted 1:100 in the blocking solution, while control slides were incubated overnight with the blocking solution, with omission of primary antibodies. In other preparations, antibody against tyrosine hydroxylase (TH), the key enzyme for noradrenaline production in sympathetic nerve endings (polyclonal rabbit, Millipore, Darmstadt, Germany), diluted to 1:500, was applied.

Subsequently, the samples were washed with PBS and 0.1% (w/v) bovine serum albumin (BSA). The secondary antibodies, goat anti-rabbit IgG tetramethylrhodamine (TRITC; Thermo Fisher Scientific, Waltham, MA U.S.A) and goat anti-mouse IgG fluorescein isothiocyanate (FITC; Thermo Fisher Scientific, Waltham, MA U.S.A) 1:500 and 1:200 respectively, diluted in PBS + 0.1% (w/v) BSA, were incubated with the samples at 37^0C for 1 hour followed by further washes with PBS + 0.1% (w/v) bovine serum albumin (BSA). Sudan Black B (Sigma-Aldrich, Dorset, UK) 1% (w/v) was then added to reduce autofluorescence, incubated for 10 min and followed by several washes. Finally DAPI, a fluorescent nuclear stain (Sigma-Aldrich, Dorset, UK) 1:1000, was added, incubated for 5 min and followed by several washes. In the preliminary study, Oil Red O (Sigma-Aldrich, Dorset, UK), a fat soluble dye was used for fat cell identification

and confirmation that nerves staining (anti-TH and anti-CGRP) were specifically distributed within PVAT. The slides were covered using Vector shield mounting solution and glass cover slips. Samples were visualised using confocal microscopy (Leica DMIRE2, Heidelberg, Germany) with objective magnification of x63 (oil).

2.6 Rat adipocyte immunoassay/Multiplex

2.6.1 Principle of rat adipocyte immunoassay/multiplex assay

The rat adipocyte immunoassay or multiplex assay that was conducted in the present study is patented as MILLIPLEX ® MAP kit (EMD Millipore, Missouri, U.S.A) and based on Luminex xMAP® technology. The Luminex applies techniques to internally colour-code microspheres with 2 fluorescent dyes. These dyes distinctly colour specific antibody-coated magnetic beads. The beads act by capturing analytes from samples, and then a biotinylated detection antibody is introduced. The reaction on the surface of each microsphere is completed by adding Streptavidin-PE conjugate (reporter molecule). Each individual microsphere is recognized and the results of its bioassay are quantified based on fluorescent reporter signals.

2.6.2 Sample preparation

Rats were killed as described in Section 2.1. MABs were dissected, prepared as either PVAT-intact or PVAT-denuded preparations and perfused with Krebs'-Henseleit buffer solution at 3 ml per min, gassed with 95 %

oxygen (O_2) and 5 % carbon dioxide (CO_2) as described in Section 2.1. After a stable baseline or raised tone had been achieved, a fraction of normoxic perfusate, 1 ml per tube, was collected for one min as a control. The process was repeated during EFS stimulation for 1 min. MABs were further perfused for another 1.5 hours with Krebs'-Henseleit solution gassed with 95 % nitrogen (N_2) and 5 % CO_2 to induce hypoxic condition of the MABs. After 1.5 hours of perfusion, fractions of hypoxic perfusate at basal level and during EFS-evoked vasocontraction or vasorelaxation were collected as in previously described steps in Sections 2.2.1 and 2.2.2 respectively. These samples were stored in a -80^{0}C freezer for 24 hours and then lyophilised using a freeze dryer (Edwards Modulyo, U.S.A) at -80^{0}C for 48 hours. Prior to assay, these samples were reconstituted with 50 µl of distilled waterper tube and then vortexed at 1000 r.p.m. for 1 min.

2.6.3 Multiplex assay procedure

All reagents and samples were allowed to warm to room temperature (20-25^{0}C) for half an hour. 200 µl of assay buffer was added into each well of the plate and vibrated for 10 min. The assay buffer was decanted and then 25 µl of assay buffer, standard, samples, Krebs'-Henseleit solution and control were added to appropriate wells as shown in Appendix 1. Each individual vial of antibody-beads was sonicated for 30 seconds and vortexed for 1 min. 150 µl of all antibody-beads were mixed and assay buffer was added to the mixture. This mixture was then added to each well. The plate was then incubated and agitated on a plate shaker overnight at 4^{0}C for 16-18 hours in the dark.

The next day, the plate was washed using a handheld magnetic separation block (EMD Millipore, Missouri, U.S.A) to allow complete settling of magnetic beads. The plate was left for 60 seconds on the handheld magnetic separation block before the well contents were removed. The plate was detached from the magnet and 200 µl of wash buffer was added to each well, followed by shaking for 30 seconds on a plate shaker. The plate was then re-attached to the magnetic plate washer again for 60 seconds and the well contents were removed as previously described. The washing steps were repeated 3 times. 50 µl of detection antibodies were added into each well. The plate was then incubated with agitation for 1 hour at room temperature (20-25^{0}C) on a plate shaker. It was important not to aspirate the well contents before adding 50 µl of Streptavidin-phycoerythrin containing detection antibodies to each well. The plate was then re-incubated with agitation for 30 min at room temperature (20-25^{0}C) on a plate shaker. After the incubation time was over, the washing protocol was carried out as described previously. 100 µl of Bio-Plex™ sheath fluid (Bio-Rad, California, USA) was added to all wells, and beads were resuspended on a shaker for 5 min. The plate was then run on Bio-Plex® instruments (Bio-Rad, California, USA). In-house automated statistical analysis (Bio-Plex Data Pro™, Version 6, California, USA) which applied t-test for data with normal distribution or Mann Whitney for data with non-parametric distribution was used to analyse multiplex data.

2.7 Porcine tissue preparation

Spleens and hearts from pigs (large white hybrid pigs, either sex, 4-6 months old, weighing ~ 50 kg) were collected on ice from a local abattoir (G Wood & Sons Ltd, Mansfield). Crude dissection was carried out to isolate

porcine splenic arteries (PSAs) from the dorsal part of the spleen or porcine coronary arteries (PCAs) that arose from the middle of the anterior aorta PSAs were used as they were known to receive substantial sympathetic innervation, therefore useful in investigation of sympathetic neurotransmission in PVAT while PCAs were chosen based on their crucial function to supply blood to the heart muscle. Both PSAs and PCAs are conduit vessels. The vessels were dissected out and placed in Krebs'-Henseleit buffer (Section 2.1) and refrigerated overnight at 4ºC. Fine dissection was conducted the following day. The PSA and PCA were prepared in two conditions, one with intact PVAT and the other with PVAT removed. The arteries were cut into rings of approximately 0.5 cm in length, suspended between two wires, and placed in organ baths containing Krebs'-Henseleit buffer maintained at 37ºC (gassed constantly, 95 % O_2, 5 % CO_2) as shown in Figure 2.6. The upper wire was inserted through the lumen of the arterial ring and was attached to a thin thread which connected to a transducer (World Precision Instruments, Sarasota, Florida, U.S.A) for isometric recording. The lower wire, which was attached to an electrode assembly, was also threaded through the lumen. The electrode assembly was connected to a Grass SD9 stimulator, while the upper wire was connected by a thread to a force transducer. The whole setup was linked to a Powerlab data acquisition system (ADInstruments Ltd., Hastings, UK) via an amplifier.

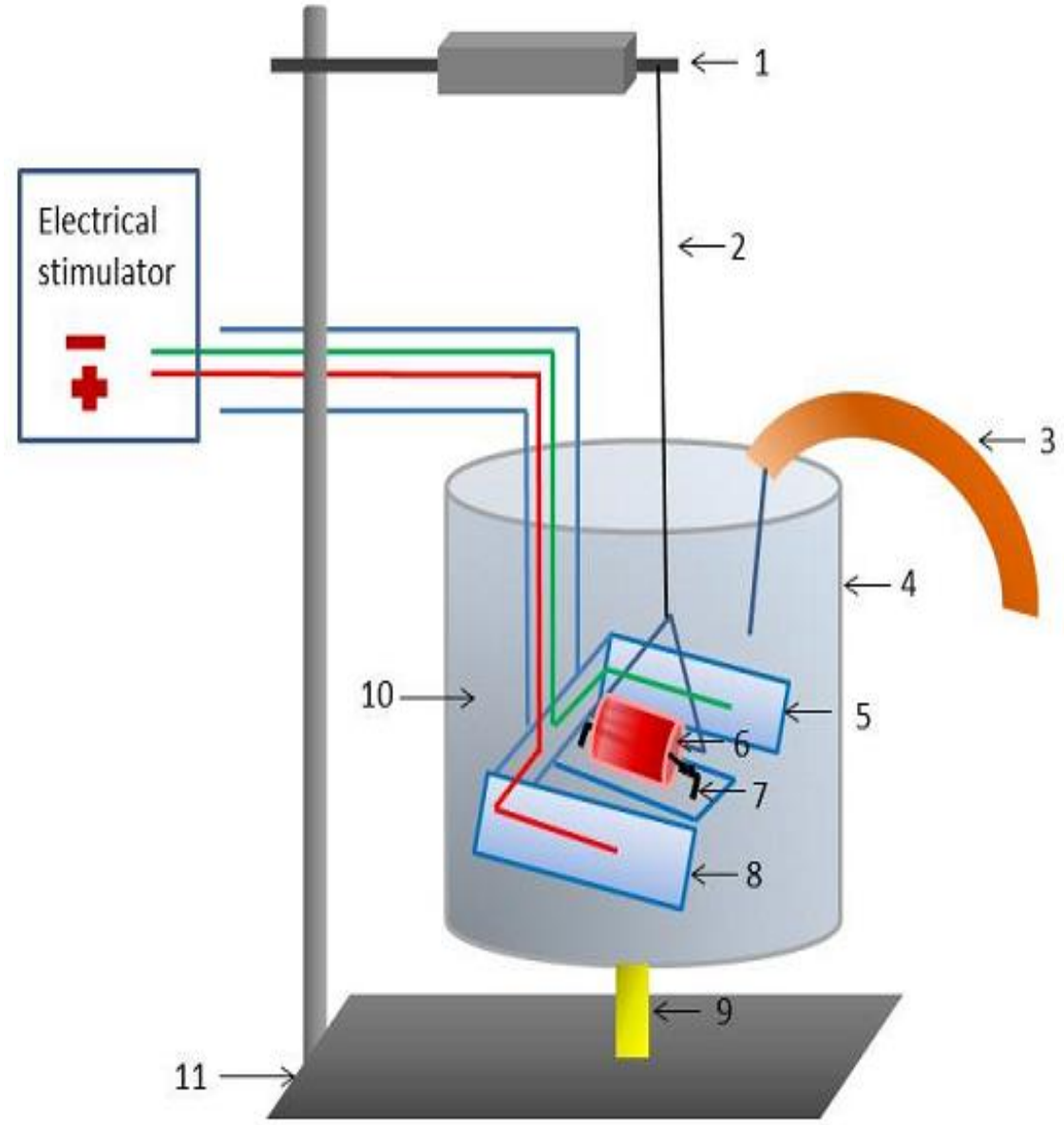

Figure 2.6: Diagrammatic representation of an isometric set up including (1) transducer, (Straub et al.) a thin thread attached to a thin wire (upper support), (Dick et al.) gas tube supplying 95 % and O_2 5 % CO_2, (4) organ bath, (5) negative electrode, (6) blood vessel mounted between upper and lower wire supports, (7) thin wire (lower support) attached to a tissue holder, (8) positive electrode, (9) drain, (10) Krebs'-Henseleit buffer, and (11) base.

2.8 Neuronal responses in porcine isolated splenic arteries

PSA rings were mounted onto wires in tissue baths (50 ml) containing warmed (37^0C) oxygenated Krebs'-Henseleit solution and were connected via isometric force transducers (ADInstruments, Sydney, Australia) to a PC running the computer program LabChart (ADInstruments, Sydney, Australia). Tension (10 g) was applied to the rings and they were allowed to equilibrate for a minimum of 30 min or until a stable baseline was achieved. The responses to EFS (1-20 Hz, 50-100 mA, 1-3 ms, 30 s) were determined after assessing viability of the arteries with two challenges of 60 mM potassium chloride (KCl). The interval between the frequencies was variable (3-10 min), and was determined by the return to baseline after each stimulation, with longer intervals at higher frequencies. Guanethidine (5 μM), a sympathetic blocker, was added immediately after the end of first frequency response curve (FRC), and after 30 min a second FRC was constructed to investigate whether the responses were sympathetically-mediated.

2.9 Drugs

Methoxamine hydrochloride, capsaicin, DL-propargylglycine (PPAG), L-aspartic acid sodium, amino oxyacetic acid (AOAA), aspartic acid, tyramine hydrochloride, acetylcholine chloride and prazosin hydrochloride were purchased from Sigma-Aldrich (Dorset, United Kingdom). Guanethidine monosulphate (Ismelin®) was purchased from Amdipharm Mercury (London, United Kingdom). HC030031, calcitonin gene-related peptide

(CGRP), endothelin-1, apelin-13, tetrodotoxin, losartan potassium, U46619 and candesartan were purchased from Tocris Bioscience (Bristol, United Kingdom). Sodium sulphide was purchased from BDH (Poole, United Kingdom). Methyl palmitate was purchased from Acros (New Jersey, U.S.A). Capsaicin, losartan potassium, and candesartan were dissolved in ethanol. Methyl palmitate and HC030031 were dissolved in dimethyl sulfoxide (DMSO) while other drugs were dissolved in distilled water.

2.10 Statistical analysis

Comparison of two groups was performed by 2-tailed, unpaired or paired Student's t-test while one-way ANOVA with Bonferroni's post-hoc test was used to analyse more than 2 groups. Two-way ANOVA with Bonferroni post-hoc test was conducted for data with 2 or more independent variables. Contractile responses of MABs are expressed as millimetre of mercury (mmHg). Contractile responses of rat mesenteric artery segments, porcine splenic and coronary arteries are expressed as gram (g) tension. Relaxant responses of the MABs were measured as mmHg and are expressed as a percentage of the methoxamine-induced contraction. The maximum contraction or relaxation response (R_{max}) was determined using non linear regression, fitted to a sigmoidal curve with a variable slope using four-parameters (top, bottom, slope factor (Hill's slope) and pEC_{50}) logistic equation. EC_{50} is the concentration of agonist required to produce a half maximal response. Locally Weighted Scatter-plot Smoother (LOWESS), a nonparametric regression focuses on the fitted curve was carried out to determine interpolated X values from all unpaired Y values. All analyses were carried out using GraphPad Prism (Version 6, GraphPad Software, California, USA) except for multiplex assay which used Bio-Plex Data Pro™

(Version 6, California, USA). P-values of less than 0.05 were considered statistically significant. Values for all figures refer to mean ± standard error mean (S.E.M) with 95% confidence. 'n' represents the number of animals used.

Chapter 3

Influence of perivascular adipose tissue on sympathetic neurotransmission

3.1 Introduction

Sympathetic nerves were first reported to innervate fat 80 years ago (Boeke, 1933). Sympathetic innervation of fat used to be a controversial subject, however, since then there has been growing evidence that sympathetic nerves innervate brown and white adipose tissue (Bartness and Bamshad, 1998; Bartness and Song, 2007; Bartness et al., 2010; Ryu and Bartness, 2014). It has been demonstrated that the sympathetic nervous system plays a role in modulating lipolysis in white adipose tissue and the degree of lipolysis appears to be determined by the relative expression of adipose adrenoceptors (Bartness and Bamshad, 1998). Adipocytes also express receptors for the sympathetic co-transmitters NPY and ATP (NPY receptors and purinoceptors respectively) and also express muscarinic receptors and receptors for peptides including angiotensin II and substance P (Bulloch and Daly, 2014).

The first study on PVAT was on that of rat aorta, carried out by Soltis and Cassis (1991), and showed the potential involvement of sympathetic nerves in modulating contractile response to electrical field stimulation. In their study, the presence of PVAT was shown to enhance EFS-evoked frequency-dependent vasocontractile responses (Soltis and Cassis, 1991). The authors concluded that this was most likely due to the presence of sympathetic nerves in the PVAT (Soltis and Cassis, 1991). However, although two decades have passed since the discovery, the role of sympathetic nerves in PVAT remains unclear and evidence for their expression in PVAT is still limited. Gao et al. (2006) was the first group to revisit the neurogenic contractile response in tissues with PVAT and their studies were carried out in rat mesenteric arteries (MA). The group produced a similar observation to that of Soltis and Cassis, (1991) in which

PVAT-potentiated the arterial contractile response to perivascular nerve stimulation (Gao et al., 2006). This was shown to occur through the production of superoxide mediated by NAD(P)H oxidase, and involved activation of tyrosine kinase and MAPK/ERK pathways (Gao et al., 2006). Lu et al. (2010a) further exposed a pro-contractile property of PVAT on perivascular nerves by providing evidence for the presence of angiotensin II in mesenteric PVAT, and this PVAT-derived compound was suggested to augment EFS-evoked neurogenic contractile responses in isolated rat MA, in line with previous observations that angiotensin II enhances sympathetic neurotransmission through pre- and post-junctional mechanisms (Dunn et al., 1991; Guimaraes and Pinheiro, 2005; Onaka et al., 1997). However, none of these functional studies specifically demonstrated the expression of nerves in PVAT. Using immunoblotting techniques, it has been demonstrated that PVAT of human saphenous vein contains a dense sympathetic innervation (Dashwood and Loesch, 2011). A more recent immunofluorescence study showed evidence of sympathetic innervation in PVAT of mice mesenteric arteries (Bulloch and Daly, 2014). Nonetheless, another recent study contradicts the role of PVAT-derived sympathetic nerves in vascular tone regulation (Ayala-Lopez et al., 2014).

The present study was conducted to further investigate the expression of sympathetic nerves in PVAT of different blood vessels, and also to examine the interaction between sympathetic nerves, PVAT, and endogenous (angiotensin II) or exogenous (methyl palmitate and apelin-13) PVAT-derived compounds. Of all the adipokines, angiotensin II was selected as it was shown to be involved in the mechanism of PVAT-potentiation of sympathetic neurogenic response in rat mesenteric arteries (Lu et al., 2010a), methyl palmitate was studied as it is a novel potential PVAT-derived vasorelaxing factor (PVATRF) (Lee et al., 2011), and apelin-13 was

chosen as there is evidence that this adipokine can stimulate sympathetic activity, in the paraventricular nucleus (Zhang et al., 2014). Furthermore, no study has previously investigated the effects of methyl palmitate or apelin-13 on sympathetic perivascular neurotransmission.

3.2 Materials and methods

3.2.1 Rat tissue preparation and experimental protocol

Male Wistar rats, weighing 180-220 g, were killed by stunning and exsanguination. Mesenteric arterial beds (MABs) were set up and perfused as described in Section 2.1. Rat abdominal aorta (AA) rings were prepared as described in Section 2.1. Neurogenic vasocontractile responses in perfused rat MABs were carried out as described in Section 2.2.1. In perfused MABs, EFS at 4-32 Hz, 90 V, 1 ms, 30 s was generated in the absence or presence of antagonists: losartan (1 μM) and candesartan (1 μM), angiotensin II receptor antagonists which were added after the first frequency response curve (FRC). MABs were incubated with the antagonists for 30 min before the second FRC was constructed. In separate experiments, methoxamine (0.1–30 μM), an α_1-adrenoceptor agonist, or tyramine (0.1–30 μM), a catecholamine-releasing agent, were added cumulatively into the perfusate in PVAT-intact and PVAT-denuded MABs. In separate experiments, MABs and AA were submaximally pre-constricted with endothelin 1 (0.01-0.1 nM) and, when a stable level of tone had been achieved, guanethidine (5 μM) was added for 30 min. Prazosin (0.3 μM) was then added following the 30 min of incubation with guanethidine in both rat MABs and AAs. In separate experiments, the effect of replacing dissected PVAT was investigated in PVAT-denuded preparations of MABs.

First, a FRC (EFS; 4-32 Hz, 90 V, 1 ms, 30 s) was carried out in PVAT-denuded preparations. After the FRC was completed, MABs were incubated with dissected PVAT; the tubing allowing outflow of the perfusate was clamped to retain Krebs'-Henseleit solution in the chamber and MABs were left incubated with dissected fat, placed on top of the MABs, for a duration of 30 min. A second FRC was then constructed.

3.2.2 Porcine tissue preparation and experimental protocol

Spleens and hearts from pigs (large white hybrid pigs, either sex, 4-6 months old, weighing approximately 50 kg) were collected on ice from a local abattoir as described in Section 2.7. Porcine splenic arteries (PSA) and porcine coronary arteries (PCA) were set up as described in Section 2.7. Neuronal vasoconstriction responses in PSA were generated as described in Section 2.8. In some experiments, guanethidine (5 μM) was added for a duration of 30 min, after stable submaximal U46619 (1-5 nM)-induced pre-constriction was achieved in both PCAs and PSAs, and then this was followed by addition of prazosin (0.3 μM) for 30 min. In separate experiments, exogenous PVAT-derived compounds, apelin-13 (0.01 μM) or methyl palmitate (0.01-0.1 μM), were added into the organ bath for a duration of 30 min after the first FRC was completed. A second FRC was then constructed when the interval was completed.

3.2.3 Immunofluorescence study to determine the location of sympathetic nerves on blood vessels and within PVAT

Segments of rat mesenteric arteries were obtained and treated as described in Section 2.5.1. Immunofluorescence staining was carried out as described in Section 2.5.2.

3.2.4 Statistical analysis

Data were analysed as described in Section 2.10.

3.3 Results

3.3.1 Immunofluorescence

3.3.1.1 Immunofluorescence characterization of perivascular tyrosine hydroxylase-containing nerves in PVAT-denuded rat mesenteric artery segments

Immunoreactivity for tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholamine biosynthesis, was observed on the surface of PVAT-denuded mesenteric arteries in all segments (SMA, 1OMA, 2OMA and 3OMA) as shown in Figure 3.1 (A, B, C, D). Fibre-like structures were observed in the longitudinal sections of SMA, 1OMA, 2OMA and 3OMA (see Figure 3.1 A, B, C, D). In the absence of anti-TH nerve antibody (negative control), no immunoreactivity was visualized in any of the artery segments as shown in Figure 3.1 (E, F, G, H).

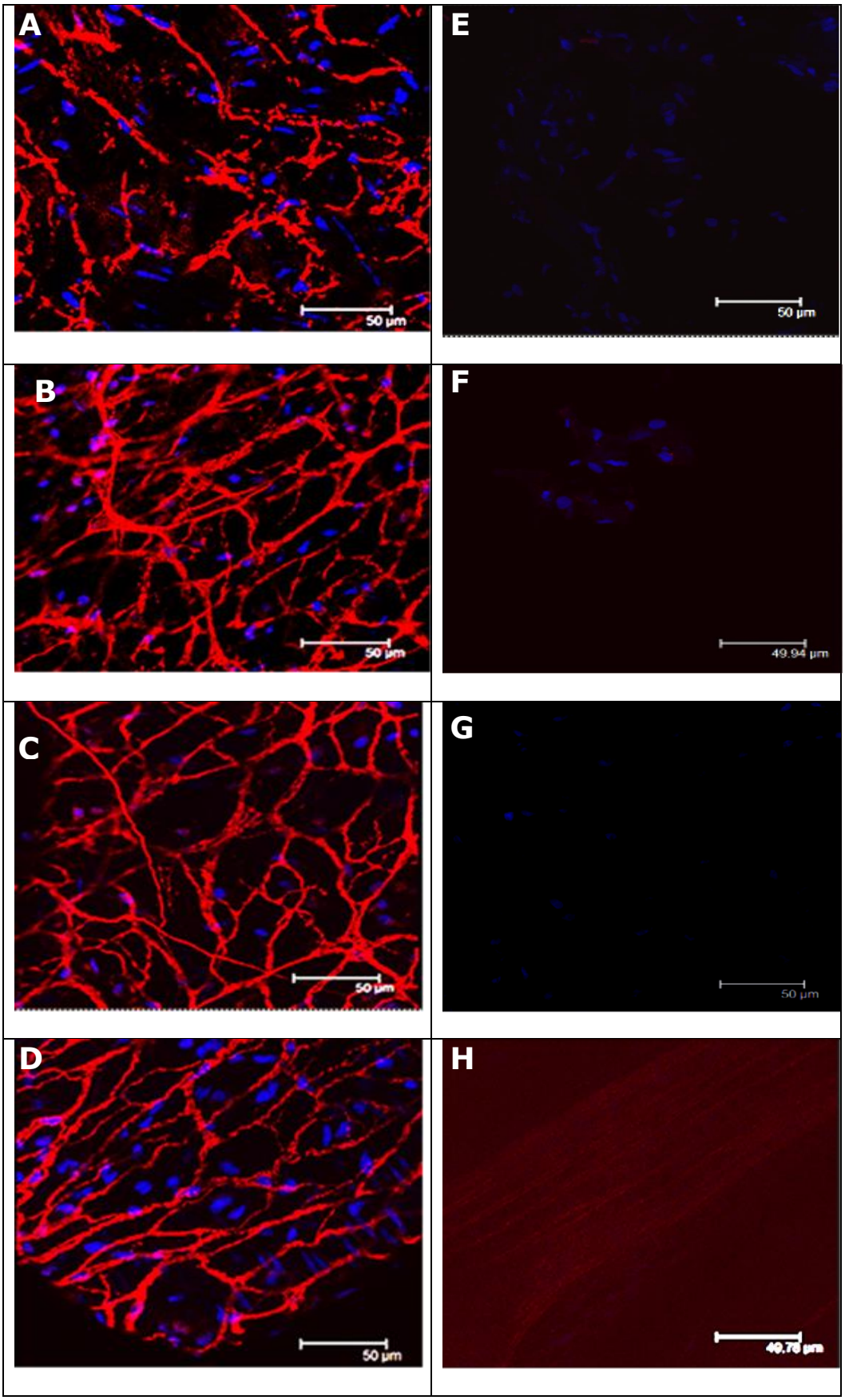

Figure 3.1: Intense tyrosine hydroxylase (TH) containing nerves (red) visualized on the surface of clean (PVAT-removed) rat mesenteric arteries

(longitudinal section): **(A)** superior mesentery artery, **(B)** first order mesentery artery, **(C)** second order mesentery artery, and **(D)** third order mesentery artery. In the absence of anti-TH antibody (negative control), no staining was observed in the longitudinal section of: **(E)** superior mesentery artery, **(F)** first order mesentery artery, **(G)** second order mesentery artery, and **(H)** third order mesentery artery. Deoxyribonucleic acid (DNA) was counterstained with DAPI (blue). Scale bar = 50 μm (n=6).

3.3.1.2 Immunofluorescence characterization of perivascular tyrosine hydroxylase-containing nerves in PVAT-intact rat mesenteric artery segments

Tyrosine hydroxylase (TH)-immunoreactivity (red) was visualized in PVAT-intact MABs (MA, 1OMA, 2OMA and 3OMA) segments which included PVAT as shown in Figure 3.2 (A, B, C, D). In the absence of anti-TH nerve antibody (negative control), no immunoreactivity was obtained in both preparations with and without PVAT as shown in Figure 3.2 (E, F, G, H). To confirm the expression of TH-containing nerves within PVAT, a co-localization protocol was employed. SMA and 2OMA segments of PVAT-intact MABs were treated with anti-TH (red) and anti-PGP9.5 (green), a neuronal marker. Intense double immunoreactivity of nerves were observed in SMA and 2OMA segments as shown in Figure 3.3 (A, B, C, E) and Figure 3.4 (A, B, C, E) respectively.

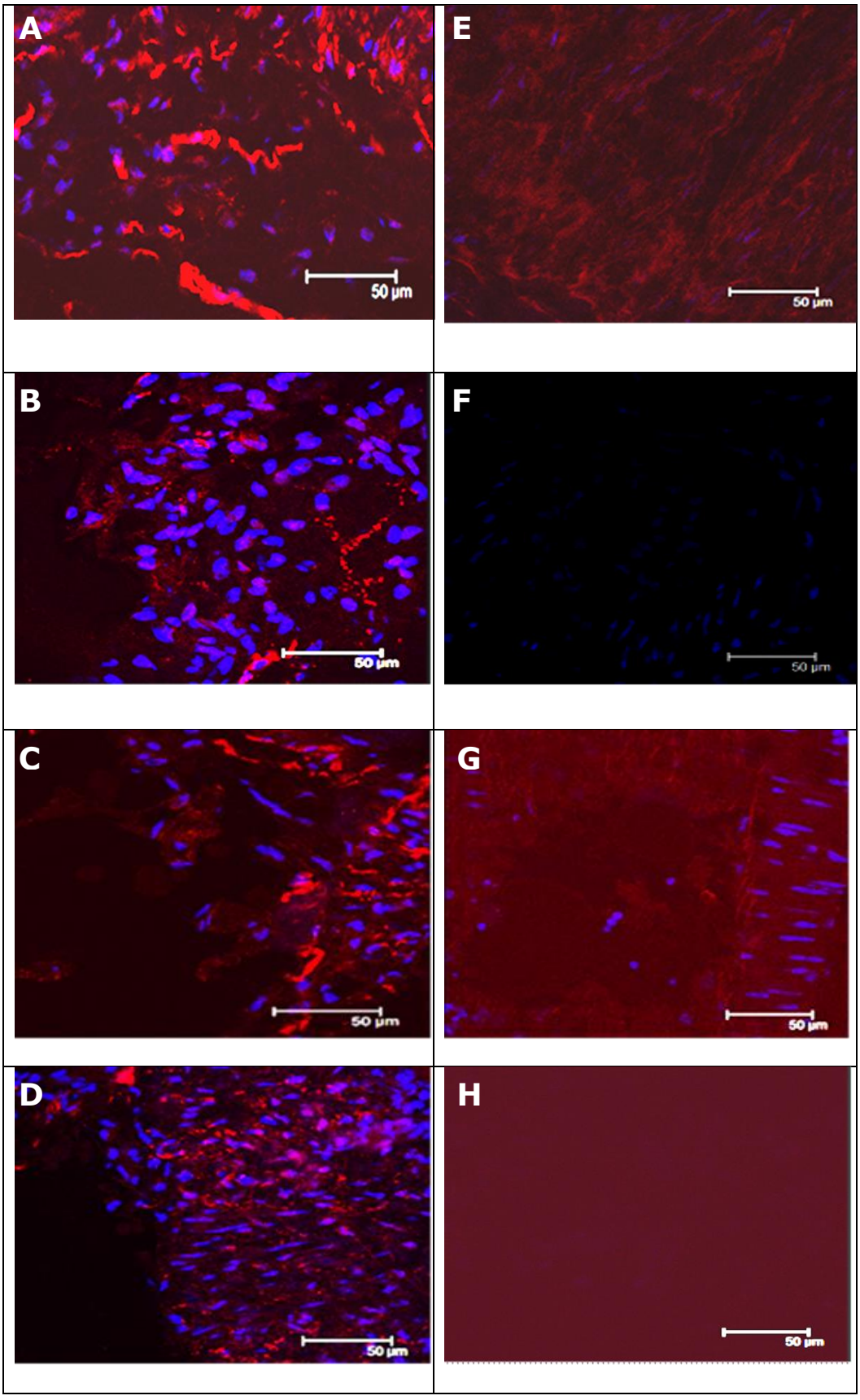
A
50 µm
E
50 µm
B
50 µm
F
50 µm
C
50 µm
G
50 µm
D
50 µm
H
50 µm

Figure 3.2: Tyrosine hydroxylase (TH)-containing nerves visualized within fat in the vicinity of (A) superior mesentery artery (B) first order mesentery artery, (C) second order mesentery artery and (D) third order mesentery artery. In the absence of anti-TH nerves antibody (negative control), no staining of nerves was observed in the longitudinal section of (E) superior mesentery artery, (F) first order mesentery artery, (G) second order mesentery artery and (H) third order mesentery artery. Deoxyribonucleic acid (DNA) was counterstained with DAPI (blue). Scale bar = 50 μm (n=5).

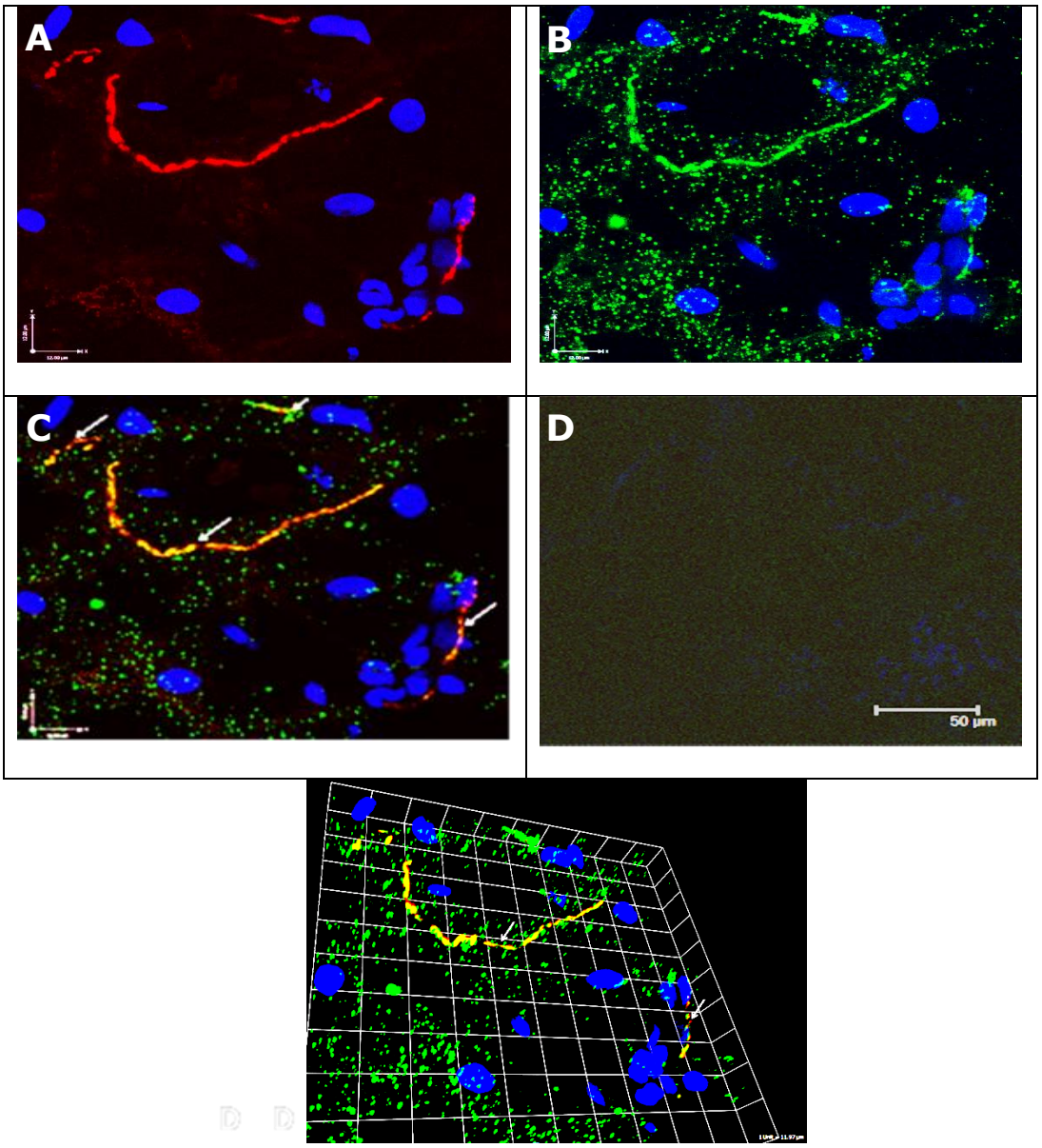

Figure 3.3: Co-localization/double labelling for tyrosine hydroxylase (TH) nerves within PVAT in PVAT-intact SMA segment. (A) Anti-TH staining (red), (B) anti-PGP 9.5 staining (green), (C) merged image shows some neurons co-expressing TH and PGP 9.5 marker in PVAT region. (D) In the absence of anti-TH and anti-PGP 9.5 antibodies (negative control), no staining was observed. (E) 3-dimensional merged image showing some neurons co-expressing TH and PGP 9.5 marker in PVAT region. DNA was counterstained with DAPI (blue). Arrows indicate double labelled nerves. Scale bars for A, B and C = 12 µM, D= 50 µM and E, 1 unit = 11.97 µM (n=2).

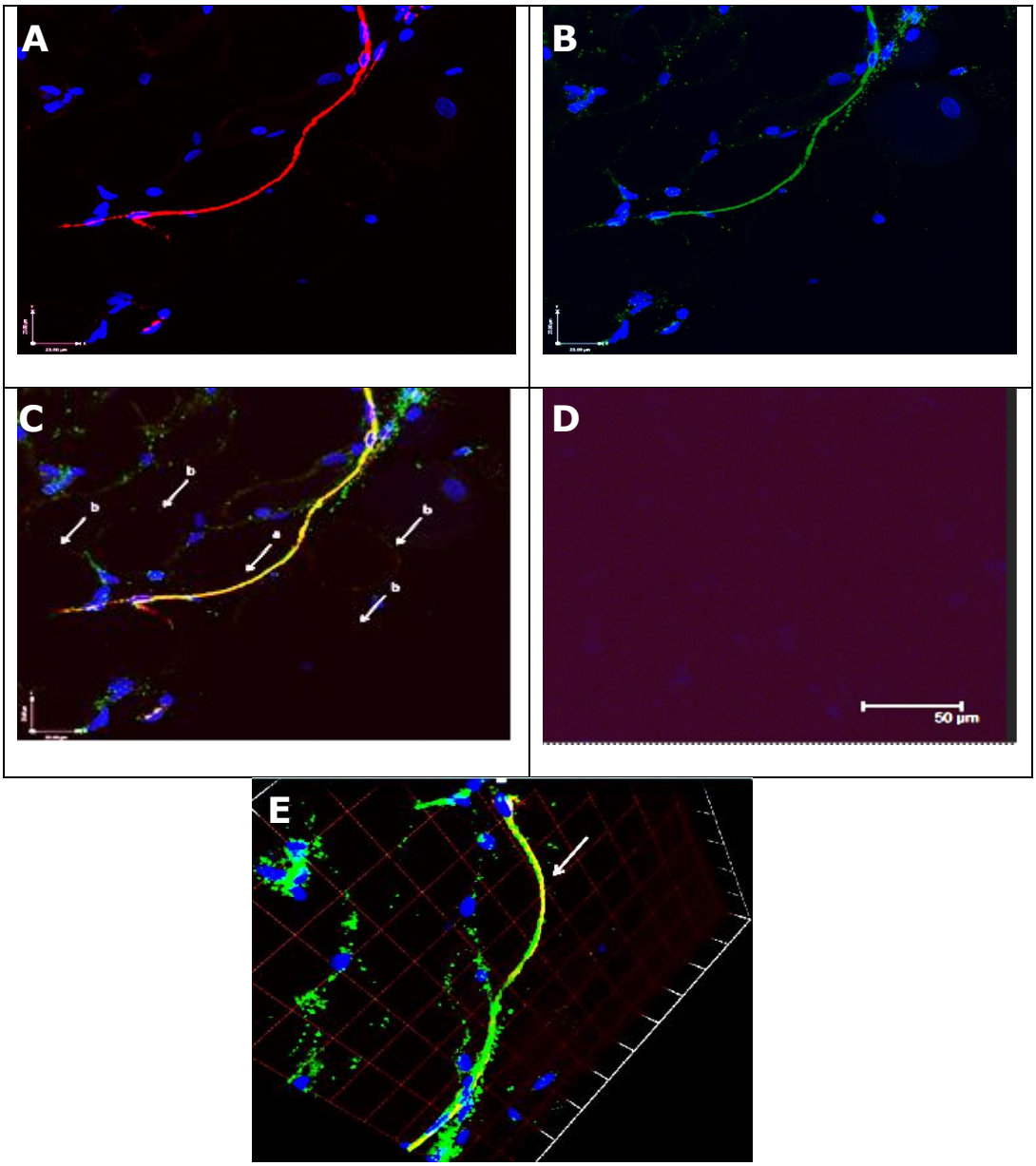

Figure 3.4: Co-localization/double labelling for tyrosine hydroxylase (TH)-containing nerves within PVAT in PVAT-intact 2OMA segment. (A) Anti-TH staining (red), (B) anti-PGP 9.5 staining (green), (C) merged image shows some neurons co-expressing TH and PGP 9.5 marker in PVAT region. D) In the absence of anti-TH and anti-PGP 9.5 antibodies (negative control), no staining was observed. (E) 3-dimensional merged image showing some neurons co-expressing TH and PGP 9.5 marker in PVAT region. DNA was counterstained with DAPI (blue). Arrows indicate double labelled nerves, a = nerve, b = adipocyte cell. Scale bars for A, B and C = 23 µM, D= 50 µM and E, 1 unit = 23.86 µM (n=2).

3.3.2 The effect of PVAT on neurogenic contractile responses in perfused rat mesenteric arterial beds

At a perfusate flow rate of 5 ml min^{-1}, there was no significant difference ($P>0.05$, Student's unpaired t-test, n=8) in basal perfusion pressure between MABs with (43 ± 1 mmHg) and without (42 ± 3 mmHg) PVAT. EFS at 4-32 Hz, 90 V, 1 ms, 30 s of perfused mesenteric vascular beds at basal tone produced frequency-dependent increases in perfusion pressure due to vasoconstriction (Figure 3.5A and Figure 3.5B). In the presence of PVAT, responses to EFS were significantly greater ($P<0.05$, $P<0.0001$, two-way ANOVA with Bonferroni's post-hoc test, n=4) than in the absence of PVAT (Figure 3.5 and 3.6). These responses were abolished by guanethidine (5 μM), a sympathetic nerve blocker, indicating the involvement of sympathetic nerves (Figure 3.7).

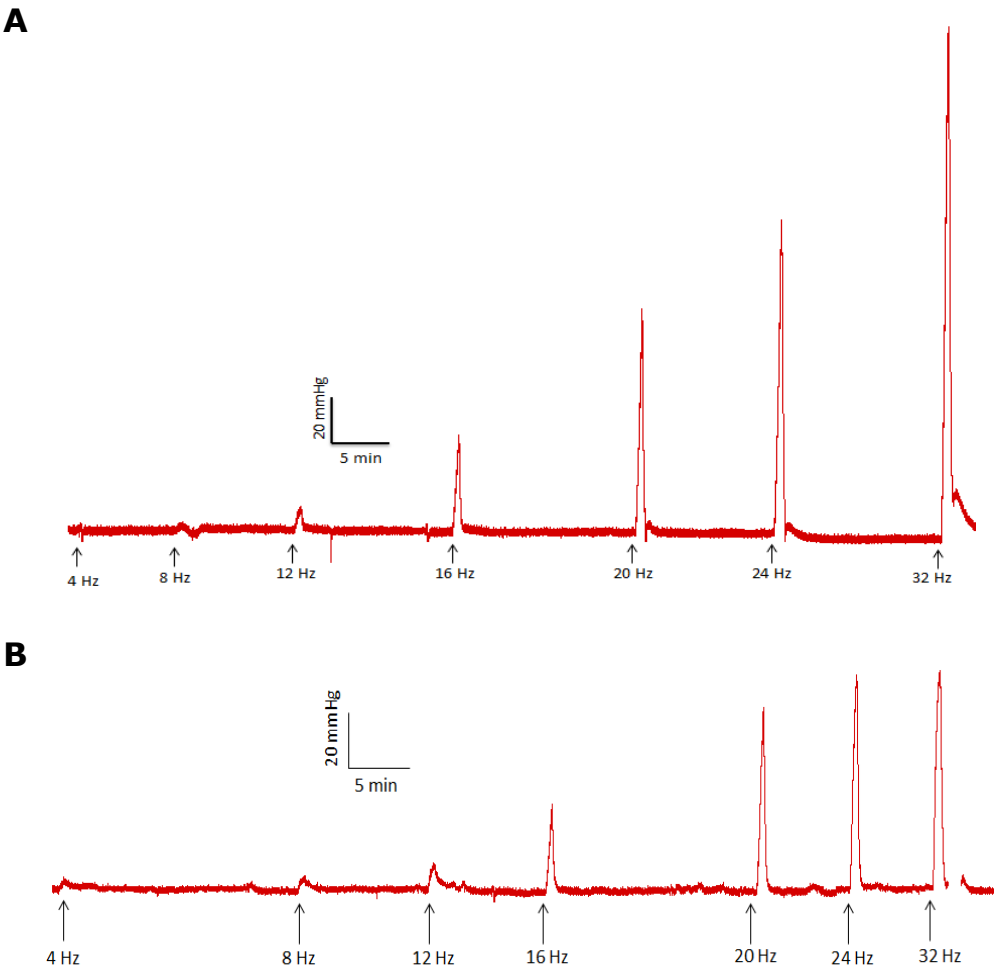

Figure 3.5: Representative traces show that electrical field stimulation (4-32 Hz, 90 V, 1 ms, 30 s) of rat perfused mesenteric vascular beds at basal perfusion pressure produced a frequency-dependent increase in perfusion pressure due to vasoconstriction in (A) the presence of perivascular adipose tissue (PVAT), and (B) in the absence of fat. Neurogenic contractile responses were attenuated in preparations without PVAT.

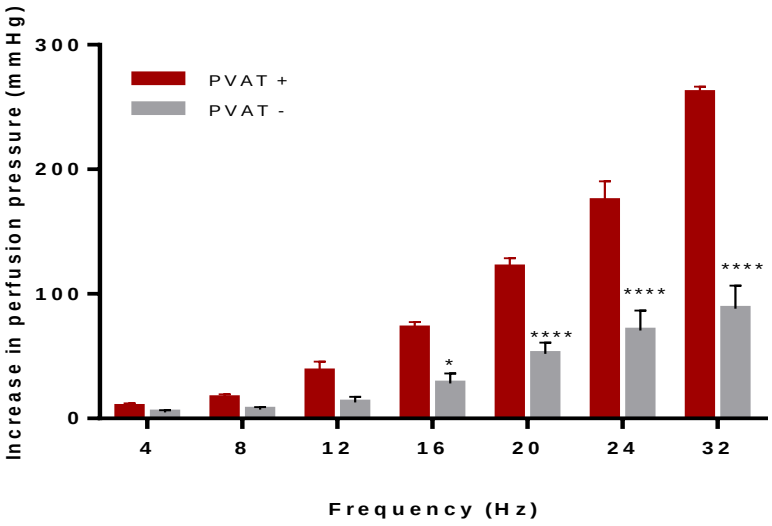

Figure 3.6: Effect of perivascular adipose tissue (PVAT) on contractile responses to electrical field stimulation (EFS; 4-32 Hz, 90 V, 1 ms, 30 s) of rat isolated mesenteric arterial beds. In the presence of PVAT, responses to EFS were significantly greater than in the absence of PVAT (*P<0.05, ****P<0.0001, two-way ANOVA with Bonferroni's post-hoc test, n=4).

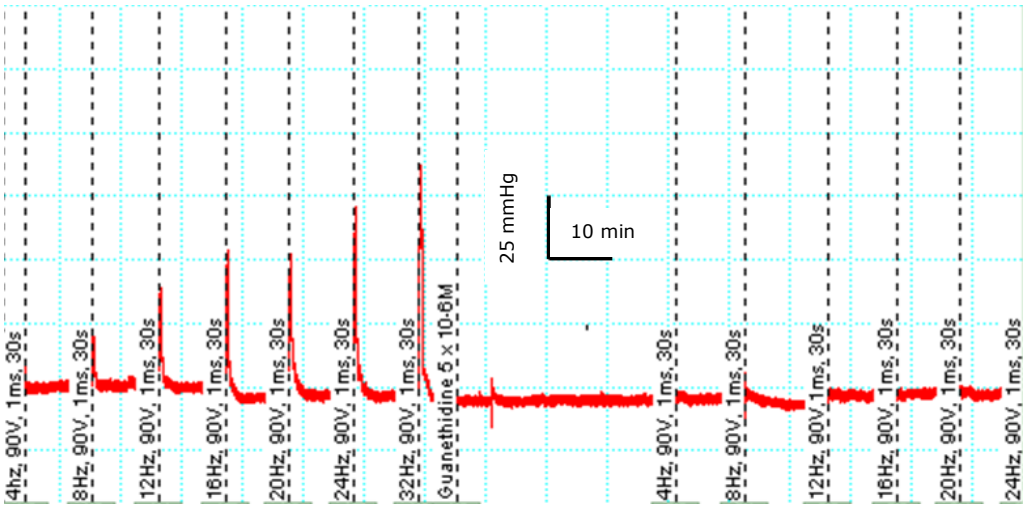

Figure 3.7: A representative trace shows electrical field stimulation (EFS; 4-32 Hz, 90 V, 1 ms, 30 s)-evoked frequency-dependent contractile responses in perfused rat mesenteric arterial bed with PVAT-intact. Contractile responses were abolished in the presence of guanethidine (5 μM), a sympatholytic agent.

3.3.3 The effect of PVAT on responses to sympathetic agonists in MABs

Since the absence or presence of PVAT affected the amplitude of sympathetic neurogenic contractile responses in MABs, experiments were carried out to investigate whether this involved pre- and/or post-junctional mechanisms. Methoxamine (0.1-30 μM), an α_1-adrenoceptor agonist, elicited concentration-dependent vasoconstrictor responses which were not different between preparations with PVAT (R_{max} = 196 ± 20 mmHg, pEC_{50} = 5.5 ± 0.10 -logM) and without PVAT (R_{max} = 191 ± 27 mmHg, pEC_{50} = 5.5 ± 0.13 -logM) ($P>0.05$, unpaired Student's t-test, n=5) as shown in Figure 3.8. Tyramine (0.1-0.3 mM), an indirect sympathomimetic agent which acts by enhancing catecholamine release, produced small concentration-dependent contractions which were not different between

preparations with PVAT (R_{max} = 9 ± 2 mmHg, EC_{50} = 5.9 ± 0.5 M) and without PVAT (R_{max} = 9 ± 2 mmHg, EC_{50} = 6.1 ± 0.4 M) (P>0.05, unpaired Student's t-test, n=5) (Figure 3.9).

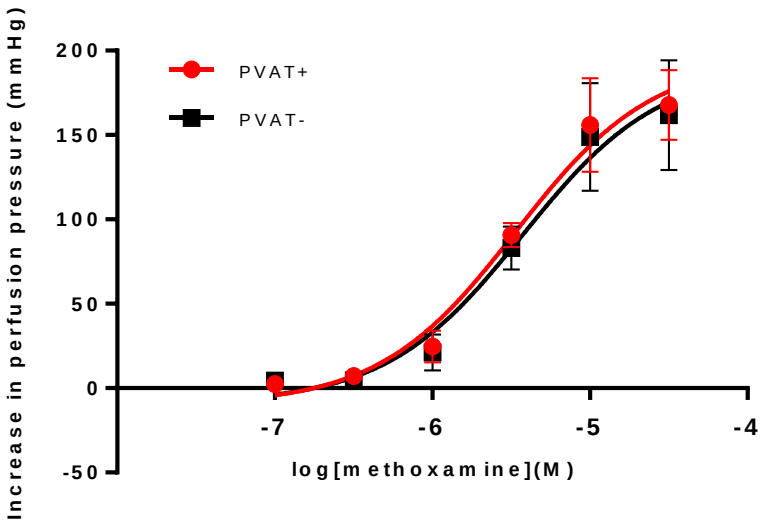

Figure 3.8: The effect of the presence and absence of perivascular adipose tissue (PVAT) on contractions to methoxamine (0.1-30 μM) in rat isolated mesenteric vascular beds. There was no difference between PVAT-intact (PVAT+) and PVAT-denuded (PVAT-) preparations with regard to the methoxamine-induced vasocontractile responses (P>0.05, unpaired Student's t-test, n=5).

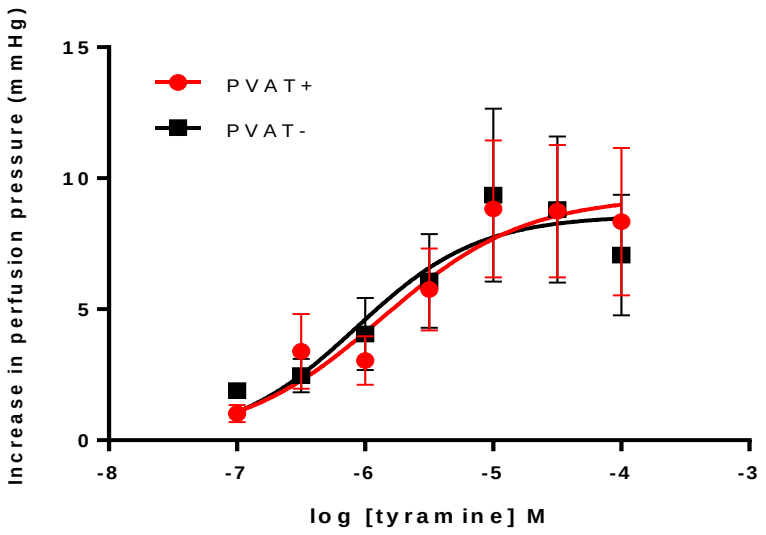

Figure 3.9: The effect of the presence and absence of perivascular adipose tissue (PVAT) on contractions to tyramine (0.1-30 μM) in rat isolated mesenteric vascular beds. There was no difference between PVAT-intact (PVAT+) and PVAT-denuded (PVAT-) preparations in tyramine-induced vasocontractile responses ($P>0.05$, unpaired Student's t-test, n=5).

3.3.4 Effects of replacing PVAT on vascular responsiveness

In separate experiments, PVAT which had been carefully dissected from the MABs was replaced on top of the MAB in order to see if there was an involvement of a diffusible factor from the PVAT during EFS. Neurogenic contractile responses to EFS (4-32 Hz, 1 ms, 90 V, 30 s) under basal tone were decreased ($P<0.0001$, two-way ANOVA with Bonferroni's post-hoc test) after dissected PVAT was transferred to PVAT-denuded preparations (n=7) (Figure 3.10).

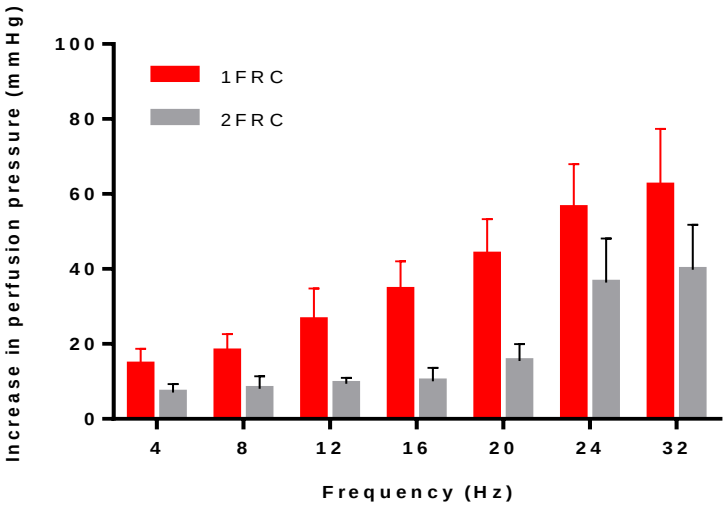

Figure 3.10: Effect of replacing dissected PVAT back to PVAT-denuded mesenteric arterial bed preparations. PVAT was removed and the first response curve (1 FRC) was generated. The dissected PVAT was placed back on the denuded preparations and the second FRC (2 FRC) was constructed. A two-way ANOVA found a main effect of replacing PVAT on the frequency response curve ($P<0.0001$, $n=7$). The replacement of PVAT greatly reduced EFS contractile responses.

3.3.5 Effect of angiotensin II receptor antagonism on EFS-induced contractile responses in rat mesenteric arterial beds with and without PVAT

In order to investigate the involvement of endogenous angiotensin II in the responses, candesartan and losartan, angiotensin II receptor antagonists, were added at the end of the first FRC in the perfused PVAT-intact MABs. Incubation with candesartan (1 μM) in the presence of PVAT significantly attenuated EFS-evoked contraction ($P<0.05$, two-way ANOVA with Bonferroni's post-hoc test, $n=5$) (Figure 3.11A). In contrast, there was no

difference between EFS-evoked vasocontractile responses in PVAT-denuded MABs in the absence or presence of candesartan ($P>0.05$, two-way ANOVA, $n=5$) as shown in Figure 3.11B. Losartan (1 μM) had no significant effect on EFS-induced contractions of MABs with PVAT (Figure 3.12). Time control experiments showed that the frequency dependent contractile responses were reproducible across two consecutive frequency response curves (Figure 3.13).

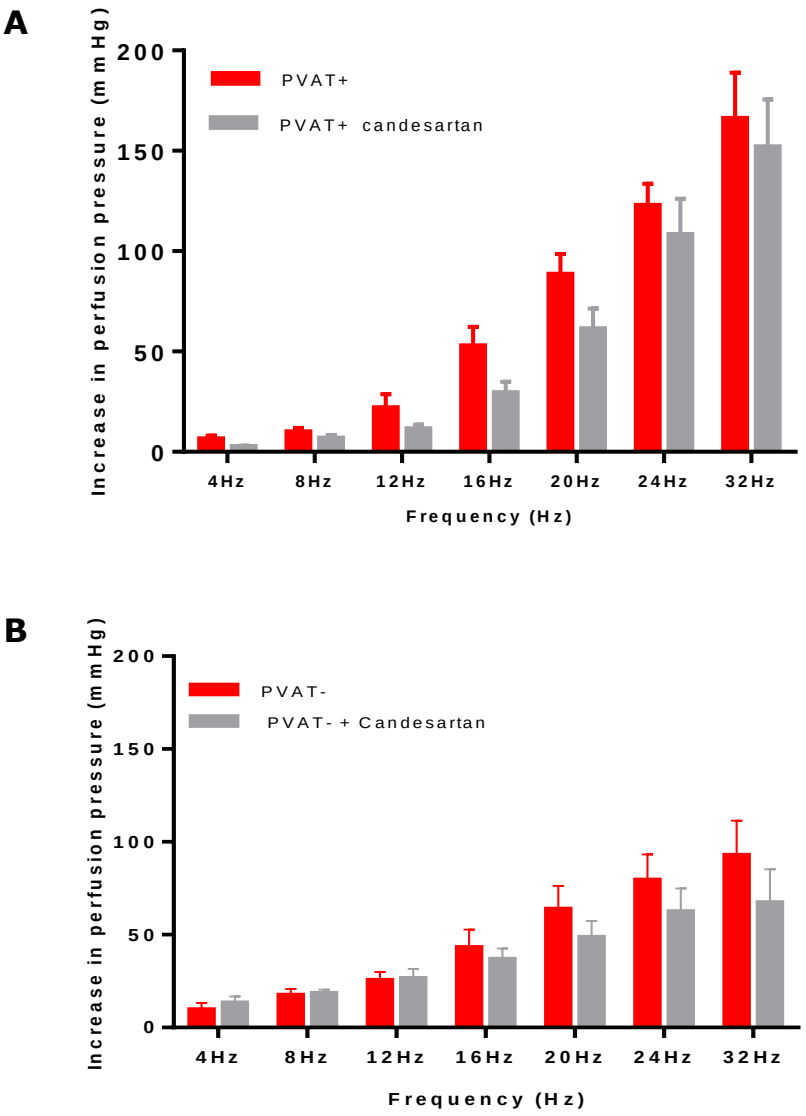

Figure 3.11: The effect of candesartan, an angiotensin II receptor antagonist, on neurogenic vasocontractile responses of rat mesenteric arterial beds (MABs) to electrical field stimulation (EFS). EFS was applied at 4-32 Hz, 90 V, 1 ms, for 30 s. (A) A two-way ANOVA found that candesartan (1 μM) significantly inhibited EFS-evoked vasocontractile responses ($P<0.05$, n=5). (B) In PVAT-denuded preparations, there was no difference in EFS-evoked vasocontractile responses in the absence and presence of candesartan (two-way ANOVA, n=5).

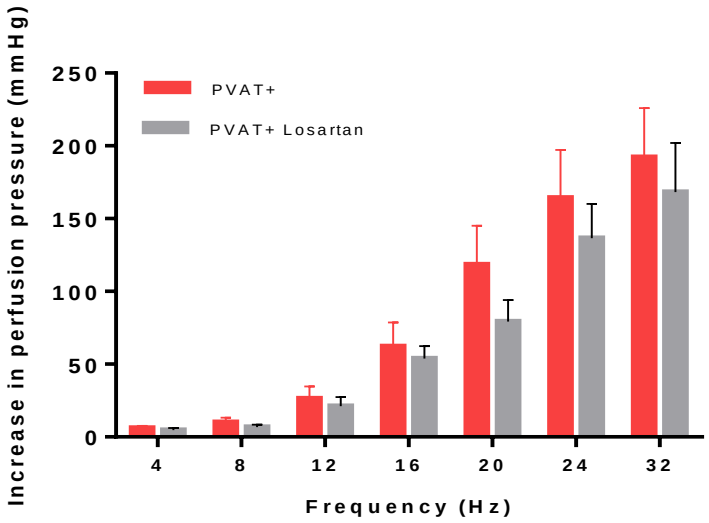

Figure 3.12: The effect of losartan, an angiotensin II receptor antagonist, on neurogenic vasocontractile responses to electrical field stimulation (EFS) in rat mesenteric arterial beds. EFS was applied at 4-32 Hz, 90 V, 1 ms, for 30 s. In perivascular adipose tissue (PVAT)-denuded preparations, there was no difference in EFS-evoked vasocontractile responses in the presence of losartan (1 μM) (two-way ANOVA, n=5).

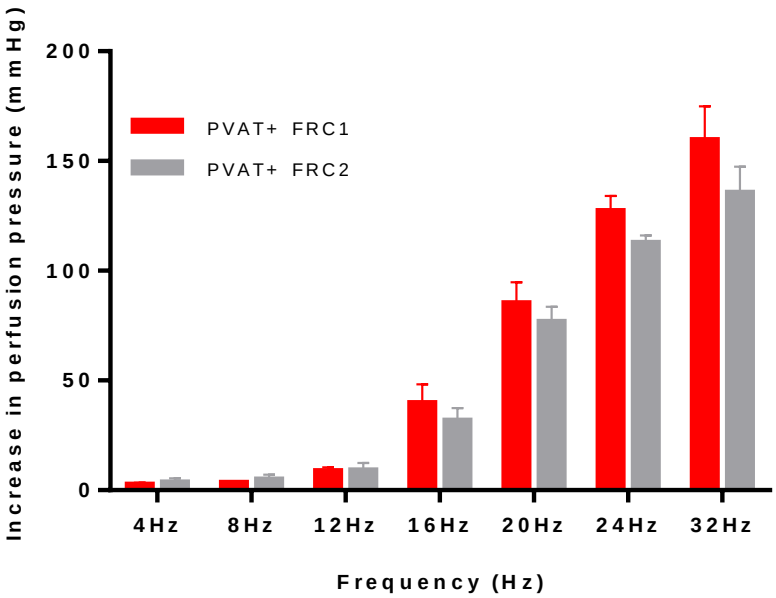

Figure 3.13: Two frequency response curves (FRC) were produced in rat mesenteric arterial beds with an interval of 30 min. Electrical field stimulation was applied at 4-32 Hz, 90 V, 1 ms, for 30 s. This reproducibility test showed that there was no difference between FRC 1 and FRC 2, indicating that these responses are reproducible ($P>0.05$, two-way ANOVA, n=5).

3.3.6 The effect of PVAT on neurogenic contractile responses in porcine isolated splenic arteries

The effect of PVAT on EFS-evoked vasocontractile responses was examined in porcine splenic arteries (PSA) in PVAT-intact and PVAT-denuded preparations. EFS at 1-20 Hz, 100 mA, 1 ms and 10 s elicited frequency-dependent vasocontractile responses in both PVAT-intact and PVAT-denuded preparations (Figure 3.14). Removal of PVAT significantly attenuated sympathetic neurogenic contractile responses ($P<0.05$, two-way ANOVA with Bonferroni's post-hoc test, n=6). These neurogenic vasocontractile responses were abolished in the presence of guanethidine

(5 μM) indicating an involvement of sympathetic nerves (P<0.0001, two-way ANOVA with Bonferroni's post-hoc test, n=5-7) as shown in Figure 3.14.

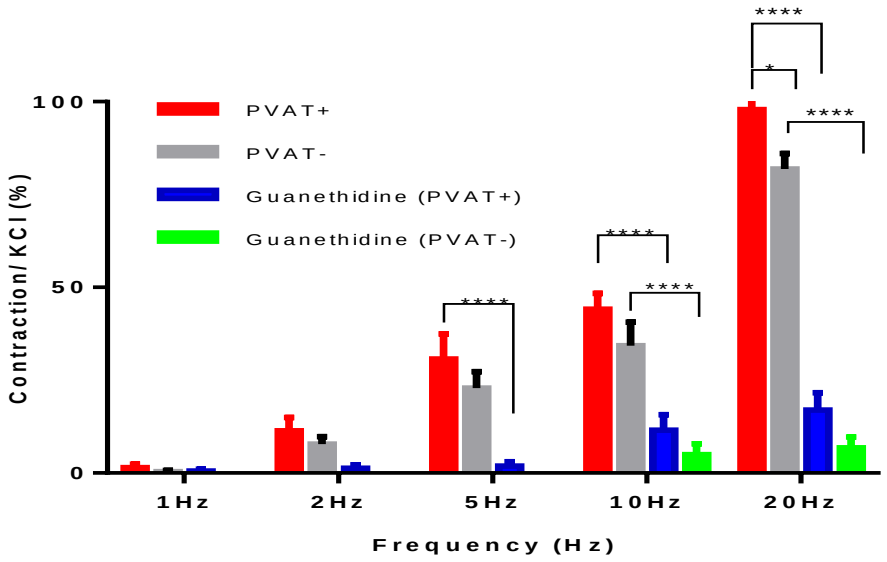

Figure 3.14: Effect of perivascular adipose tissue (PVAT) on contractile response to electrical field stimulation (EFS; 1-20 Hz, 100 mA, 1 ms, 10 s) in porcine splenic arteries. In the presence of PVAT (PVAT+), responses to EFS were significantly greater than in the absence of PVAT (PVAT-) (n=6). The responses were greatly attenuated in the presence of guanethidine (5 μM). Data are expressed as a percentage change of the potassium chloride (KCl, 60 mM)-induced contraction (*P<0.05, ****P<0.0001, two-way ANOVA with Bonferroni's post-hoc test, n=5-7).

3.3.7 Effect of methyl palmitate on electrically-evoked contractile responses in porcine splenic arteries

Methyl palmitate is a novel adipokine, which has recently been found to be expressed within PVAT (Lee et al., 2011). However, little is known about its possible influence on neurogenic responses. Under basal tone conditions, EFS (1-20 Hz, 50 mA, 1 ms and 10 s) produced contractile responses which were frequency-dependent in the PSA. Methyl palmitate (0.1 μM and 0.01 μM) had no significant effect on EFS-evoked frequency-dependent vasoconstriction in the presence (n=8) and absence (n=6) of PVAT ($P>0.05$, two-way ANOVA) as shown in Figure 3.15A and Figure 3.15B, respectively.

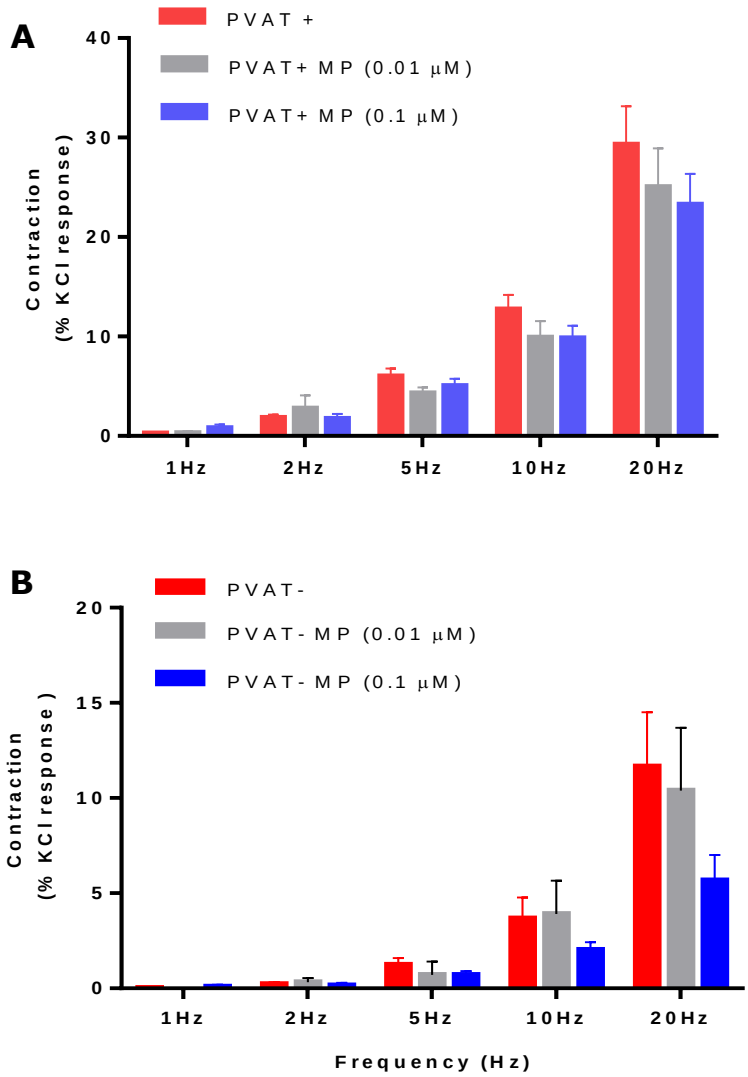

Figure 3.15: The effect of methyl palmitate on electrical field stimulation (EFS)-evoked contractions of porcine splenic arteries in **(A)** the presence of PVAT (PVAT+) and **(B)** the absence of PVAT (PVAT-). Methyl palmitate (MP) was added at 0.01 µM and 0.1 µM. EFS was applied at 1-20 Hz, 50 mA, 1 ms and 10 s. The contractile responses were not different in the presence or absence of methyl palmitate in both PVAT-intact and PVAT-denuded preparations (two-way ANOVA, n=6-8).

3.3.8 Effect of apelin-13 on neurogenic vasocontractile responses in PSA

Apelin-13, a novel adipokine, has recently been detected within PVAT (Maenhaut and Van de Voorde, 2011), but its role in sympathetic control of the vasculature remains unknown. EFS (1-20 Hz, 50 mA, 3 ms and 10 s) evoked frequency-dependent neurogenic vasoconstriction in both PVAT-intact (Figure 3.16) and PVAT-denuded (data not shown). Apelin-13 (0.01 μM) significantly inhibited EFS-evoked neurogenic vasoconstriction in both PVAT-intact ($P<0.001$, two-way ANOVA, n=4) (Figure 3.16 and Figure 3.17A) and PVAT-denuded ($P<0.0001$, two-way ANOVA with Bonferroni's post-hoc test, n=3) (Figure 3.17B) preparations of PSA. In both preparations, apelin-13 significantly inhibited neurogenic contractile responses of PVAT-intact preparations ($P<0.001$) and PVAT-denuded preparations ($P<0.0001$) at 20 Hz as shown in Figure 3.17A and Figure 3.17B respectively.

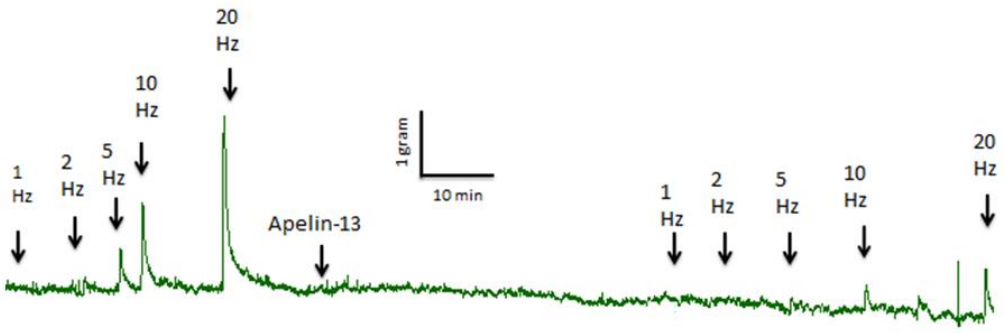

Figure 3.16: A representative trace shows that electrical field stimulation (1-20 Hz, 50 mA, 3 ms and 10 s) of porcine splenic artery under isometric tension condition produced a frequency-dependent increase in contractility due to vasoconstriction in the presence of perivascular adipose tissue. These responses were attenuated in the presence of apelin-13.

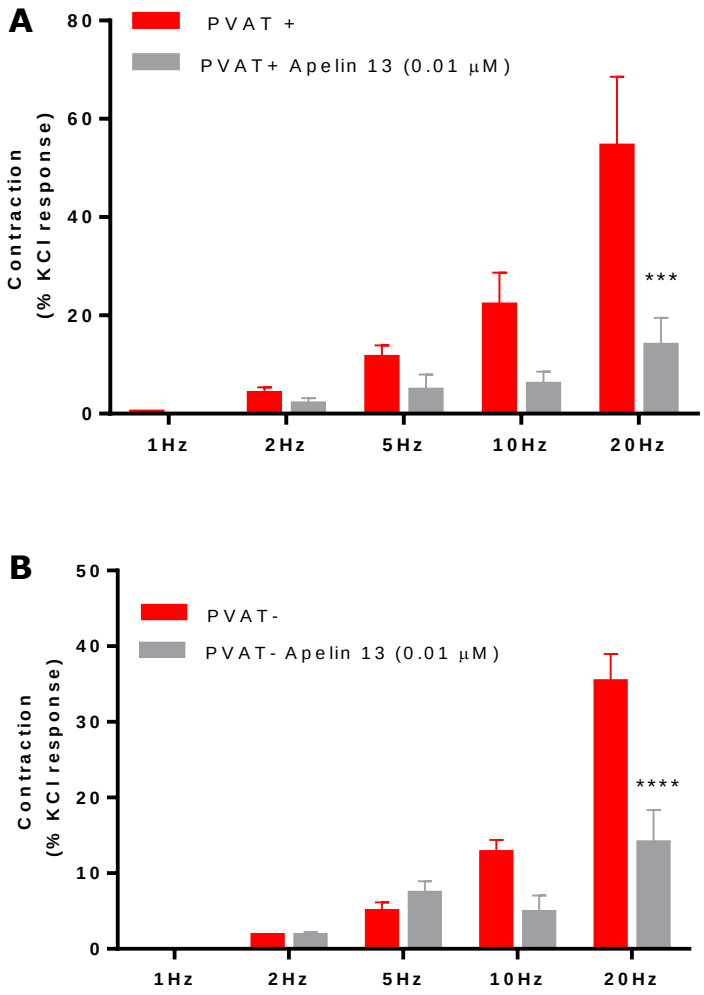

Figure 3.17: The effect of apelin-13 on electrical field stimulation (EFS)-evoked contractions of porcine splenic arteries in (A) the presence of perivascular adipose tissue (PVAT+) and (B) the absence of PVAT (PVAT-). EFS was applied at (1-20 Hz, 50 mA, 1 ms and 10 s). The contractile response at 20 Hz was significantly reduced in both PVAT-intact and PVAT-denuded preparations (***P<0.001, ****P<0.0001, respectively, two-way ANOVA with Bonferroni's post-hoc test) in the presence of apelin-13 with (n=4) and without (n=3) PVAT.

3.3.9 Effect of guanethidine on tone of rat mesenteric arterial beds, rat abdominal aorta, porcine splenic arteries and porcine coronary arteries in the presence and absence of PVAT

Guanethidine is a sympatholytic which may also release catecholamines at sympathetic nerve endings (Kadzielawa, 1962). In PVAT-intact perfused rat MABs pre-contracted with endothelin 1 (ET_A agonist, 0.01-0.1 nM), the addition of guanethidine (5 μM) produced a contractile response of 17.1 ± 3 mmHg (n=7), but this contractile effect was absent (-4.5 ± 2 mmHg) in PVAT-denuded preparations ($P<0.001$, one-way ANOVA with Bonferroni's post-hoc test, n=5) (Figure 3.18). The contractile response to guanethidine in PVAT-intact preparations was abolished in the presence of prazosin (0.3 μM), an α_1-adrenoceptor antagonist ($P<0.0001$, one-way ANOVA with Bonferroni's post-hoc test, n=7) as shown in Figure 3.18.

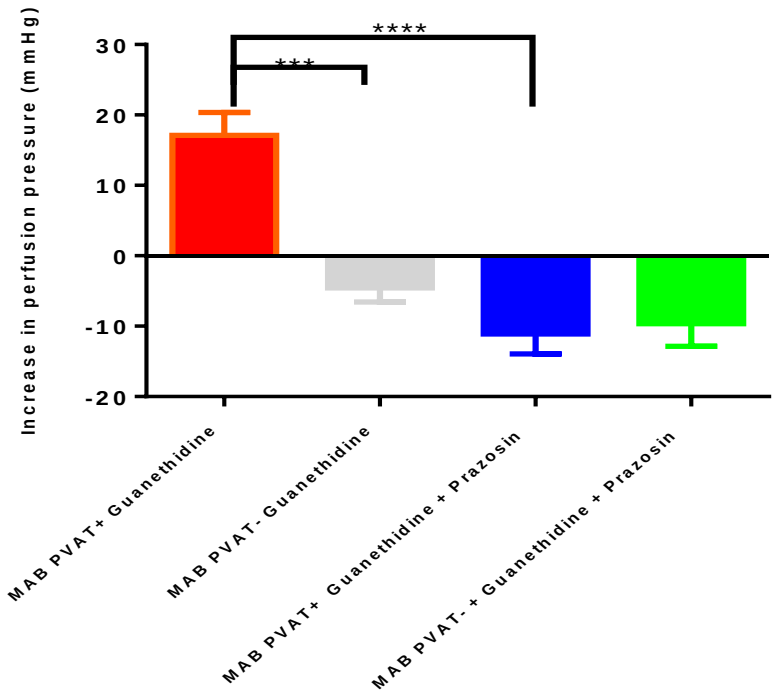

Figure 3.18: The effect of perivascular adipose tissue (PVAT) on the vasocontractile response to guanethidine (5 μM) and the effect of prazosin, an α_1-adrenoceptor antagonist, on this contractile response in rat isolated mesenteric arterial beds (MAB). All preparations were submaximally pre-contracted with endothelin. Guanethidine produced a vasocontractile response in PVAT-intact, but not in PVAT-denuded preparations (***P<0.001, one-way ANOVA with Bonferroni's post-hoc test, n=5-7). The response was reversed by prazosin (****P<0.0001, one-way ANOVA with Bonferroni's post-hoc test, n=7).

In PVAT-intact rat abdominal aortas pre-contracted with endothelin-1 (0.01-0.1 nM), the addition of guanethidine (5 μM) produced a contractile response of 0.46 ± 0.05 g, which was almost absent in PVAT-denuded preparations (0.09 ± 0.07 g) (P<0.001, one-way ANOVA with Bonferroni's post-hoc test, n=13) (Figure 3.19). Prazosin (0.3 μM), an α_1-adrenoceptor antagonist, abolished the guanethidine-induced contraction in PVAT-intact

preparations (P<0.0001, one-way ANOVA with Bonferroni's post-hoc test, n=4-6) as shown in Figure 3.19.

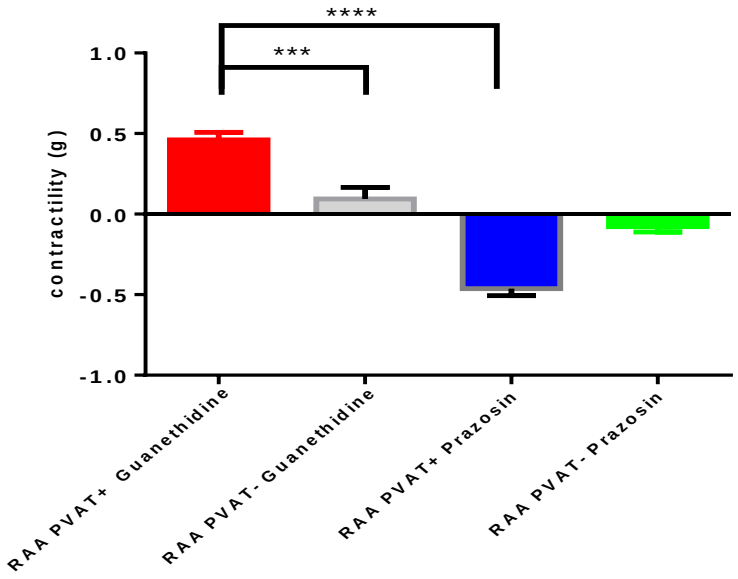

Figure 3.19: The effect of perivascular adipose tissue (PVAT) on the vasocontractile response to guanethidine (5 μM) and the effect of prazosin, an α_1-adrenoceptor antagonist, on this contractile response in rat abdominal aortas (RAA). All preparations were submaximally pre-contracted with endothelin-1 (0.01-0.1 nM). Guanethidine produced a vasocontractile response in PVAT-intact which was greater than PVAT-denuded preparations (***P<0.001, one-way ANOVA with Bonferroni's post-hoc test, n=13). The response was reversed by prazosin (****P<0.0001, one-way ANOVA with Bonferroni's post-hoc test, n=4-6).

The addition of guanethidine (5 μM) in pre-constricted porcine splenic arteries (PSAs) produced contraction in PVAT-intact preparations but not in PVAT-denuded preparations (Figure 3.20). In PVAT-intact PSAs pre-contracted with U46619 (1-5 nM), the addition of guanethidine (5 μM) produced greater contractile responses of 0.56 ± 0.05 g compared to PVAT-denuded PSAs where only a very small contraction was observed (0.15 ± 0.15 g) ($P<0.05$, one-way ANOVA with Bonferroni's post-hoc test, n= 10 and 8, respectively) (Figure 3.21). Prazosin (0.3 μM) abolished the guanethidine-induced contraction in PVAT-intact preparations ($P<0.0001$, one-way ANOVA with Bonferroni's post-hoc test, n= 5-9) as shown in Figure 3.21.

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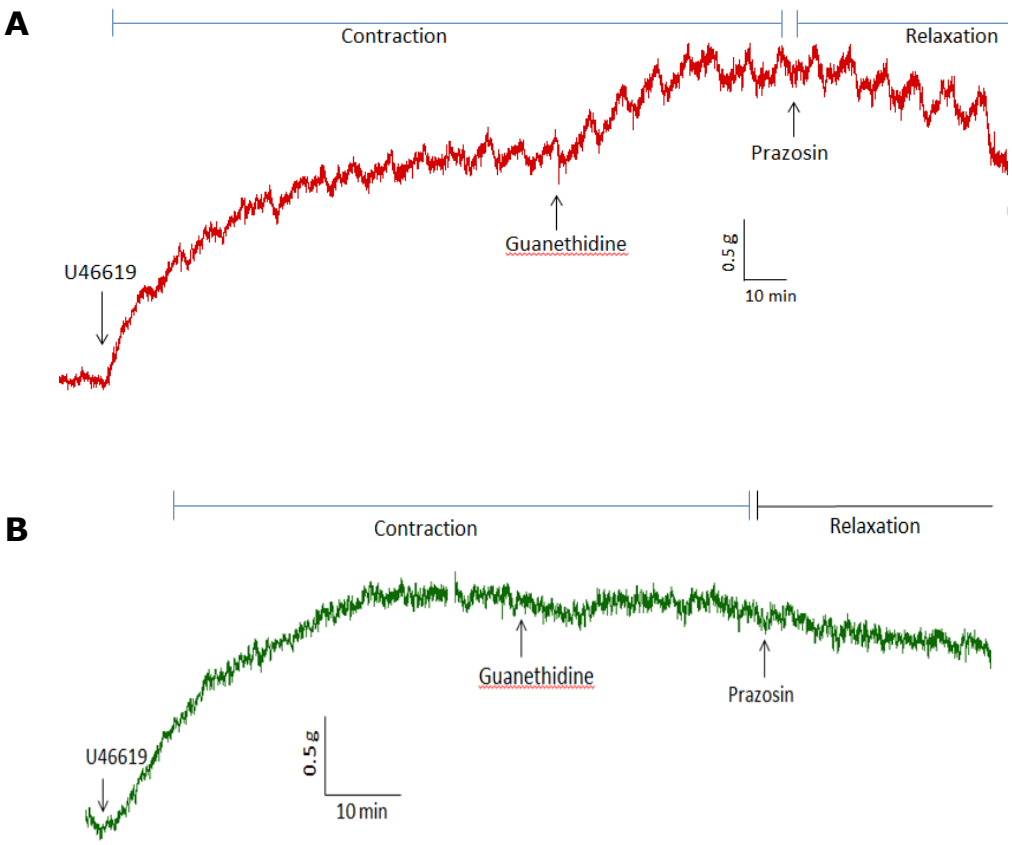

Figure 3.20: Representative traces show that addition of guanethidine (5 μM) in U46619-evoked pre-constricted porcine splenic arteries produced a contractile response in the (A) presence of perivascular adipose tissue (PVAT) but not in the (B) absence of PVAT. The presence of prazosin (0.3 μM) reversed the contractile response in both preparations.

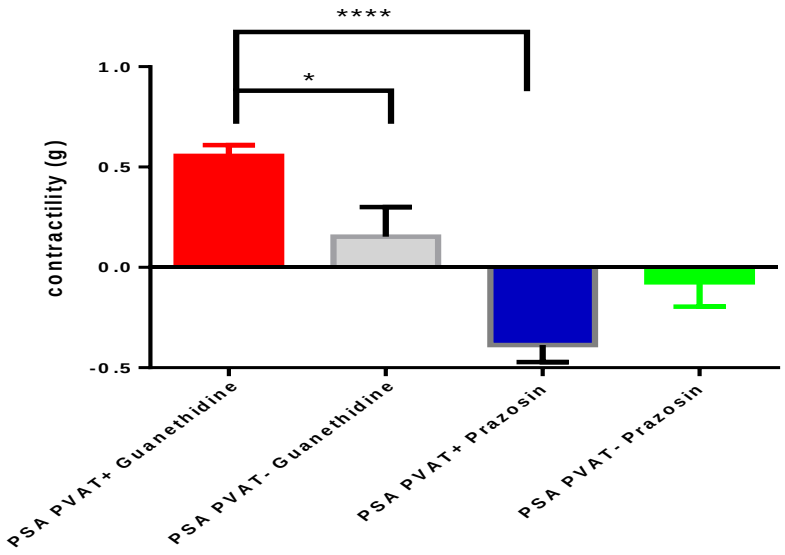

Figure 3.21: The effect of perivascular adipose tissue (PVAT) on the vasocontractile response to guanethidine (5 μM) and the effect of prazosin, an α_1-adrenoceptor antagonist, on this contractile response in porcine splenic arteries (PSA). All preparations were submaximally pre-contracted with U46619 (1-5 nM). Guanethidine produced a vasocontractile response in PVAT-intact arteries which was greater than in PVAT-denuded preparations (*P<0.05, one-way ANOVA with Bonferroni's post-hoc test, n=8-10). The response was reversed by prazosin (****P<0.0001, one-way ANOVA with Bonferroni's post-hoc test, n =5-9).

The addition of guanethidine (5 μM) to PVAT-intact PCA pre-contracted with U46619 (1-5 nM) also induced vasoconstriction (0.59 ± 0.12 g), however, this effect was similar to that in PVAT-denuded preparations (0.60 ± 0.59 g) (P>0.05, one-way ANOVA, n=11-12). Prazosin (0.3 μM) attenuated, but not significantly, the guanethidine-induced contraction in both PVAT-intact and PVAT-denuded preparations (P>0.05, one-way ANOVA, n = 5-6) (Figure 3.22).

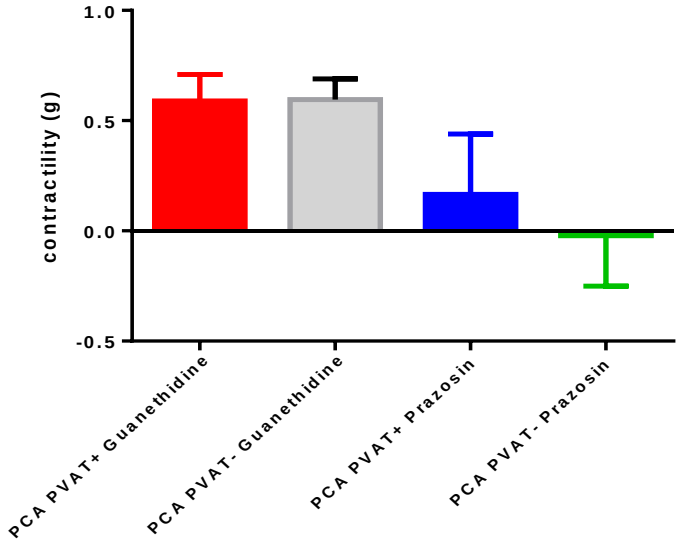

Figure 3.22: The effect of perivascular adipose tissue (PVAT) on the vasocontractile response to guanethidine (5 μM) and the effect of prazosin, an α_1-adrenoceptor antagonist, on this contractile response in porcine coronary arteries (PCA). All preparations were submaximally pre-contracted with U46619 (1-5 nM). Guanethidine produced a vasocontractile response in PVAT-intact arteries which was not different compared to that in PVAT-denuded preparations ($P>0.05$, one-way ANOVA, n=11-12). The response was not significantly reversed by prazosin ($P>0.05$, one-way ANOVA with Bonferroni's post-hoc test, n=5-6).

3.4 Discussion

The present chapter shows: (1) the existence of sympathetic nerves within PVAT of rat MABs; (Straub et al.) PVAT modulates neurogenic responses in rat MABs, in which it enhanced sympathetic neurogenic contraction; (Dick et al.) the data with candesartan in rat MABs appears to indicate a possible involvement of endogenous angiotensin II in PVAT-enhanced neurogenic

contractile responses, consistent with previous findings in the rat superior mesenteric artery (Lu et al., 2010); (4) The data with guanethidine and prazosin in rat MABs, rat aortas and PSA may indicate that sympathetic nerves innervate PVAT in blood vessels in different species, and these nerves are recruited during EFS, accounting for the larger sympathetic neurogenic contractile responses in the presence of PVAT. Collectively, these data provide vital insight into the distribution and *in vitro* role of PVAT-derived sympathetic nerves.

3.4.1 The presence of sympathetic nerves within PVAT of MABs

Immunofluorescence staining of rat MABs in the preparations without PVAT showed the presence of extensive innervation with perivascular sympathetic nerves where fibres immunoreactive to TH were identified. This observation is consistent with previous studies which showed that TH-containing nerves densely innervate blood vessels of MABs; the TH-immunoreactivity was eliminated by cold-storage denervation and 6-hydroxydopamine (adrenergic toxin), but not capsaicin (CGRP depletor) demonstrating that these fibres are sympathetic adrenergic nerves (Hobara et al., 2004a; Kawasaki et al., 2009; Kawasaki et al., 2011). In the presence of PVAT, immunofluorescence staining showed the presence, but with sparser innervation, of PVAT-derived sympathetic nerves, where fibres immunoreactive to TH were recognized. Co-staining with PGP 9.5 revealed the sympathetic nerves within PVAT. In PVAT-intact preparations, the longitudinal sections contain both arteries and PVAT, with DAPI was reactive to both nerves and fat cells.

Sympathetic nerves have been detected immunohistochemically in other fat depots such as inguinal fat pads of wild mice (Zeng et al., 2015). Transneuronal retrograde tract tracer, pseudorabies virus, a common technique to detect sympathetic neuroanatomy in fat, has revealed the existence of these nerves in fat by labelling the sympathetic nerves outflow from brain to white adipose tissue (WAT) in laboratory rats and Siberian hamsters (Bamshad et al., 1998; Cao et al., 2004; Oldfield et al., 2002). Using the same strategy, the same groups also showed the presence of sympathetic nerves in brown adipose tissue (BAT) of the brain of Siberian hamsters (Bartness et al., 2010). Functional studies including the application of a "unilateral denervation" approach in a few species, in which one of a pair of WAT or BAT pad is denervated, with its contralateral mate serving as a within-animal neurally intact control, also revealed sympathetic innervation in both WAT and BAT (Bartness and Song, 2007; Bartness et al., 2010; Desautels and Dulos, 1990; Foster et al., 1982).

Recently, the crosstalk between sympathetic nerves and fat is under intense investigation as it has been identified to play a role in pathological conditions including hypertension and obesity (Hall et al., 2010; Xiong et al., 2014). Despite this, knowledge of sympathetic neurotransmission in PVAT is still at its infancy. Although there are a few reports that suggest the involvement of PVAT-derived sympathetic nerves in vasoregulation based on functional evidence (Gao et al., 2006; Lu et al., 2010a), the presence of sympathetic nerves in PVAT by immunofluorescence has only recently been demonstrated in PVAT of mice mesenteric arteries (Bulloch and Daly, 2014). The present study provides additional evidence for the existence of sympathetic nerves within PVAT, and using immunofluorescence demonstrates the presence of the nerves in PVAT of another species. This study also clearly shows that sympathetic nerves

innervate conduit (SMA) and different orders of small mesenteric arteries of rat.

3.4.2 Effects of PVAT on MAB contractile responses to EFS

The present study investigated the role of PVAT in perivascular sympathetic neurotransmission. EFS elicited frequency-dependent (4-32 Hz) contractile responses which were abolished by guanethidine, a sympathetic nerve blocker, indicating an involvement of sympathetic nerves, consistent with previous studies (McGregor, 1965; Shatarat et al., 2014). The principal neurotransmitter involved in these EFS-induced contractions under basal tone conditions is noradrenaline (NA) since the responses have been shown to be abolished by prazosin, an α_1-adrenoceptor antagonist, although in mesenteric artery preparations at raised tone, the additional involvement of ATP as a sympathetic co-transmitter can be demonstrated (Pakdeechote et al., 2007). Contractility studies were carried out to investigate the effect of PVAT on responses to EFS. EFS elicited frequency-dependent contractile responses due to vasoconstriction in both PVAT-intact and PVAT-denuded preparations. Notably, in the absence of PVAT there was a markedly reduced vasoconstriction, which is in agreement with the studies of Gao et al. (2006), Lu et al. (2010a) and Ayala-Lopez et al. (2014) using rat isolated mesenteric artery rings.

Soltis and Cassis were the first to describe the effects of PVAT on perivascular neurotransmission when they showed that removal of PVAT abolished contractions to EFS in rat aortic rings (Soltis and Cassis, 1991). Gao et al. (2006) suggested that the presence of PVAT augments EFS-

induced vasoconstriction through the release of superoxide by PVAT and activation of tyrosine kinase and the mitogen-activated protein kinases/ extracellular signal-regulated kinases (MAPK/ERK) pathway. This group suggested that EFS releases NA from perivascular nerves to mediate vasoconstriction and simultaneously enhances superoxide generation from PVAT and arteries. The rise of superoxide level potentiates NA-induced vasoconstriction and thus results in a greater contraction of PVAT-intact mesenteric arteries than PVAT-denuded mesenteric arteries (Gao et al., 2006). The study neither includes nor excludes the presence of sympathetic nerves in PVAT. On the other hand, Ayala-Lopez et al. (2014) suggested that PVAT components are independent of sympathetic nerves, based on the EFS-evoked contractile response in rat aorta which was not different in the absence and presence of PVAT. The authors suggested that PVAT contains a functional pool of catecholamines that potentially regulates vascular tone (Ayala-Lopez et al., 2014). This finding is in contrast to the seminal study by Soltis and Cassis (1991), also in rat aorta, which showed that EFS resulted in no response in PVAT-denuded aorta but elicited frequency-dependent contraction in intact vessels. This discrepancy probably arises as a result of experimental approach; Ayala-Lopez et al. (2014) carried out the EFS-evoked contraction experiments under raised tone conditions while Soltis and Cassis (1991) conducted the EFS experiments under basal tone conditions. At raised tone, there is a possibility that EFS caused maximal, or close-to-maximal, vessel contraction. Therefore, any additional recruitment of sympathetic nerves from PVAT did not cause any further contractile response. The authors also demonstrated that the removal of the celiac ganglion, a sympathetic source for superior mesenteric arteries, did not reduce concentration-dependent contraction to tyramine in the present of PVAT (Ayala-Lopez et al., 2014). However, the study did not provide an explanation as to whether

adipocytes of PVAT are capable of releasing catecholamines and what stimulates their release.

The majority of the literature identifies PVAT as an important contributor to vasculature relaxation (Aghamohammadzadeh et al., 2012; Gollasch, 2012; Szasz and Webb, 2012) in vessels including mesenteric arteries (Galvez et al., 2006; Verlohren et al., 2004) and aorta (Lohn et al., 2002; Soltis and Cassis, 1991). Various mechanisms and factors have been postulated on how PVAT promotes vasorelaxation, including the involvement of PVAT-derived vasorelaxing compounds and activation of K_v channels (Gollasch, 2012; Lohn et al., 2002; Verlohren et al., 2004). In contrast, the EFS experiments in the present study show that the contractile response in PVAT-intact MABs is significantly greater than in PVAT-denuded MABs. Although the reason for this difference is still unclear, there are a number of possibilities. The simplest explanation is that sympathetic nerves are present in PVAT, and so by removing the PVAT, the nerves also were removed. Another explanation is that substances released from PVAT affect, pre-junctionally, the release of neurotransmitter from the perivascular sympathetic nerves; specifically, they augment neurotransmitter release. A possible candidate is angiotensin II, which has been shown to cause greater potentiation of contractile responses of the rat mesenteric bed to EFS than to exogenous noradrenaline, suggesting possible pre-junctional effects (Kawasaki et al., 1982; Malik and Nasjletti, 1976). Lu et al. (2010a) suggested an involvement of angiotensin II in PVAT-induced augmentation of contractile responses to EFS in rat mesenteric arteries, but they did not investigate whether the effect was pre- or post-junctional (Lu et al., 2010). Our experiments investigating the possible involvement of angiotensin II are described below. The present

study did not specifically quantify the density of the sympathetic nerves on adventitia or in PVAT, however, the immunofluorescence data showed that the nerves appeared to be sparser in PVAT. Since the removal of PVAT had a great impact on neurogenic contractile responses, a third possibility is that the process of removing the PVAT causes damage to the function of the perivascular nerves. Further study, which can include viral tract tracing (as discussed in Chapter 7) is warranted to understand this phenomenon and to elucidate the role of PVAT-derived sympathetic nerves.

3.4.3 The effect of PVAT on responses to sympathetic agonists in MABs

Alpha$_1$ (α_1)-adrenoceptors are G protein-coupled receptors. There are three α_1 adrenoceptors subtypes: α-$_1$A, -$_1$B and -$_1$D, all of which signal through the $G_{q/11}$ family of G-proteins (Peng and Chang, 2000). α_1-adrenoceptors are found on blood vessels and their activation causes vasoconstriction. The vasoconstriction response in mesenteric arteries has been shown to be mediated by α_{1D} and possibly α_{1B}-adrenoceptors (Hussain and Marshall, 2000). In the present study, methoxamine, an α_1-adrenoceptor agonist, was used to investigate contractility of blood vessels in PVAT-intact and PVAT-denuded preparations in MABs. Methoxamine elicited concentration-dependent vasoconstrictor responses which were not different between preparations with and without PVAT. The results indicate that α_1-adrenoceptors on smooth muscle layer remain intact in both preparations and that the removal of PVAT did not damage blood vessels of mesenteric arteries and therefore testify that the reduced contractile responses to EFS was unlikely due to vascular smooth muscle damage.

Most of the literature reports that because of the anticontractile effect of PVAT, the removal of PVAT leads to a significant increase in vascular contractility in response to exogenous agonists (Galvez et al., 2006; Lohn et al., 2002; Verlohren et al., 2004). Thus, it is perhaps surprising that we did not also observe an increase in contractility in PVAT-denuded preparations. However, the effect of PVAT on contractions to exogenous agonists seems to depend on the agonist. In rat mesenteric arteries, the contractile responses generated to KCl and U46619 were not different in the absence and presence of PVAT, but responses to 5-HT, endothelin and phenylephrine were reduced (Verlohren et al., 2004; Galvez et al., 2006; Gao et al., 2006). Verlohren et al. (2004) attributed the lack of effect of PVAT on contractions to U46619 to the lesser involvement of K^+ channels in these responses (compared to responses to 5-HT, endothelin and phenylephrine), and showed that the PVAT-intact mesenteric arteries were more hyperpolarised. In rat aortic rings, Soltis and Cassis (1991) showed that contractions to KCl and phenylephrine, an α_1-adrenoceptor agonist, were similar in the absence and presence of PVAT, but the sensitivity of contractions to NA were reduced in the presence of PVAT (which they attributed to increased uptake of the NA by the PVAT).

In the present study, I also investigated the effect of an indirect sympathomimetic agent, tyramine, in the presence and absence of PVAT in MABs. It has been reported that tyramine releases NA from sympathetic neurons to exert effects including vasoconstriction and increases in BP (Trendelenburg, 1972). Thus, in principle, the responses should have been influenced by the presence or absence of PVAT in the same way as those for guanethidine; in other words, a greater tyramine contractile response should have been observed in preparations with PVAT. In fact, tyramine produced small and very variable concentration-dependent contractions,

which were not different between PVAT-intact and denuded preparations. An earlier study by Elliot et al. (1989) observed a contractile response to tyramine in rat MABs, but they used a single concentration (as we did with guanethidine), which may be more appropriate for observing vasocontraction when it is mediated secondary to the release of sympathetic neurotransmitters, as multiple doses of tyramine can lead to sympathetic nerves desensitization. The small responses to tyramine observed in the present study are consistent with a recent study by Anwar et al. (2012) who found that tyramine had no contractile effect when applied as doses in rat MABs. This result is not in agreement with previous studies which observed that contractile responses of rat aortic and mesenteric arteries rings to tyramine were reduced in rings with PVAT removed (Ayala-Lopez et al., 2014; Soltis and Cassis, 1991). The discrepancy between the present study and previous studies may be accounted for by differences in tissue used and experimental approaches.

3.4.4 Effects of replacing PVAT on vascular responsiveness

To obtain further information about the possible role of PVAT in mediating vascular function, PVAT was removed and the dissected PVAT was placed back on denuded vessels of MABs. EFS elicited frequency-dependent contractile responses in the first response curve, however, replacing dissected PVAT on PVAT-denuded preparations abolished EFS-evoked vasoconstriction. This suggests that PVAT releases transferrable vasorelaxing and/or inhibitory factors and is consistent with other studies which were carried out the in rat aorta (Dubrovska et al., 2004; Lohn et al., 2002; Takemori et al., 2007) and rat mesenteric arteries (Verlohren et al., 2004). The amount of dissected PVAT replaced on PVAT-denuded

vessels has been shown to play a role in mediating the degree of vasorelaxation, in which greater amounts of PVAT generated larger relaxation of serotonin pre-constriction vessels (Dubrovska et al., 2004; Verlohren et al., 2004). Takemori et al. (2007) demonstrated that transfer of solution incubated with PVAT-intact aorta to PVAT-denuded aorta led to a significant increase in the relaxation response in wild type mice but not in lipoatrophic A-ZIP/F1 transgenic mice aorta, therefore suggesting that a vasorelaxing factor can only be produced from perivascular fat of wild type mice.

Although it seems like the data with replaced PVAT contradicts the present EFS experiment with intact PVAT (see Section 3.4.2), these observations might be due to an alteration of sympathetic nerve function when they were removed from the vessels. There are few possible explanations. First, there is possibility that the signalling connection between sympathetic nerves at the adventitia and sympathetic nerves within PVAT was modified and resulted in a reduced sympathetic response in the second FRC. The immunofluorescence data here as well as in other studies showed that there were still substantial amount of intact sympathetic nerves on the adventitia of rat mesenteric arteries in which PVAT had been removed as discussed above (Gradin et al., 2003; Ralevic et al., 1991). Hence, this observation indicates the importance of interactions between sympathetic nerves at the adventitia and in PVAT in regulating their normal function. Second, it is possible that the PVAT-derived sympathetic nerve network has been altered and loss of inhibitory control has led to enhanced release of vasodilator adipokines (Rayner and Trayhurn, 2001), thus resulting in attenuation of the second FRC. Third, the dissected PVAT was left in cold Krebs'-Henseleit solution for 30 min which might lead to deterioration of sympathetic nerve fibres in the dissected PVAT. These results also suggest

that PVAT-derived vasorelaxing factors could be conserved, while sympathetic nerves or their interaction with PVAT-derived compounds was altered by removing PVAT. The data also indicates that the effect of PVAT-derived vasorelaxing compounds may become dominant in a circumstance where PVAT-derived sympathetic nerves are altered.

3.4.5 Involvement of angiotensin II in neurogenic vasoconstriction responses in rat MABs with the presence of PVAT

PVAT is reported to contain substantial levels of angiotensinogen, angiotensin 1-converting enzyme mRNA, and abundant angiotensin II (Engeli et al., 2003). Angiotensin II has been identified to play a role in PVAT-mediated potentiation of vascular contraction to perivascular neuronal stimulation (Lu et al., 2010a; Soltis and Cassis, 1991). Therefore, the present study investigated the involvement of angiotensin II derived from PVAT in the greater contractile response to EFS of PVAT-intact mesenteric arteries. In these particular experiments, candesartan but not losartan, both angiotensin II receptor antagonists, significantly reduced the contractile responses to EFS in the mesenteric arteries preparations with PVAT. These results indicate a possible involvement of angiotensin II in increasing the contractile response. The reproducibility tests that were conducted as controls for the losartan and candesartan studies showed that the responses were comparable between the first and the second EFS response curves generated consecutively in the same preparation. Hence, these data appear to include a possible involvement of angiotensin II in the EFS-increased contractile response in the presence of PVAT. In the present study, the effect of vehicle (ethanol) for angiotensin II antagonists was not

investigated. The concentration of ethanol used in angiotensin II receptor antagonist experiments was 0.001 % v/v. A previous study showed ethanol (0.1 %) enhanced the electrically-evoked contractile response (Shatarat, 2011), however, the concentration of ethanol used was 100 folds higher than the concentration of ethanol in the present study. In contrast, Sangsiri et al. (2013) demonstrated that ethanol at the highest concentration (0.01% v/v) did not affect neurogenic or agonist-induced blood vessel contraction in rat mesenteric arteries. Another study showed that effect of ethanol vehicle in rats small mesenteric arteries (up to 0.6% v/v final concentration in myograph bath) had a negligible relaxant effect (Hoi and Hiley, 2006). Based on the previous data, although it is less likely that the concentration of vehicle used in the current study affected the present data, however, future study with specific investigation on ethanol vehicle effect is warranted.

There is an increasing interest among researchers in comparing the properties of angiotensin II type 1 receptor antagonists including losartan and candesartan. Gschwend et al. (2003) showed that increased myogenic constriction in mesenteric resistance arteries from rats with chronic heart failure was fully reversed after candesartan (100 nM) and losartan (10 μM) treatment. Another study showed losartan displayed competitive antagonism with a pA_2 value of 6.50 while candesartan exhibited non-competitive antagonism with a pA_2 value of 8.71 in rat tail artery (Pinheiro et al., 2002). In rat mesenteric arteries, losartan demonstrated significant inhibition of exogenous angiotensin II-enhanced electrically-evoked sympathetic neurotransmission (Balt et al., 2001). In contrast, losartan has been reported to be ineffective in blocking the pre-junctional effect of angiotensin II in rabbit vas deferens and rat left ventricle (Moura et al.,

1999; Trachte and Heller, 1990). In hypertensive patients, candesartan was found to be superior in lowering BP compared to losartan (Bakris et al., 2001; Gradman et al., 1999; Grosso et al., 2011) although Kjeldsen et al. (2010) showed the effects of these drugs were comparable. These findings clearly show specific properties of each agent may explain the divergent experimental outcomes. In addition, Michel et al. (2013) reported that the chemical structure of each angiotensin II receptor antagonist is unique and this has implications for a wide range of physicochemical, pharmacological, and pharmacokinetic properties.

The outcomes of the present study are broadly in agreement with the findings of Lu et al. (2010a). This group, as well as the present study, produced two main pieces of evidence to support the involvement of angiotensin II in enhanced EFS-mediated neurogenic contractile responses. Firstly, in both studies neurogenic contraction was enhanced in PVAT-intact tissue, but not in PVAT-denuded tissue, thus suggesting that PVAT contains a vasocontractile mediator, or a mediator which can augment sympathetic neurogenic contraction. Secondly, in the present study candesartan, an angiotensin II receptor antagonist, attenuated EFS contraction in PVAT-intact tissue, but not that in PVAT-denuded tissue. Lu et al. (2010a) also found that exogenous angiotensin II elicited an increased contractile response in PVAT-denuded tissue compared to PVAT-intact tissue, suggesting that angiotensin II was indeed involved in augmenting EFS contraction in MA of rats. These findings support a previous study by the same group showing that PVAT promoted vasoconstriction to EFS through superoxide production (Gao et al., 2006) as angiotensin II is identified as a potent stimulator for nicotinamide adenine dinucleotide phosphate-oxidase NAD(P)H oxidase to produce superoxide (Rahman et al., 2004).

Conversely, lipoatrophic A-ZIP/F1 transgenic mice aorta showed an increased contractile response mediated by angiotensin II type 1 receptors compared to wild type mice (Takemori et al., 2007). The reason why the present result was different than Takemori et al. (2007) might be due to the different tissues used, in which Takemori et al (2007) used aorta of mice, while the present study used MAB of rat. In the present study, losartan had no significant effect on the sympathetic neurogenic contractile responses, probably because of differences in chemical structure, affinity, allosteric and/or potency compared to candesartan.

3.4.6 Effect of methyl palmitate and apelin-13 on neurogenic vasocontractile responses in porcine splenic arteries (PSA)

In the present study, it has been shown that the presence of PVAT enhanced EFS-induced vasocontractile responses in MABs (see Section 3.4.2), next the effect of PVAT on sympathetic neurotransmission in another vessel from a different species was further investigated. EFS experiments in isolated PSA were carried out as this vessel is well known to possess robust sympathetic innervation. Similar to my observation in MABs, removal of PVAT significantly attenuated EFS-evoked neurogenic vasoconstriction in PSA. Further steps were taken to investigate the modulation of identified PVAT-derived metabolites on sympathetic neurotransmission. In the present study, the effects of exogenous PVAT-derived metabolites, methyl palmitate and apelin-13 on sympathetic neurotransmission in PSA were investigated. Methyl palmitate is a vasodilator which acts by opening voltage-dependent potassium channels on smooth muscle cells, and has recently been detected in PVAT of rat aorta (Lee et al., 2011). The relaxation effect of methyl palmitate has also

been reported in retina (Lee et al., 2010). However, a more recent study showed that exogenous methyl palmitate did not relax *in vitro* contraction of human pregnant myometrium (Crankshaw et al., 2014). The present study showed that exogenous methyl palmitate had no effect on sympathetic neurogenic contractions in PSAs in the presence and absence of PVAT, although there was a trend for the contractile responses to be reduced with increasing concentration of methyl palmitate. To the best of my knowledge, the present study provides the first data on the effect of methyl palmitate on sympathetic activation in vasculature. It is unclear whether this trend for a reduction in the EFS-mediated neurogenic vasocontractile response is a pre-junctional effect or due to functional antagonism as methyl palmitate is known to possess vasorelaxant actions.

In the present study, the effect of vehicle dimethyl sulphoxide (DMSO) was not investigated. The concentrations of DMSO used in the methyl palmitate experiments were 0.00001 % v/v and 0.0001 % v/v. A study by our group previously found that DMSO (0.15% of final bath volume) did not cause a vascular response (Hopps, 2013). Previous studies demonstrated that DMSO at the final concentration (0.1 % v/v and 0.001 % v/v) did not affect electrically-evoked contractions of rat small mesenteric arteries and rabbit isolated pulmonary arteries respectively (Jackson et al., 2002; Kun et al., 2014). Based on the previous data, although it is less likely that the concentration of DMSO used in the current study affected the present data, however, future study to investigate the DMSO vehicle effect is warranted.

Apelin can be classified as an adipokine which has vasorelaxing and vasocontractile properties (see general introduction). Little is known about the effect of apelin on sympathetic nerve activity. Apelin-13 was shown to enhance mean arterial pressure, plasma NA and arginine vasopressin in the

paraventricular nucleus of spontaneously hypertension rats (SHR) compared to Wistar-Kyoto rats (WKY) (Zhang et al., 2014). Hence, the group suggested that apelin-13 in the paraventricular nucleus contributes to hypertension via sympathetic activation and arginine vasopressin release in SHR (Zhang et al., 2014). Paraventricular nucleus is one of the most important autonomic control centres in the brain (Ferguson et al., 2008) and regulates sympathetic nerve discharge (SND) frequency components (Kenney et al., 2003). Zhang et al. (2014) also suggested that the mechanism involves N-methyl-D-aspartate receptors (NMDAR) and non-NMDAR in the paraventricular nucleus. Consistent with Zhang et al. (2014), another study on the effect of apelin-13 on sympathetic activity by Masaki et al. (2012) showed that microinjection of apelin-13 into the paraventricular nucleus increased brown adipose tissue sympathetic nerve activity. In the present study, apelin-13 reduced EFS-induced vasocontractile responses in both PVAT-intact and PVAT-denuded PSAs. Although EFS elicited frequency-dependent vasocontractile responses may suggest that the responses were neurogenic, however, further experiments with the presence of tetrodotoxin and NA release analysis can support the present data, that these vasoconstrictions were nerve-mediated. Apelin inhibited the electrically evoked vasoconstrictions, but it is unclear whether this involves a pre-and/or post junctional mechanism. In human mammary arteries, it has been shown that under raised tone condition, apelin acts as a vasodilator (Maguire et al., 2009), and a study by Tatemoto et al. (2001) showed that apelin-12, apelin-13 and apelin-36 reduced the blood pressure of rats via a nitric oxide-dependent mechanism, which raises the possibility that the action of apelin involves a post junctional mechanism. To investigate the possible involvement of pre-junctional mechanisms, further study measuring NA release under basal tone and during EFS activation, in the absence and presence of apelin is required. In the current study, apelin

had no observable effect on the basal tone. Future study should be carried out to explore the effect of apelin in pre-contracted arteries.

3.4.7 Effect of guanethidine on rat mesenteric arteries and aorta and porcine splenic and coronary arteries with and without PVAT

The addition of guanethidine to pre-constricted rat MABs, rat aortas and PSA produced a robust contractile response in PVAT-intact preparations, but had little or no effect in PVAT-denuded preparations. It was not my first intention to use guanethidine as a contractile agent, but rather to use it as sympathetic blocker in my experiments. Thus, these were unexpected findings which appear to support the present immunofluorescence data on the expression of sympathetic nerves within PVAT. An earlier study suggested that the sympathetic atony after administration of guanethidine was due to the loss of catecholamines from the sympathetic nerve endings or to a possible blocking action on the adrenoceptors (Maxwell et al., 1960). It has been demonstrated that guanethidine decreases the noradrenaline content of heart muscle, intestine and spleen of rabbits and rats as well in the heart and aorta of dogs (Butterfield and Richardson, 1961; Cass et al., 1960; Cass and Spriggs, 1961). Kadzielawa (1962) found that the early effects of guanethidine are mainly due to the release of endogenous catecholamines. Thus, in the present study, the contractile response to guanethidine could be due to the release of catecholamines from sympathetic nerves; the contractile response was greater with PVAT across different vessels and in different species, consistent with the expression of sympathetic nerves within PVAT. However, further experiments such as with the application of tetrodotoxin, a potent

neurotoxin, need to be carried out to confirm this hypothesis, that the guanethidine-induced vasoconstriction is nerve-related. In addition, analysis of NA release into Krebs'-Henseleit during vasoconstriction before and after guanethidine treatment, using chromatography techniques, also can be carried out to determine that the response was neurogenic.

The guanethidine-produced contractile response observed in PVAT-intact preparations was reversed by prazosin, an α_1-adrenoceptor antagonist, in PVAT-intact preparations of rat MABs, rat aortas and PSA, and there was a trend for prazosin to reduce the contraction to guanethidine in the PCA. This observation indicates the contractile responses were due to α_1-adrenoceptors activation by NA, released by sympathetic nerves activation and also is in line with the study of Kadzielawa (1962), which indicates that guanethidine may release catecholamines at sympathetic nerve endings. The data with prazosin suggest that the catecholamine that is involved in the guanethidine-generated contractile response is NA, which acts predominantly on α_1-adrenoceptors on the vascular smooth muscle to cause vasoconstriction. In the present study, guanethidine had a comparable effect on the PCA in the absence and presence of PVAT, perhaps because metabolic regulation is more dominant than autonomic nerve control in this vessel.

The immunofluorescence and guanethidine data strongly indicate the presence of sympathetic nerves in PVAT of MABs. However, there was a mismatch between the two experimental approaches with regard to the density of sympathetic innervation that they suggest exists within PVAT. The immunolocalisation data suggests that the majority of the sympathetic nerves are perivascular (sympathetic innervation was sparse in PVAT), while the guanethidine data places most of the sympathetic nerves within

PVAT (contraction was robust only in the presence of PVAT). It is possible that stores of catecholamines exist within PVAT, as suggested in a recent study by Ayala-Lopez et al. (2014) as discussed above. Previous research has shown the potential expression of α_1-adrenoceptors within PVAT (Lohn et al., 2002; see review by Bulloch and Daly, 2014). If so, perhaps the NA that is released by guanethidine from sympathetic nerves within PVAT, acts on α_1-adrenoceptors expressed on adipocytes to cause a release of NA from PVAT and amplification of the vasocontractile response. However, contractile responses to methoxamine were not different between preparations with and without PVAT (discussed above), suggesting that this mechanism may not contribute significantly. Further studies which can include determination of nerves density in both PVAT-intact and PVAT-denuded using immunofluorescence and western blotting techniques and tetrodotoxin pre-treatment experiments are needed to understand the mechanism involved in guanethidine-induced contractions of vascular preparations with PVAT.

3.5 Conclusions

Sympathetic nerves are present in PVAT of rat MABs and these nerves play a significant role in modulating *in vitro* MABs vascular tone. Activation of sympathetic derived PVAT enhanced neurogenic vasoconstriction in rat MABs accounting for the greater contractile responses in the presence of PVAT. PVAT-derived angiotensin II appears to be involved in the enhanced sympathetic neurogenic contractile responses in the presence of PVAT. This study on PVAT-derived sympathetic nerves adds new perspectives to the understanding of PVAT physiology in the vascular system since the majority of studies have focussed on the anti-contractile role of PVAT.

Chapter 4

Expression and vasomotor role of sensory nerves in PVAT

4.1 Introduction

In addition to the sympathetic system as the main neurogenic vasoregulator, the vascular system is also influenced by nonadrenergic noncholinergic (NANC) nerves (Burnstock, 2007; Lundberg, 1996). It has been shown that EFS of pre-constricted preparations from various species and vascular beds causes vasodilatation (Bevan and Brayden, 1987) which can be abolished by tetrodotoxin but not by a β-adrenoceptor antagonist or muscarinic cholinoceptor antagonist (Kawasaki et al., 1988; Scott et al., 1996; Simonsen et al., 1999) and thus indicates the involvement of NANC nerves. In the rat mesenteric arterial bed, treatment with $CGRP_{8-37}$, a CGRP receptor antagonist, CGRP receptor desensitization and pre-treatment with capsaicin (depletes CGRP from primary afferent nerves) result in abolishment of NANC-mediated vasodilatation (Han et al., 1990; Kawasaki et al., 1988), thus indicating an involvement of CGRPergic nerves. The capsaicin sensitivity of these nerves identifies them as "capsaicin sensitive sensory nerves" (Holzer, 1988; Maggi, 1991; Maggi and Meli, 1988). CGRP has been shown to be the main vasodilator neurotransmitter of sensory nerves in the rat MAB, and it works antagonistically with NA to regulate rat MAB vascular tone (Kawasaki et al., 2011).

It is now well established that perivascular adipose tissue (PVAT) has an important role in the vascular system as it releases vasoactive metabolites including vasorelaxants such as adiponectin, leptin and hydrogen sulfide. These substances exert their effects in a paracrine manner and can cause vasodilatation by acting on targets located on either smooth muscle cells or endothelial cells (Szasz and Webb, 2012). Despite extensive research on PVAT, the possible expression of sensory nerves in PVAT, their interaction

with PVAT-derived vasoactive compounds, and role in controlling vascular tone remain unexplored.

Sensory nerves are abundant in white adipose tissue (WAT) (Bartness and Bamshad, 1998; Bartness and Song, 2007), but their expression in PVAT has not been investigated. Sensory innervation of WAT was first shown neuroanatomically using an anterograde tract tracer, true blue, which resulted in labelled neurons in the dorsal root ganglia (DRG) (Fishman and Dark, 1987). Further experiments including histology and a transneuronal viral tract tracer, the H129 strain of herpes simplex virus-1 (HSV) (Rinaman and Schwartz, 2004), convincingly corroborated the existence of CGRP and substance P containing nerves within WAT. Brown adipose tissue (BAT) also has significant sensory innervation, as revealed by immunohistochemical staining for CGRP and substance P (Giordano et al., 1996; Norman et al., 1988). Interestingly, more than a decade after sensory nerves were discovered in WAT and BAT, the function of the nerves remains elusive in both types of adipose tissues.

The diversity of mediators and roles of adipose tissue depending on the fat depot has been reported (Bjorndal et al., 2011; Claria et al., 2013). PVAT appears to be anatomically and physiologically distinct from other fat depots (as discussed in the Introduction). The aims of the present study were to investigate the existence of sensory nerves within PVAT of mesenteric arteries and to examine the effect of PVAT on sensory neurotransmission in resistance and conduit arteries (2OMA and SMA).

4.2 Materials and methods

4.2.1 Rat mesenteric arterial bed preparation

Male Wistar rats weighing 180-220 g were killed by stunning and exsanguination. Perfused mesenteric arterial beds were set up as described in Section 2.1.

4.2.2 Perfusion protocol

MABs in the presence and absence of PVAT were perfused with Krebs'-Henseleit solution (Section 2.1) containing guanethidine (5 μM), a sympathetic nerve blocker, for 30 min, and then methoxamine (1- 10 μM) was added to pre-constrict the preparations (to 30-70 mmHg above baseline). Two consecutive response curves to EFS, at 1-12 Hz, 60 V, 0.1 ms and 30 s, were constructed with a 30 min interval between them, in methoxamine-pre-constricted MABs, as described in Section 2.2.2. The effect of PVAT on relaxation of the mesenteric arteries was further investigated in methoxamine-pre-constricted MABs, in the presence of guanethidine (5 μM), using various agonists and antagonists added either into the perfusate (as cumulative concentrations): capsaicin (0.01-30 μM), or as bolus injections (50 μl doses; injected into neoprene rubber tubing proximal to the preparation): capsaicin ($1.5x10^{-12}$-$5x10^{-10}$ moles) and exogenous CGRP ($1.5x10^{-13}$-$1.5x10^{-11}$ moles).

4.2.3 Isometric tension recording in conduit and resistance arteries

Rings of mesenteric arteries (superior and second order) (approximately 2 mm in length) were obtained as described in Section 2.1 and were mounted in chambers (5 ml) of a wire myograph. EFS at 1-12 Hz, 60 V, 0.1 ms and 30 s was applied in methoxamine-pre-constricted mesenteric arterial rings in the presence of guanethidine (5 μM) as described in Section 2.3. In other experiments, capsaicin (0.01-10 μM), was cumulatively added after raising the tone of the arterial rings with methoxamine. Finally, tetrodotoxin (1 μM) was added after the stable raised tone was achieved and responses to EFS repeated.

4.2.4 Enzyme immunoassay (EIA) for measuring CGRP release

Segments of mesenteric arteries (superior mesenteric, first order, second order and third order, in the absence and presence of PVAT) and PVAT (dissected from MABs) were prepared, and incubated in microcentrifudge tubes at 37 ºC for 15 min in the absence and presence of capsaicin (10 μM) as described in Section 2.4.2. CGRP was extracted from the Krebs'-Henseleit solution and the assay was conducted as described in Section 2.4.2. To compare the CGRP expression of artery segments with and without PVAT, the concentration of CGRP was normalized per segment length.

4.2.5 Immunofluorescence study to locate sensory nerves on blood vessels and within PVAT

Segments of rat mesenteries were prepared as described in Section 2.5.1. and immunofluorescence staining was carried out as described in Section 2.5.2. MAB segments were observed under confocal scanning microscopy (Leica DMIRE2, Heidelberg, Germany) as described in Section 2.5.2. Velocity image software (Perkin Elmer, Massachusetts, U.S.A) was used in some experiments to visualize 3 dimensional images.

4.2.6 Statistical analysis

Data were analysed as described in Section 2.10.

4.3 Results

4.3.1 Immunofluorescence study

4.3.1.1 Immunofluorescence characterization of perivascular CGRP-containing nerves in PVAT-denuded mesenteric artery segments

Immunoreactivity for CGRP was observed on the surface of PVAT-denuded mesenteric arteries in all segments (MA, 1OMA, 2OMA and 3OMA) as shown in Figure 4.1 (A, B) and Figure 4.2 (A, B, C). In the SMA, the longitudinal section showed fibre-like structures and some of them appeared to have neuronal somata on the surface of the vessel (Figure

4.1A), while the transverse section of the SMA showed the predominant localisation of CGRP-immunoreactivity in the adventitia (Figure 4.1B). Fibre-like structures and cell bodies were also observed in the longitudinal sections of 1OMA, 2OMA and 3OMA (Figure 4.2 A, B, C). In the absence of anti-CGRP nerve antibody (negative control), no immunoreactivity was visualized in any of the artery segments, as shown in Figure 4.1 (C, D) and Figure 4.2 (D, E, F).

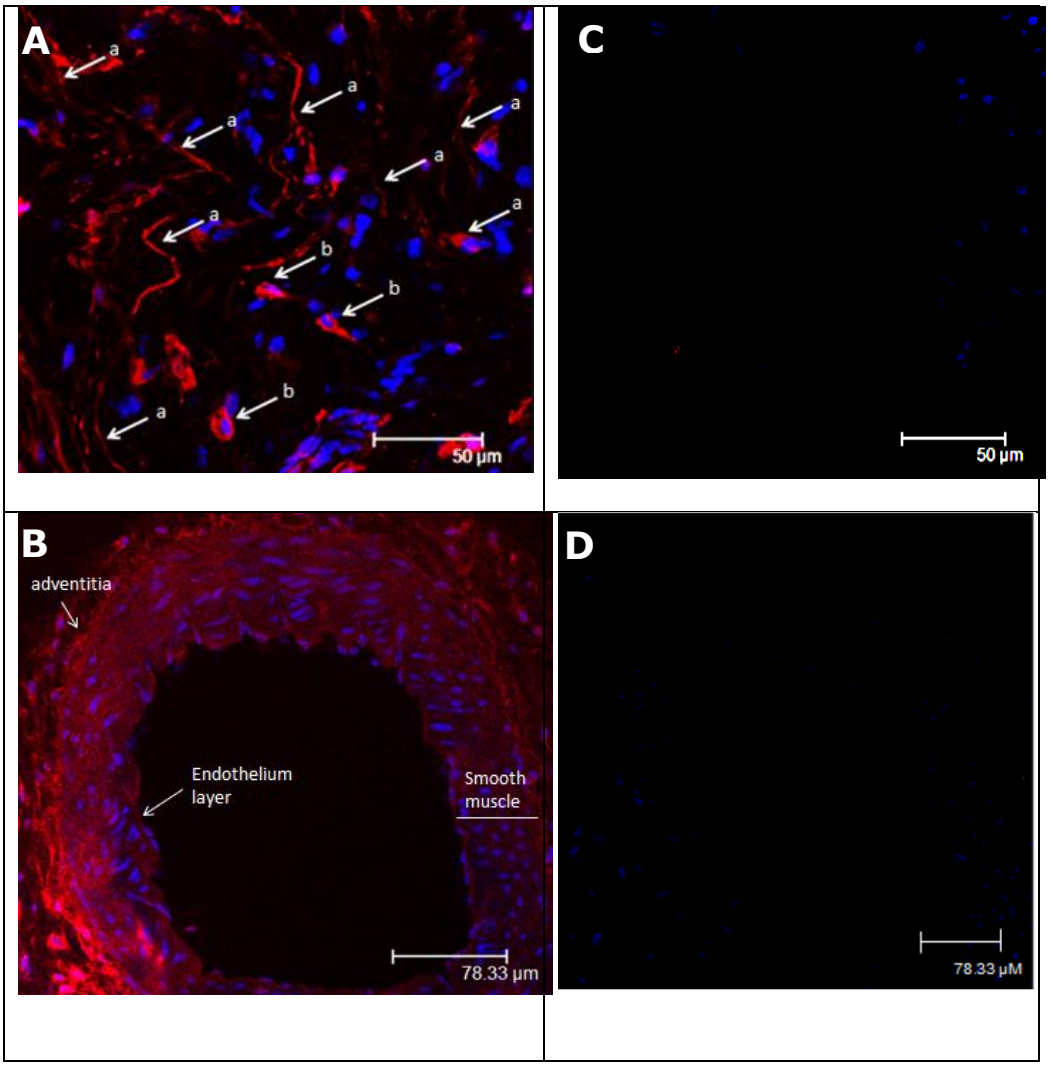

Figure 4.1: Calcitonin gene-related (CGRP) immunoreactivity (red) visualized on the surface of PVAT-denuded rat superior mesenteric artery: **(A)** longitudinal section and **(B)** transverse section. In the absence of anti-CGRP antibody (negative control), no staining was observed on the surface of the superior mesentery artery in **(C)** longitudinal section and **(D)** transverse section. Deoxyribonucleic acid (DNA) was counterstained with DAPI (blue). Arrows indicate (a) immunoreactive fibre-like structures (b) adventitial neuronal somata. Scale bar for **A** and **C** = 50 µm, **B** and **D** = 78.33 µM (n=6).

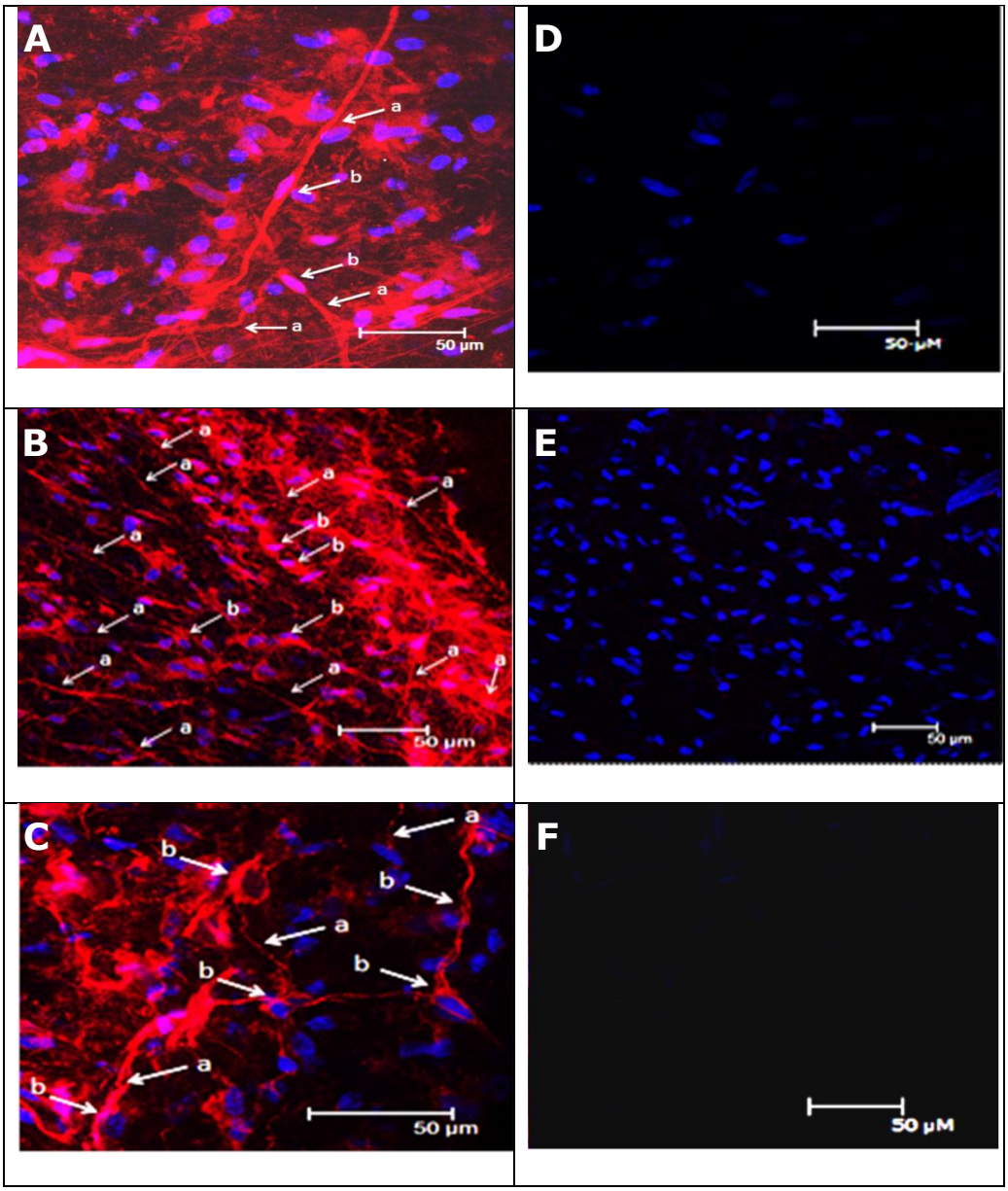

Figure 4.2: Calcitonin gene-related (CGRP) immunoreactivity (red) on the surface of longitudinal sections of PVAT-denuded mesenteric artery segments: **(A)** first order mesentery artery, **(B)** second order mesentery artery and **(C)** third order mesentery artery. In the absence of anti-CGRP antibody (negative control), no staining on nerves was observed on the surface of PVAT-denuded vessels of **(D)** first order mesentery artery, **(E)** second order mesentery artery and **(F)** third order mesentery artery. Deoxyribonucleic acid (DNA) was counterstained with DAPI (blue). Arrows indicate (a) immunoreactive fibre-like structures (b) adventitial neuronal somata. Scale bar = 50 µm (n=6).

4.3.1.2 Immunofluorescence characterization of perivascular CGRP-containing nerves in PVAT-intact mesenteric artery segments

CGRP-immunoreactivity was visualized in PVAT-intact mesenteric artery segments as shown for the SMA in Figure 4.3 (A, B) and for the 1OMA, 2OMA and 3OMA in Figure 4.4 (A, B, C). In the absence of anti-CGRP antibody (negative control), no immunoreactivity was observed as shown in the SMA in Figure 4.3 (C, D) and in the 1OMA, 2OMA and 3OMA in Figure 4.4 (D, E, F). To confirm the expression of CGRP nerves within PVAT, a co-localization protocol was employed. SMA and 2OMA segments of PVAT-intact MABs were co-treated with anti-CGRP and anti-PGP9.5, a neuronal marker. Intense double immunoreactivity was observed as shown in Figure 4.5 (C, E) and Figure 4.6 (C, E).

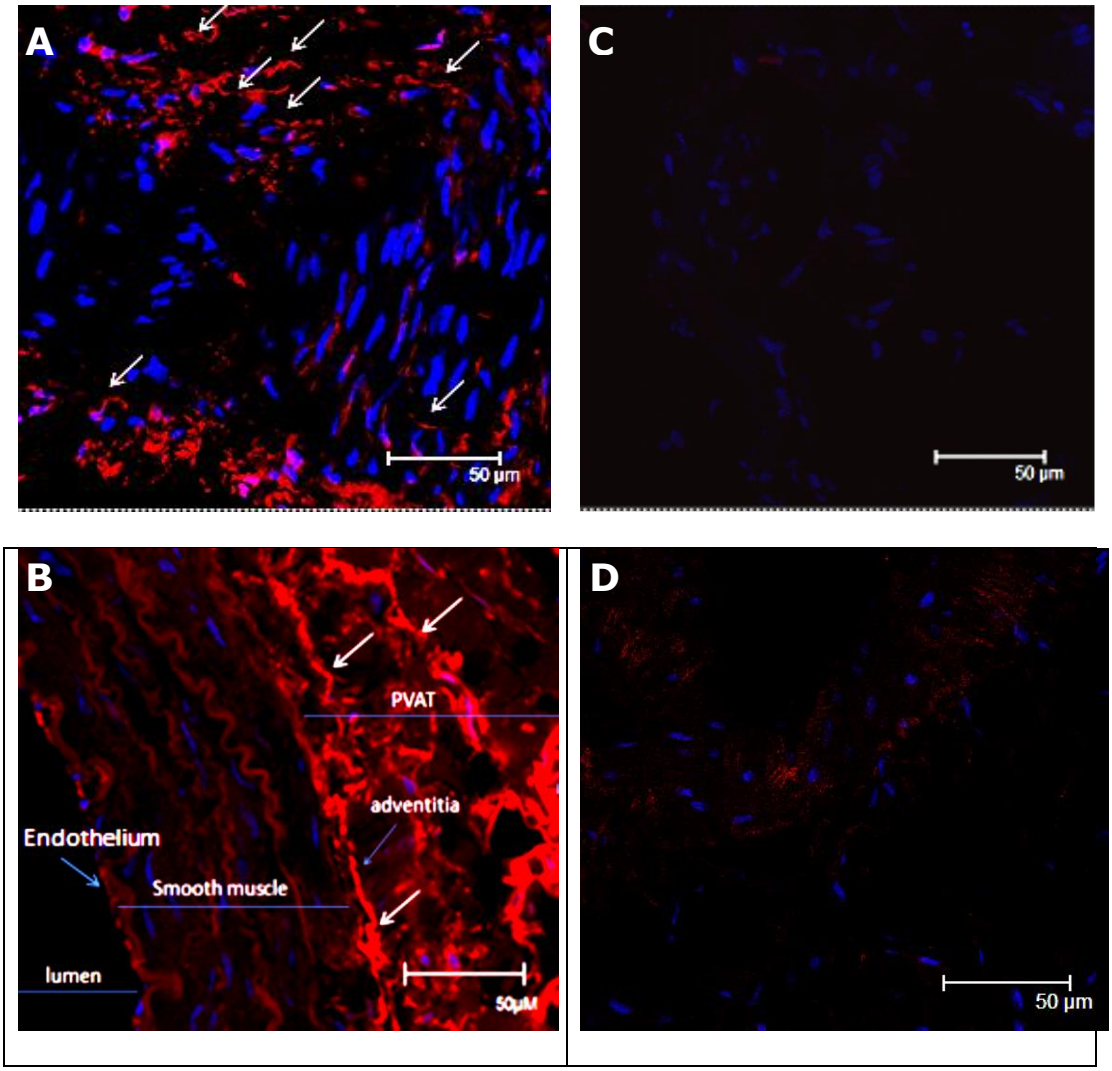

Figure 4.3: Calcitonin gene-related peptide (CGRP) immunoreactivity (red) visualized within fat (PVAT) in the vicinity of the superior mesentery artery (A) longitudinal section and (B) transverse section. In the absence of anti-CGRP nerves antibody (negative control), no staining was observed in (C) superior mesenteric artery (longitudinal section) and (D) superior mesenteric artery (transverse section). Deoxyribonucleic acid (DNA) was counterstained with DAPI (blue). Arrows indicate immunoreactive fibre-like structures. Scale bar = 50 µm (n=6).

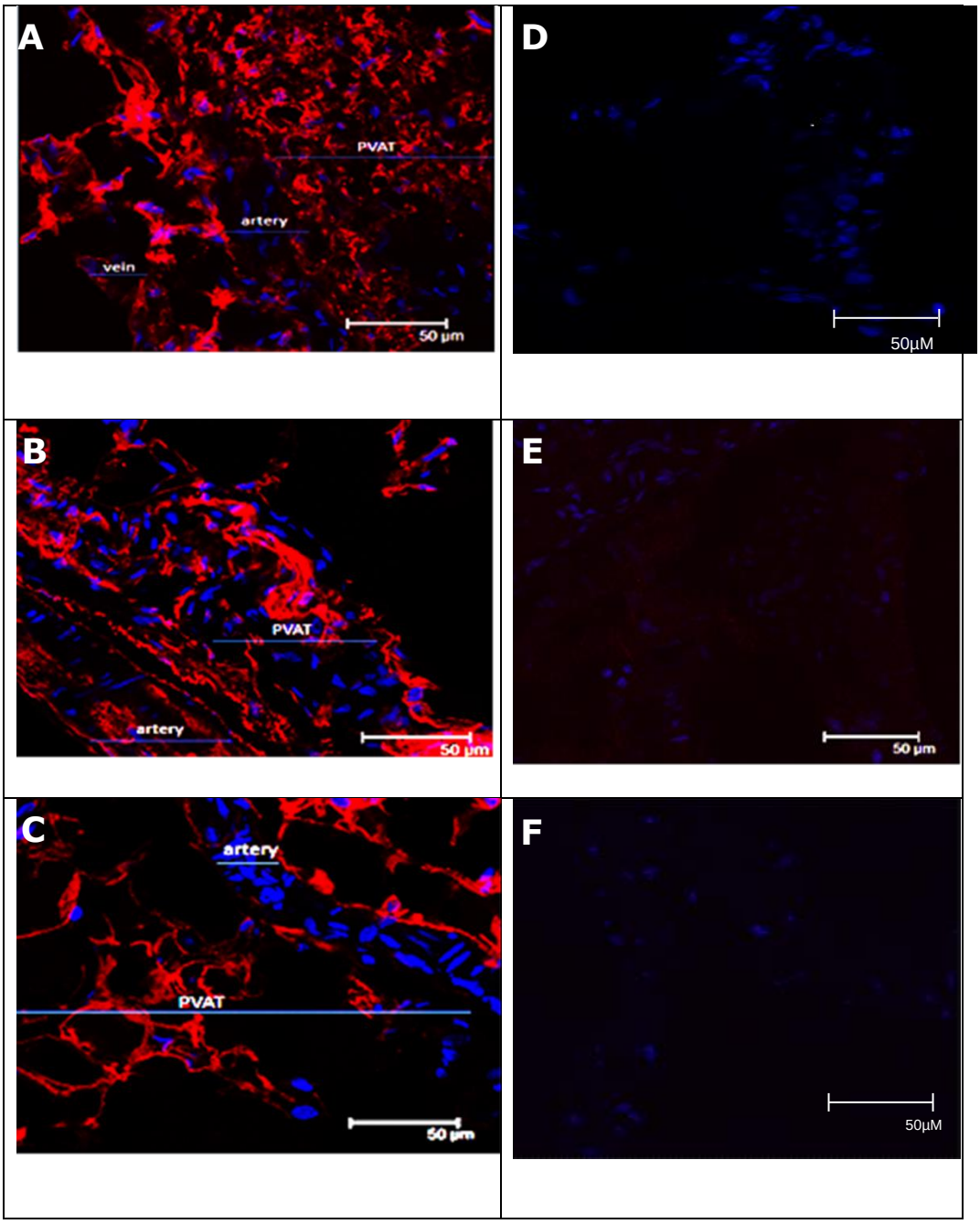

Figure 4.4: Calcitonin gene-related peptide (CGRP) immunoreactivity (red) visualized within longitudinal sections of mesenteric arteries with fat (PVAT) in **(A)** first order mesentery artery, **(B)** second order mesentery artery and **(C)** third order mesentery artery. In the absence of anti-CGRP antibody (negative control), no staining was observed in **(D)** first order mesentery artery, **(E)** second order mesentery artery and **(F)** third order mesentery artery. Deoxyribonucleic acid (DNA) was counterstained with DAPI (blue). Scale bar for **A** and **C** = 50 µm (n=6).

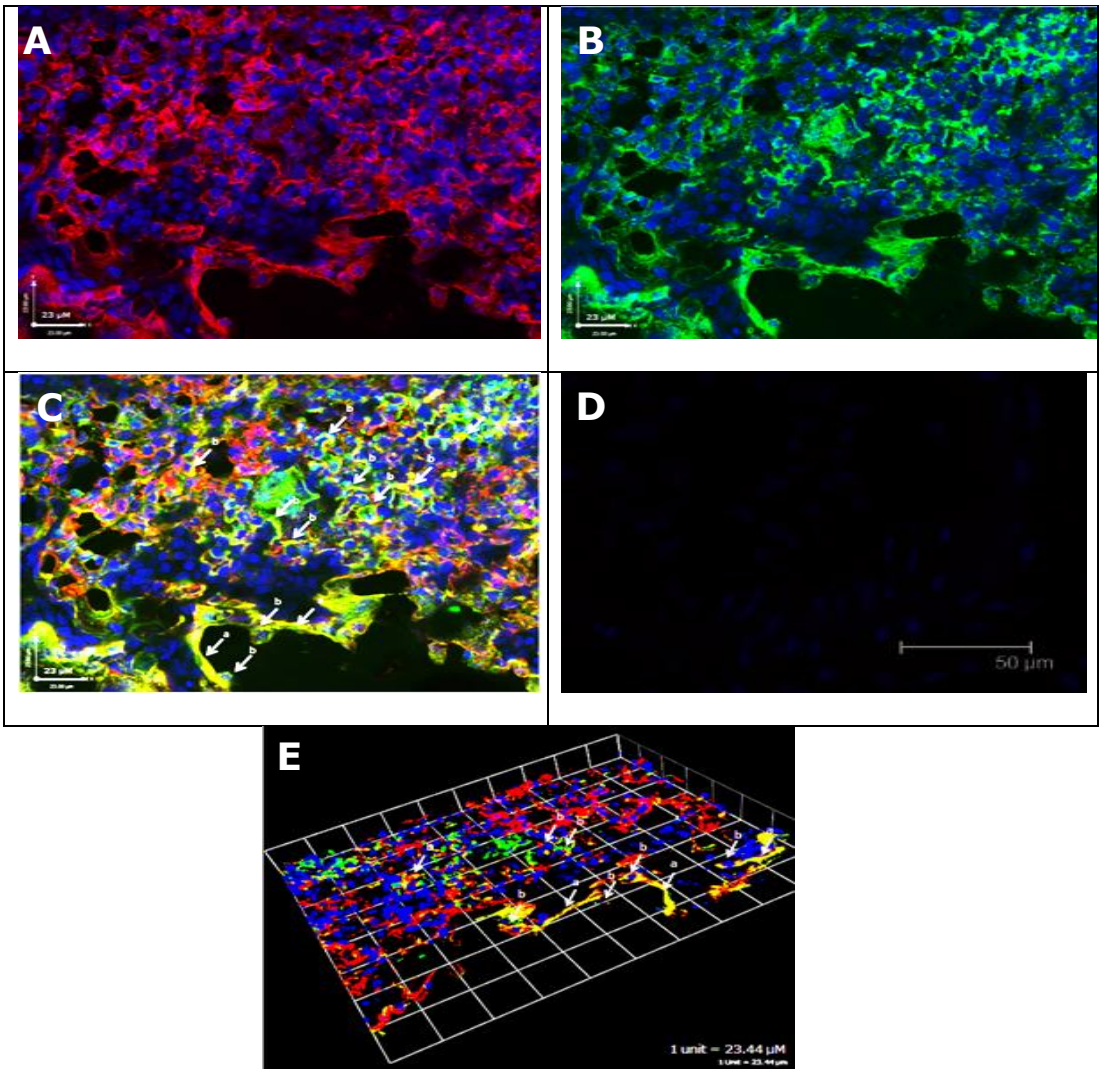

Figure 4.5: Co-localization/double labelling for calcitonin gene-related (CGRP) and PGP 9.5 (neuronal marker) within perivascular adipose tissue (PVAT) in PVAT-intact rat superior mesenteric artery. (A) Anti-CGRP staining (red), (B) anti-PGP 9.5 staining (green), (C) merged image shows some neurons co-expressing CGRP and PGP 9.5 in PVAT. (D) In the absence of anti-CGRP and anti-PGP 9.5 antibodies (negative control), no staining was observed. (E) 3-dimensional merged image showing some neurons co-expressing CGRP and PGP 9.5 marker in PVAT region. Deoxyribonucleic acid (DNA) was counterstained with DAPI (blue). Arrows indicate CGRP and PGP 9.5 double labelled: a = nerve, b =cell body/neuronal somata. Scale bars for A, B and C = 23 µM, D= 50 µM and E, 1 unit = 23.44 µM (n=2).

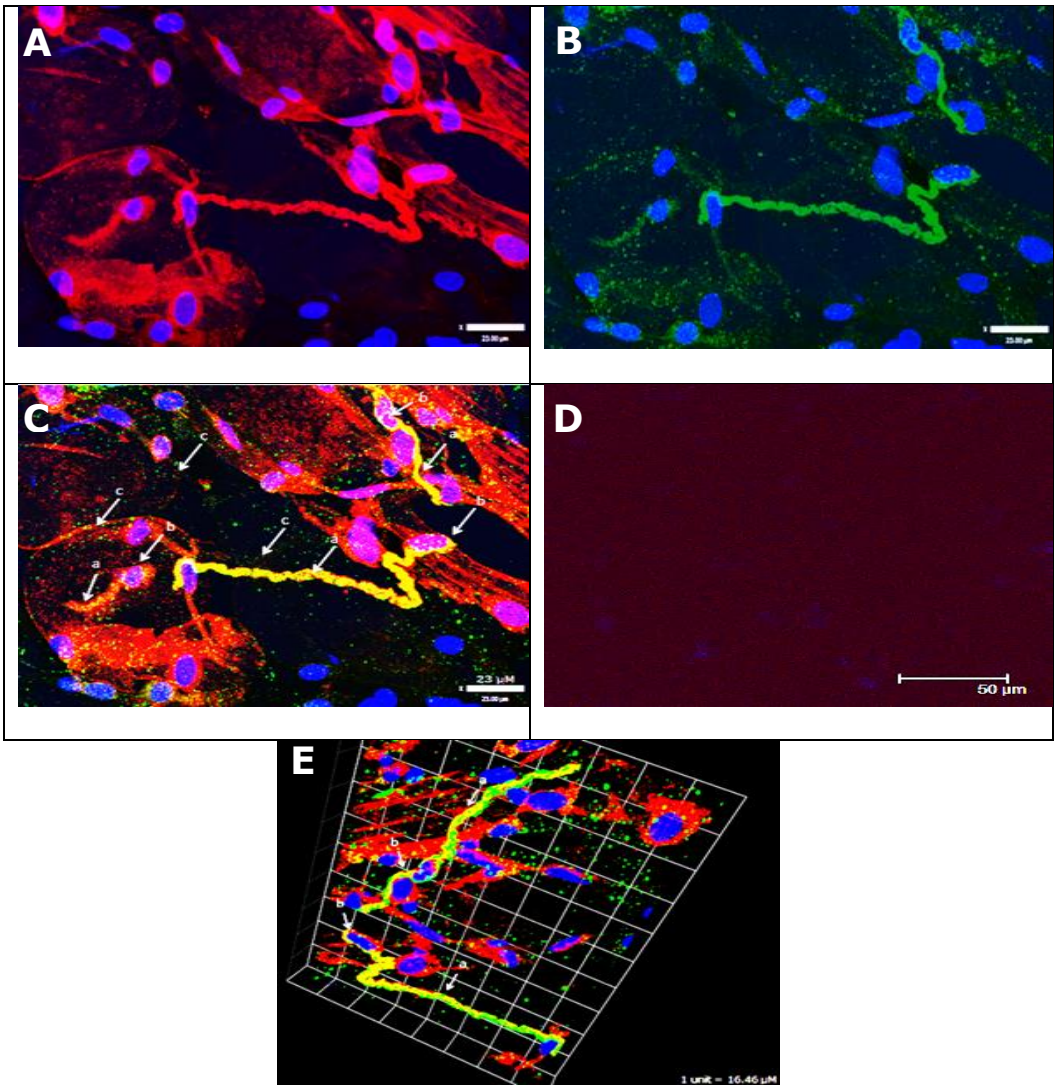

Figure 4.6: Co-localization/double labelling for CGRP immunoreactivity and PGP 9.5 within perivascular adipose tissue (PVAT) in PVAT-intact second order mesenteric artery (2OMA) segment. (A) Anti-CGRP staining (red), (B) anti-PGP 9.5 staining (green), (C) merged image shows some neurons co-expressing CGRP and PGP 9.5 marker in PVAT region. (D) In the absence of anti-CGRP and anti-PGP 9.5 antibodies (negative control), no staining was observed. (E) 3-dimensional merged image showing some neurons co-expressing CGRP and PGP 9.5 marker in PVAT region. Deoxyribonucleic acid (DNA) was counterstained with DAPI (blue). Arrows indicate CGRP and PGP 9.5 double labelled: a = nerve, b = cell body/neuronal somata, c = adipocyte cell. Scale bars for A, B and C = 23 µM, D= 50 µM and E, 1 unit = 16.46 µM (n=2).

4.3.2 Enzyme immunoassay (EIA) study

Having shown the presence of sensory nerves in PVAT, EIA was then used to see if capsaicin (10 μM) could evoke a greater release of CGRP from mesenteric vessels with intact PVAT than in vessels without PVAT. SMA, 1OMA, 2OMA and 3OMA were prepared with and without PVAT, for experiments to measure the capsaicin-evoked release of CGRP; there was no significant difference between the levels of CGRP in the Krebs'-Henseleit solution bathing preparations in the presence of absence of PVAT for any of the SMA, 1OMA, 2OMA or 3OMA ($P>0.05$, unpaired Student's t-test, $n=6$) as shown in Figure 4.7A-D. However, the concentration of CGRP was consistently higher in all of the PVAT-intact groups compared to PVAT-denuded groups, especially in 3OMA in which the level of CGRP was 2-fold higher in the presence of PVAT compared to in the absence of PVAT preparations as shown in Figure 4.7D. These data are in line with the immunofluorescence studies which suggest that while sensory nerves are expressed in PVAT, they are more abundant in the perivascular region.

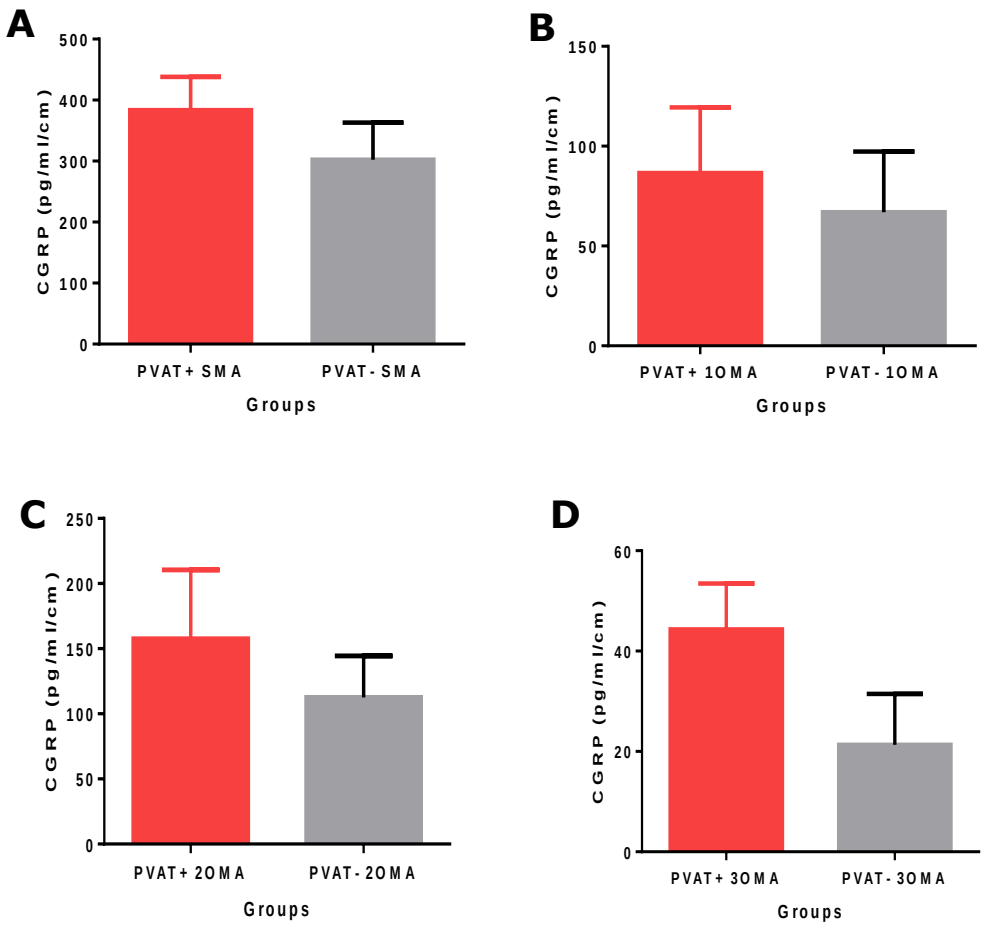

Figure 4.7: Capsaicin-evoked calcitonin gene-related peptide (CGRP) release in artery segments from the rat mesentery. Segments of (A) superior mesenteric artery (SMA), (B) first order mesenteric artery (1OMA), (C) second order mesenteric artery (2OMA) and (D) third order mesenteric artery (3OMA) were prepared with (PVAT+) and without (PVAT-) PVAT. There was no difference in the amount of capsaicin-evoked CGRP released between PVAT-intact and PVAT-denuded arteries (unpaired Student's t-test, n=6).

In separate experiments, PVAT was dissected from SMA and 2OMA and the level of CGRP in the bathing Krebs'-Henseleit solution, with and without capsaicin (10 μM) treatment, was measured using EIA. CGRP levels in the Krebs'-Henseleit solution bathing dissected fat from SMA were similar with and without capsaicin treatment (Figure 4.8). In contrast, the CGRP level in the Krebs'-Henseleit solution bathing dissected PVAT from 2OMA was significantly greater with capsaicin treatment than in the absence of capsaicin ($P<0.01$, one-way ANOVA followed by Bonferroni post-hoc test, n=4) (Figure 4.8). These data provide further evidence for the presence of sensory nerves in PVAT, specifically in the PVAT of resistance arteries.

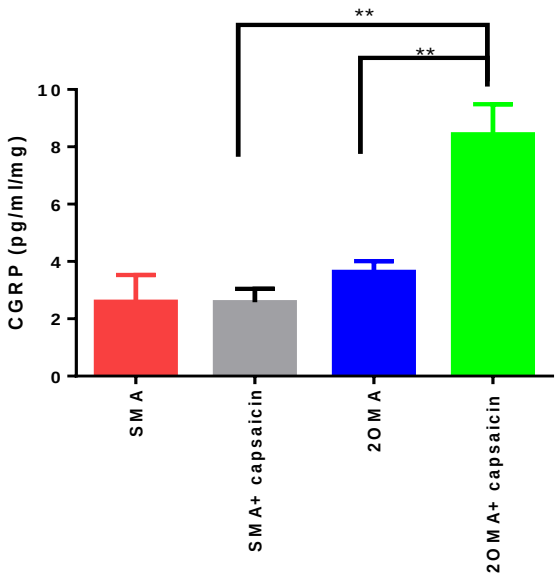

Figure 4.8: Comparison of the concentration of calcitonin gene-related peptide (CGRP) in the Krebs'-Henseleit solution bathing dissected perivascular adipose tissue from segments of superior mesenteric artery (SMA) and second order mesenteric artery (2OMA) in the presence and absence of capsaicin (10 μM). The presence of capsaicin enhanced CGRP release in 2OMA. The release of CGRP in the presence of capsaicin was greater in 2OMA compared to SMA (**P<0.01, one-way ANOVA followed by Bonferroni post-hoc test, n=4).

4.3.3 Responses to electrical field stimulation on SMA and 2OMA in the presence and absence of PVAT

The possible contribution of sensory nerves in PVAT to the control of vasomotor tone was then investigated. Isometric tension recording was used to investigate the effect of PVAT on sensory transmission in isolated conduit (superior mesenteric arteries; SMA) and small arteries (second order mesenteric arteries; 2OMA). EFS (1-12 Hz, 60 V, 0.1 ms, 30 s) was applied in the presence of guanethidine (5 μM) and methoxamine (1-10 μM), to block sympathetic neurotransmission and pre-constrict the preparations respectively. Ring segments of arteries are classically prepared without PVAT for studies employing isometric tension recording. In the presence of PVAT, EFS elicited frequency dependent vasodilatation in both 2OMA and SMA (Figure 4.9). The relaxation responses to EFS (expressed as a percentage of methoxamine-induced tone) were greater in 2OMA compared to SMA ($P<0.01$, $P<0.001$, $P<0.0001$, two-way ANOVA with Bonferroni's post-hoc test, $n=6$) as shown in Figure 4.9.

The presence of PVAT significantly enhanced vasodilatation in 2OMA compared to the absence of PVAT ($P<0.05$, $P<0.001$, $P<0.0001$, two-way ANOVA with Bonferroni's post-hoc test, $n=6$) (Figure 4.10A). The EFS-evoked relaxation in PVAT-intact 2OMA was abolished in the presence of tetrodotoxin (TTX, 1 μM), a neurotoxin which acts by blocking sodium channels ($P<0.001$, two-way ANOVA with Bonferroni's post-hoc test, $n=6$) (Figure 4.10A). In the SMA with PVAT, the small relaxation responses to EFS were abolished by removal of PVAT ($P<0.001$, two-way ANOVA with Bonferroni's post-hoc test, $n=6$) and in the presence of TTX ($P<0.001$, two-way ANOVA with Bonferroni's post-hoc test, $n=6$) (Figure 4.10B). These

data identify a contribution of sensory nerves in PVAT to vasorelaxant control of rat small mesenteric arteries.

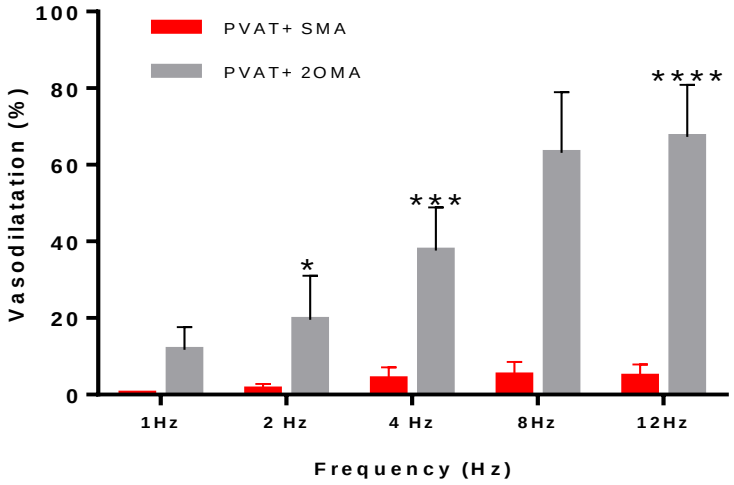

Figure 4.9: The responses to EFS at 1-12Hz, 60V, 0.1ms, 30s in methoxamine (1-10 μM) pre-constricted rat mesenteric arteries in the presence of guanethidine (5 μM). In the presence of perivascular adipose tissue (PVAT+), the responses to EFS were greater in second order mesenteric arteries (2OMA) compared to superior mesenteric arteries (SMA) (*P<0.05, ***P<0.001, ****P<0.0001, two-way ANOVA with Bonferroni's post-hoc test, n=6).

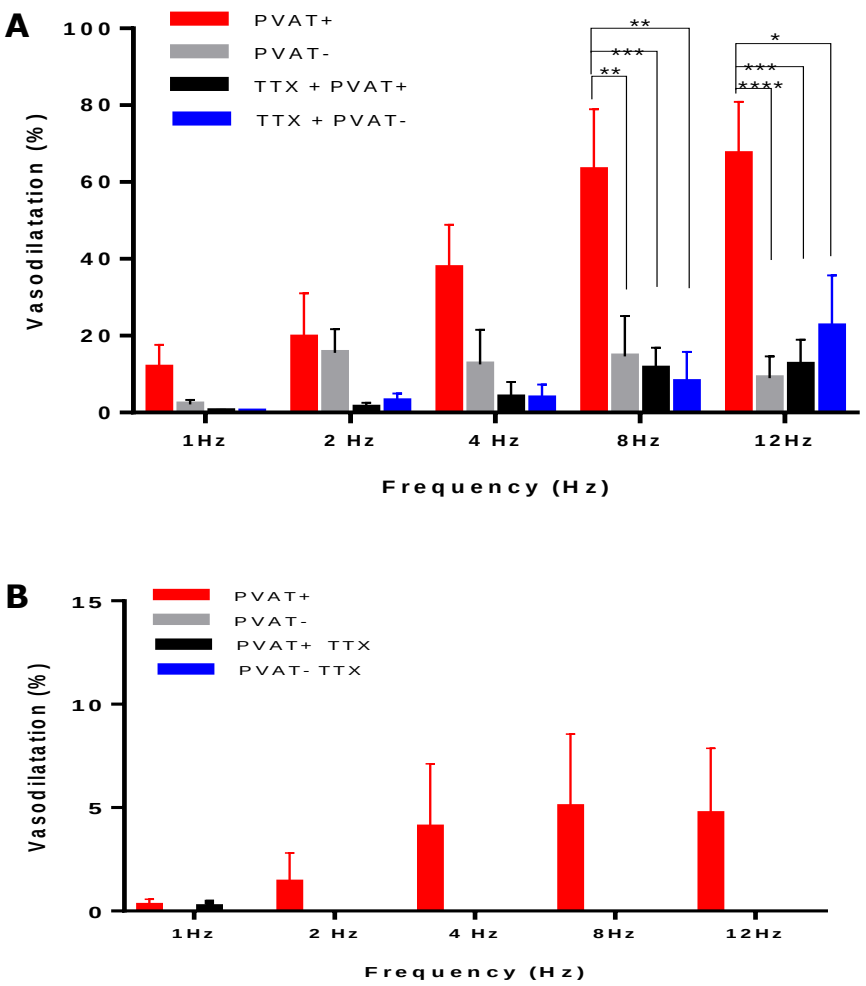

Figure 4.10: Responses to electrical field stimulation (EFS at 1-12 Hz, 60 V, 0.1 ms, 30 s) in methoxamine (1-10 μM) pre-constricted mesenteric arterial rings in the presence of guanethidine (5 μM). Comparison of EFS-induced vasorelaxation in the presence (PVAT+) and absence (PVAT-) of perivascular adipose tissue in second order mesenteric arteries (2OMA) and superior mesenteric arteries (SMA). **(A)** EFS-evoked vasorelaxation effect was abolished in the 2OMA and attenuated in the SMA with the addition of tetrodotoxin (TTX, 1 μM). (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, two-way ANOVA with Bonferroni's post-hoc test, n=6). **(B)** A two-way ANOVA found a significant difference in neurogenic vasorelaxation responses between PVAT-intact SMA, PVAT-denuded SMA and SMA with the addition of TTX (1 μM) (P<0.001, n=6).

4.3.4 The effect of PVAT on neurogenic vasodilatation of perfused MABs

The rat MAB is a preparation with extensive PVAT and the present study investigated if removal of PVAT had the opposite effect to that observed when PVAT was retained in the isolated mesenteric arteries (whether PVAT removal in the MAB would attenuate sensory neurotransmission). EFS (0.5 -12 Hz, 60 V, 0.1 ms and 30 s) of perfused MABs in the presence of guanethidine and methoxamine (to block sympathetic neurotransmission and pre-constrict the preparations respectively), elicited frequency dependent vasodilatation in PVAT-intact preparations (Figure 4.11A). These responses are due to stimulation of sensory nerves since they are abolished by capsaicin and tetrodotoxin treatment (Kawasaki et al., 1988; Ralevic et al., 1995). Neurogenic vasodilatation was abolished in preparations in which PVAT had been removed ($P<0.001$, $P<0.0001$, two-way ANOVA with Bonferroni's post-hoc test, n=4) (Figure 4.11B and Figure 4.12). Two consecutive frequency response curves were carried out, with an interval of 30 min, and were reproducible in PVAT-intact preparations as shown in Figure 4.13 ($P>0.05$, two-way ANOVA, n=4). These data are consistent with the expression of sensory nerves within PVAT, however it was surprising that the neurogenic responses were abolished, since mesenteric arterial sensory nerves are also present as a network in the adventitia (see 4.3.1.1).

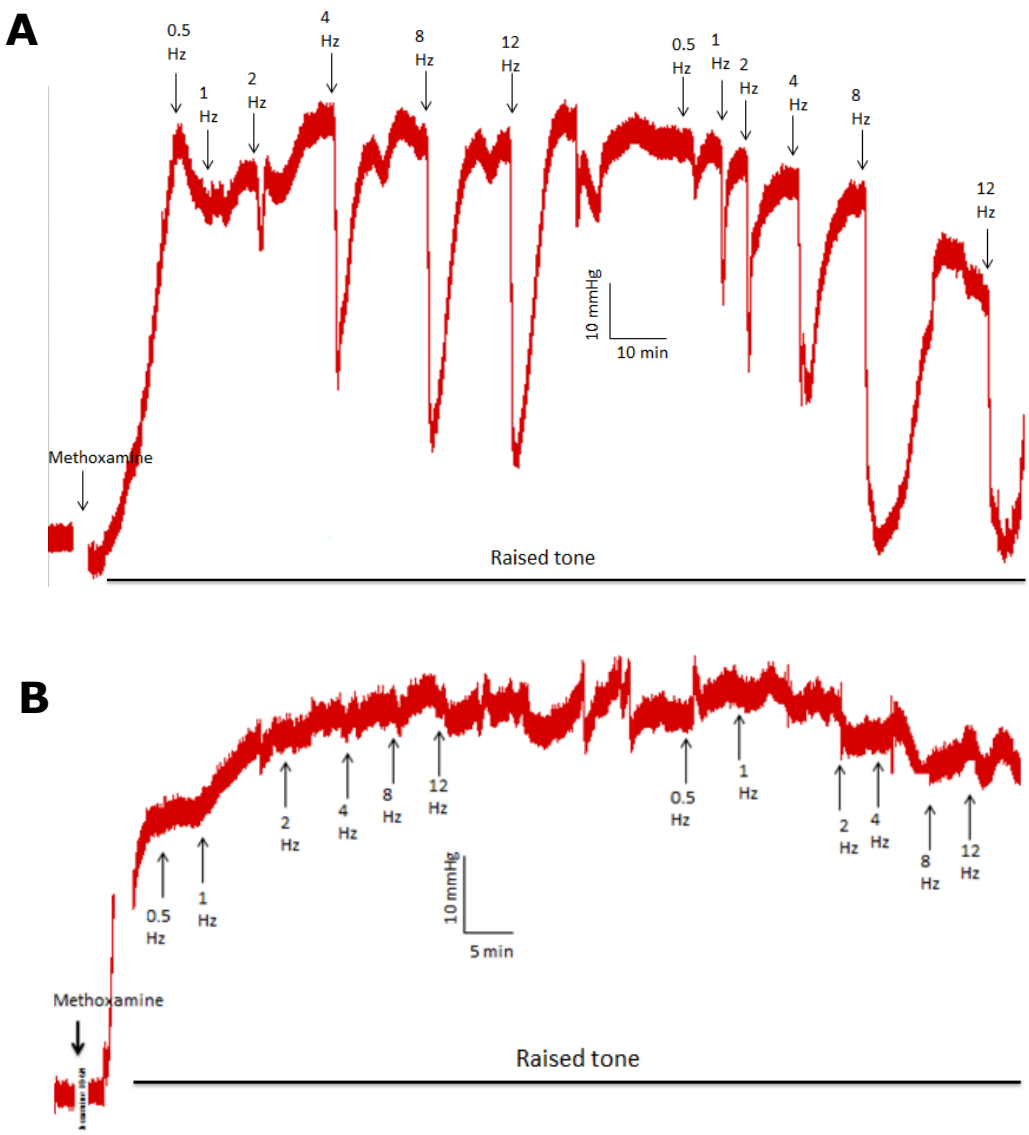

Figure 4.11: Representative traces show that electrical field stimulation (0.5-12 Hz, 60 V, 0.1 ms, 30 s) of rat perfused mesenteric vascular beds with Krebs'-Henseleit solution containing guanethidine (5 μM) and methoxamine (1-5 μM), at raised tone produced a frequency-dependent decrease in perfusion pressure due to vasodilatation in **(A)** the presence of perivascular adipose tissue (PVAT), and **(B)** in the absence of PVAT. In the absence of PVAT the neurogenic vasorelaxant responses were attenuated. Two consecutive frequency response curves with a 30 minute interval in (A) and (B) were reproducible.

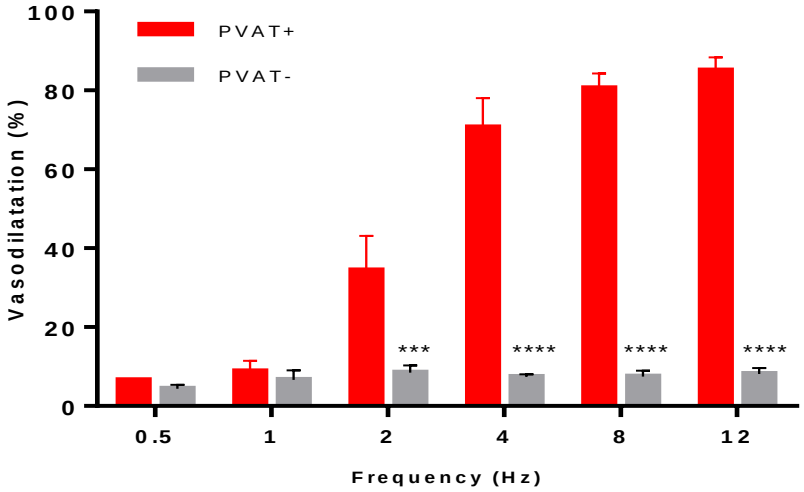

Figure 4.12: Effects of perivascular adipose tissue (PVAT) on neurogenic vasodilatation of rat mesenteric arterial beds. Preparations were submaximally pre-constricted with methoxamine (1-10 μM) in the presence of guanethidine (5 μM) to block sympathetic neurotransmission. In the presence of PVAT (PVAT+) electrical field stimulation (EFS; 0.5 -12 Hz, 60 V, 0.1 ms and 30 s) elicited frequency-dependent vasodilatation. In the absence of PVAT (PVAT-) the EFS vasodilator response was abolished (***P<0.001, ****P<0.0001, two-way ANOVA with Bonferroni's post-hoc test, n=4).

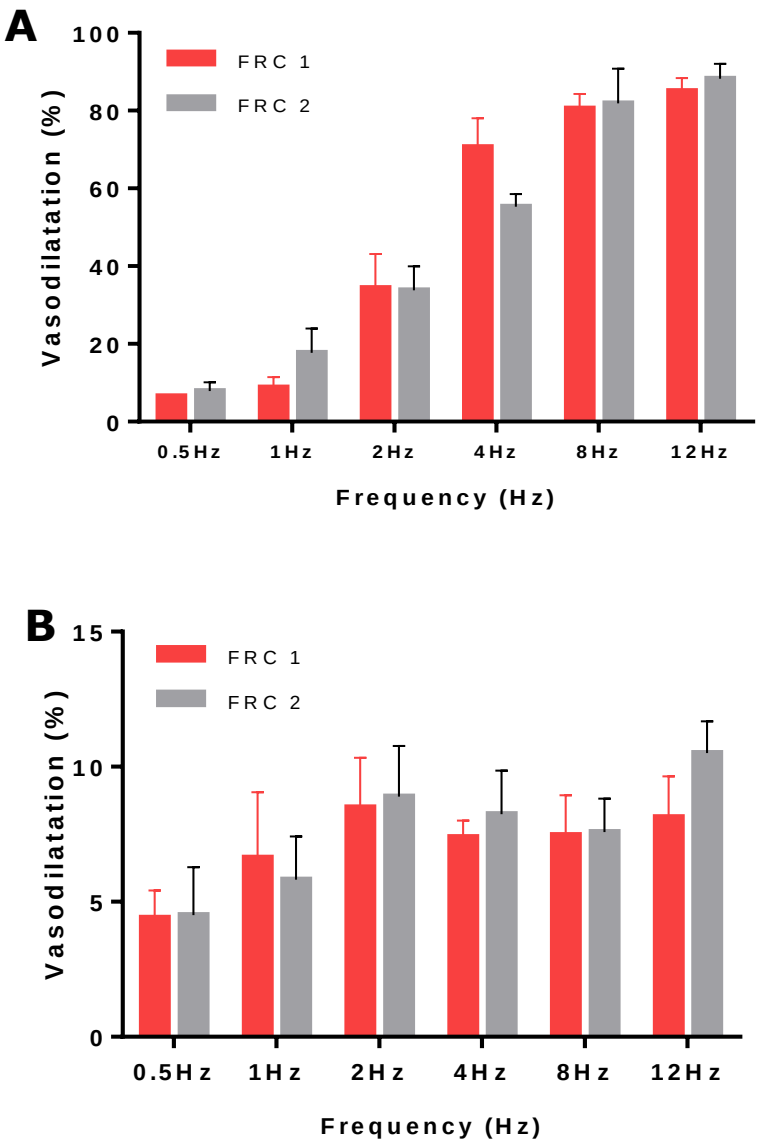

Figure 4.13: Reproducibility tests show there was no difference between two consecutive frequency response curves (FRC 1 and FRC 2) in the rat perfused mesenteric arterial bed. Preparations were submaximally pre-constricted with methoxamine (1-10 μM) in the presence of guanethidine (5 μM) (to block sympathetic neurotransmission) and electrical field stimulation (0.5 -12 Hz, 60 V, 0.1 ms and 30 s) was applied to generate FRC1 and FRC2 with a 30 min interval. Data shown are of preparations in the **(A)** presence and **(B)** absence of perivascular adipose tissue ($P>0.05$, two-way ANOVA, n=4).

To examine the integrity of vessels in both PVAT-intact and PVAT-denuded preparations, bolus injection of exogenous CGRP ($1.5x10^{-13}$-$5x10^{-11}$ moles) was carried out in both preparations. CGRP was used since it is the principle vasodilator neurotransmitter of sensory nerves in rat mesenteric arteries (Kawasaki et al., 1988). Bolus injection of CGRP elicited dose-dependent vasodilatation of pre-constricted MAB in both PVAT-intact and PVAT-denuded preparations (Figure 4.14). There was no difference in vasodilator responses to CGRP between the preparations (P>0.05, two-way ANOVA) (Figure 4.14) showing that the ability of the smooth muscle to relax was similar between the preparations with and without PVAT.

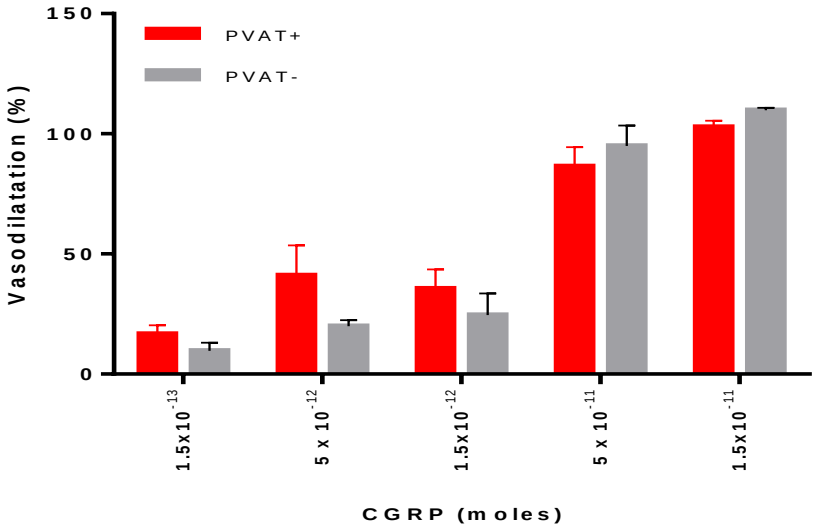

Figure 4.14: Effect of the presence and absence of perivascular adipose tissue (PVAT) on vasodilatation to calcitonin gene-related peptide (CGRP) ($1.5x10^{-13}$-$1.5x10^{-11}$ moles) in rat mesenteric arterial beds. Responses were assessed in the presence of guanethidine (5 μM) in preparations sub-maximally pre-constricted with methoxamine (1-10 μM). There was no significant difference between preparations with (PVAT+) and without (PVAT-) PVAT with regard to their relaxation responses to bolus doses of CGRP (P>0.05, two-way ANOVA, n=5).

4.3.5 Effects of PVAT on vasorelaxant responses to capsaicin in mesenteric vascular beds

To further investigate the possible expression of sensory nerves within PVAT, capsaicin, a TRPV1 agonist, was used. It was anticipated that relaxant responses to capsaicin might be attenuated in PVAT-denuded preparations, because of the absence of sensory nerves, which are contained within removed PVAT. Capsaicin was added in methoxamine pre-constricted vessels as cumulative concentrations (0.1-30 μM) and as bolus injections ($1.5x10^{-12}$-$5x10^{-10}$ moles) (Figure 4.15). Unexpectedly, the concentration-dependent relaxations to capsaicin were not significantly different between preparations with and without PVAT (Figure 4.15A). The dose-dependent relaxations to capsaicin were also not significantly different between the preparations with and without PVAT (Figure 4.15B).

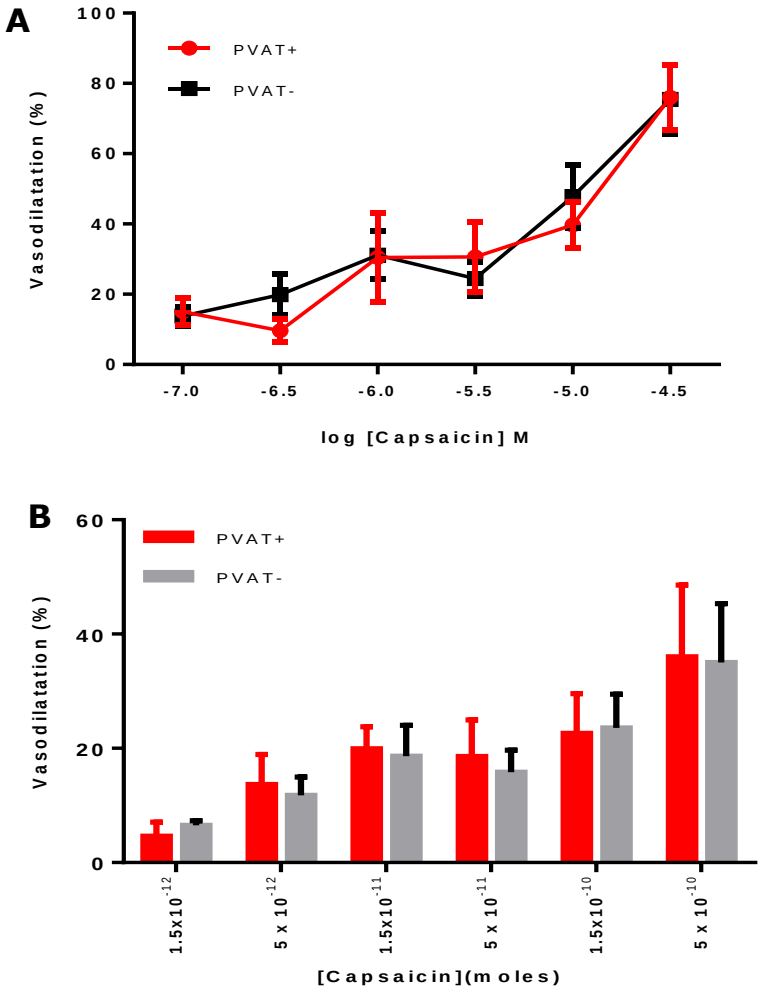

Figure 4.15: Effects of the presence and absence of perivascular adipose tissue (PVAT) on vasodilatation to capsaicin of rat isolated mesenteric vascular beds. Preparations were submaximally pre-constricted with methoxamine (1-10 μM) and relaxation responses were expressed as a percentage of this response. (A) Capsaicin was applied as cumulative concentrations (0.1-30 μM); there was no significant difference between the presence (PVAT+) and absence (PVAT-) of PVAT with regard to concentration-dependent relaxations to capsaicin ($P>0.05$, Student's t-test, n=5). (B) Similarly, there was no significant difference between the presence and absence of PVAT with regard to dose-dependent relaxations to bolus injections of capsaicin ($1.5x10^{-12}$-$5x10^{-10}$ moles) ($P>0.05$, two-way ANOVA, n=5).

4.3.6 Relaxations to capsaicin in SMA and 2OMA in the presence and absence of PVAT

In methoxamine pre-constricted isolated vessels, capsaicin (0.1-10 μM) elicited concentration-dependent relaxation in SMA and 2OMA (in both PVAT-intact and PVAT-denuded preparations. There was no significant difference in vasodilator responses to capsaicin between preparations with and without PVAT ($P>0.05$, unpaired Student's t-test) as shown in Figure 4.16.

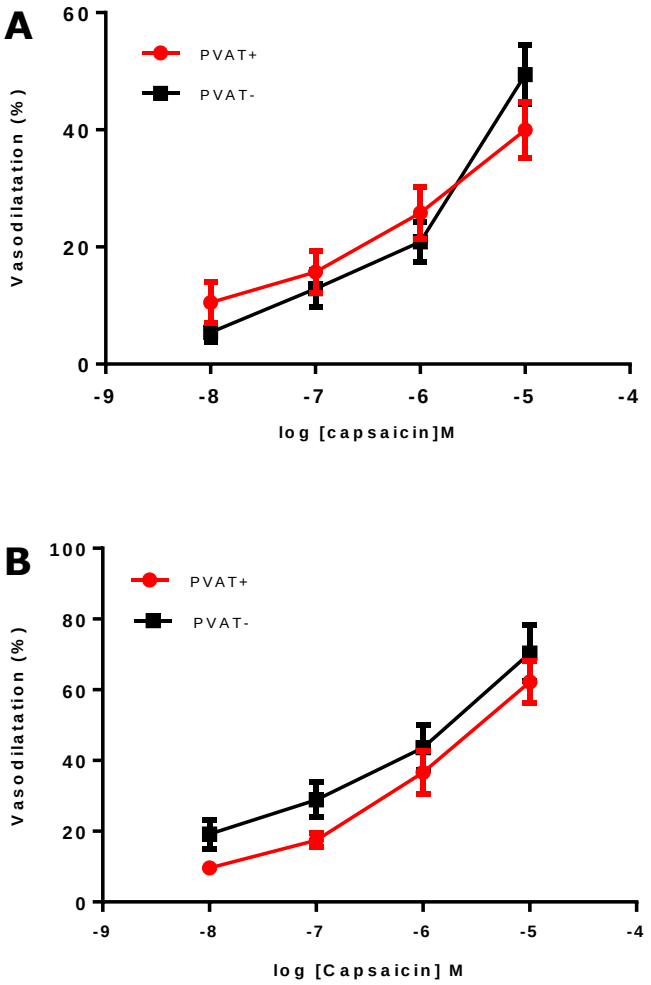

Figure 4.16: The effects of perivascular adipose tissue (PVAT) on responses to capsaicin in rat isolated superior mesenteric arteries (SMA) and second order mesenteric arteries (2OMA). Artery segments were prepared with (PVAT+) and without (PVAT-) PVAT. Vasodilator responses to capsaicin (0.1-10 μM) are expressed as a percentage of the methoxamine-induced pre-constriction. Guanethidine (5 μM) was present throughout to mimic the conditions used for electrical field stimulation. There was no difference between the responses to capsaicin in either the SMA (PVAT+, n=9; PVAT-, n=14) or (B) 2OMA (PVAT+, n=10; PVAT-, n=14).

4.4 Discussion

The present study provides evidence for the functional expression of sensory nerves within PVAT of rat mesenteric arteries. This is the first report to show the distribution of sensory nerves in PVAT, and that these sensory nerves in PVAT play a direct role in vasoregulation of rat MABs. There are several novel findings in this study: (1) The immunofluorescence study revealed that sensory nerves are expressed in PVAT; (Straub et al.) The enzyme immunometric assay (EIA) provided further evidence that the sensory nerves are distributed within PVAT; (Dick et al.) The isometric tension experiments of isolated SMA and 2OMA indicated that PVAT enhanced neurogenic vasorelaxation, and this was greater in smaller arteries (2OMA) compared to conduit arteries (SMA); (4) The perfusion experiments showed that the removal of PVAT greatly attenuated EFS-evoked neurogenic vasorelaxation in rat MABs.

4.4.1 The presence of sensory nerves within PVAT of MABs

Immunofluorescence staining of rat mesenteric arterial SMA, 1OMA, 2OMA and 3OMA in PVAT-denuded segments showed the presence of extensive innervation with perivascular nerves where fibres immunoreactive to anti-CGRP were identified. Similar observations in mesenteric arterial preparations without PVAT have been reported elsewhere (Kawasaki et al., 2009; Kawasaki et al., 1988; Kawasaki et al., 2011). CGRP-immunoreactivity was also visualized within PVAT in PVAT-intact mesenteric artery segments, which indicates the presence of sensory nerves within PVAT of MABs. In order to confirm my observation, a double staining protocol with the presence of anti-CGRP and anti-PGP 9.5

antibodies was applied, and co-localization of the staining was evident. In PVAT-intact preparations, the longitudinal sections contain both vessels and PVAT, with DAPI was reactive to both nerves and fat cells too.

Interestingly, cell bodies or neuronal somata were visualized in all segments of both PVAT-intact and PVAT-denuded preparations. Traditionally, cell bodies of CGRP were believed to be located only in the dorsal root ganglia (DRG) (Supowit et al., 1995) which terminate peripherally on blood vessels and other tissues innervated by the sensory nervous system and centrally in laminae I/II of the dorsal horn of the spinal cord (Marti et al., 1987). However, a more recent study showed that adventitial neuronal somata are present in small rat mesenteric arteries (MA) (Somasundaram et al., 2006), which is in line with the current observation. The group used DNA sequence analysis to show 100 % homology with the β-CGRP cDNA, indicating that mRNA encoding β-CGRP is expressed in the vessel. The study demonstrated the possibility of adventitial neuronal somata as a sensory origin in small MA. A more recent study from the same group revealed that the adventitial neuronal somata express a number of genes including paladin which participate in response to injury/stress and vasodilatation (Somasundaram et al., 2012).

4.4.2 Enzyme immunoassay (EIA) study

EIA is a sensitive and quantitative method to measure CGRP levels in biological samples and has widely been used in previous studies (Frobert et al., 1999; Kessler et al., 1999). EIA was employed to compare the CGRP levels between PVAT-intact and PVAT-denuded preparations. Capsaicin at a high concentration (10 μM) was used as it is well known that at this concentration capsaicin can deplete CGRP and substance P from sensory nerves (Gamse et al., 1980). Although there was no difference in the CGRP expression level in all segments between preparations with and without PVAT, there was a trend that the CGRP concentration was consistently greater in all PVAT-intact segments compared to denuded segments. This observation suggests that CGRP-containing nerves are distributed in both PVAT and in the vessel wall, consistent with the immunohistochemical staining.

In the present study, CGRP release from dissected PVAT in the presence and absence of capsaicin was investigated. By investigating just the dissected PVAT, the aim was to reduce the relatively high background CGRP level provided by CGRP released from sensory nerves in the adventitia. CGRP levels in the Krebs'-Henseleit solution bathing dissected fat from the SMA were similar with and without capsaicin (10 μM) treatment. In contrast, the CGRP level in the Krebs'-Henseleit solution bathing dissected PVAT from 2OMA was significantly greater with capsaicin treatment than in the absence of capsaicin. The CGRP level in the presence of capsaicin was higher in 2OMA than in SMA. It is postulated that the level of CGRP release is determined by the sensory nerves distribution. This observation suggests that sensory nerves are distributed in PVAT, and

shows that the density of sensory nerves in PVAT of mesenteric small arteries is greater than in PVAT of conduit mesenteric arteries. This outcome is consistent with the present immunofluorescence and neurogenic vasorelaxation study in both 2OMA and SMA, which suggest the density and the relaxation responses to EFS were greater in 2OMA compared to SMA (see Section 4.3.1 and 4.3.3). Human adipocytes from abdominal fat have been shown to express mRNA for CGRP, but CGRP protein expression was absent under control conditions and was only induced after treatment with LPS (Radimerski et al., 2010). Thus, a source of CGRP in PVAT in structures other than in sensory nerves seems unlikely.

4.4.3 Responses to electrical field stimulation on SMA and 2OMA in the presence and absence of PVAT

The present study investigated the role of PVAT in modulating sensory nerve-mediated vasodilatation in mesenteric arteries. To the best of my knowledge, there is no existing literature on the effect of PVAT on neurogenic vasodilation. In isometric tension experiments in methoxamine-pre-constricted mesenteric arterial rings (SMA and 2OMA) in the presence of guanethidine, EFS elicited frequency-dependent (0.5-12 Hz) vasodilatation. The endothelium was not removed as previous studies have shown that endothelium removal did not affect both pro and anti-contractile effects of PVAT in rat mesenteric arteries (Verlohren et al., 2004) and rat aorta (Dubrovska et al., 2004). In rat mesenteric arterial rings, the presence of PVAT-enhanced neurogenic vasodilatation; removal of PVAT in both SMA and 2OMA segments reduced EFS-evoked vasodilatation responses. Tetrodotoxin (1 μM) added at the end of experiments abolished EFS responses, which indicates that the responses

were neurogenic. Relaxation in 2OMA was significantly larger than that in the SMA, raising the possibility that neurogenic vasorelaxation is more dominant in smaller or resistant arteries compared to conduit arteries. It is known that smaller arteries tend to be more densely innervated than conduit arteries (Burnstock, 1975). A different sensitivity to CGRP could contribute since previous studies have indicated that human α-CGRP is more potent in mesenteric resistance arterioles than in mesenteric conduit arteries in both rats and humans (Marshall et al., 1986; 1988). Although immunohistochemistry and EIA data from the current study showed that large arteries are also innervated by sensory nerves, EFS-evoked sensory vasodilatation measured in SMA under isometric tension indicates that sensory nerves have little or no role in regulating vasodilatation in large arteries. Classically, artery ring preparations are prepared with PVAT removed, and the present study may explain why there are so few reports of sensory neurogenic relaxations in isolated vessel ring preparations.

4.4.4 The effect of PVAT on neurogenic vasodilatation of perfused MABs

The rat perfused mesenteric arterial bed preparation was originally described for use by McGregor (1966) and has widely been used to investigate sensory neurotransmission. It is a preparation that is extensively covered in PVAT, although this is never specifically mentioned by users of the preparation. In the converse experiment to that in the MA rings, I wanted to see if, in the MABs, neurogenic responses would be reduced after removal of PVAT. Consistent with the isometric tension experiments in rat isolated 2OMA and SMA, electrically-evoked neurogenic vasodilatation responses in perfused rat MAB preparations were

significantly attenuated in preparations in which PVAT was removed, which supports the notion that the presence of PVAT enhances neurogenic vasodilatation.

There are a number of possibilities that may contribute to this observation. The simplest explanation is that sensory nerves are expressed within PVAT and contributed to the neurogenic vasodilation of the MABs due to EFS. This is in line with the immunohistochemical staining and EIA data. Moreover, it can be postulated that perivascular nerves act synergistically with PVAT-derived relaxing factors (PVATRF) in inducing MABs vasorelaxation and removal of PVAT reduces this effect. It is well known that the adventitia of MABs is densely innervated by sensory nerves and PVAT contains numerous vasorelaxing compounds. It is possible that the CGRP that is released from the sensory nerves acts at adjacent adipocytes to release adipokines. With regard to the possible involvement of factors released from adipocytes, PVAT-derived angiotensin II was suggested to augment sympathetic neurotransmission in rat mesenteric arteries (Lu et al., 2010a), but angiotensin II is unlikely to be involved here since it is a vasoconstrictor which inhibits CGRP release from sensory nerves in rat MABs (Kawasaki et al., 1998) and thus PVAT removal would have been expected to augment sensory neurogenic vasorelaxation.

Unexpectedly, removal of the PVAT abolished sensory neurogenic relaxation, rather than just attenuating it. In the present study, immunofluorescence staining clearly showed that sensory nerves densely innervate blood vessels in all segments (SMA, 1OMA, 2OMA and 3OMA) of PVAT-denuded preparations. Confocal scanning also showed that despite the removal of PVAT, there was an abundance of sensory nerves at the adventitia. This observation is consistent with many publications which

show a high density of CGRP-containing nerves on the surface of clean rat mesenteric arteries (Kawasaki et al., 1988; Kawasaki et al., 2011). Based on these findings, it is intriguing why removal of PVAT abolished vasodilatation in PVAT-denuded preparations while there were still substantial amount of CGRP-containing nerves remaining in the adventitia. Further study, including viral tract tracing to investigate the connection between PVAT-derived sensory nerves and adventitial-sensory nerves is warranted to elucidate this observation.

4.4.5 The integrity of vessels and the involvement of TRPV1 channels and CGRP receptors in mediating EFS-evoked neurogenic vasodilation responses

In order to further investigate the possible functional expression of sensory nerves within PVAT, and to address the possibility that removal of PVAT caused damage to the neuronal network, capsaicin, an agonist at vanilloid receptor subtype 1 (TRPV1), was used. The aim was to see if capsaicin had an enhanced vasorelaxant response in arteries with PVAT, because these have more sensory nerves than arteries without PVAT. Moreover, since capsaicin can directly activate sensory nerves via TRPV1 channels on the nerve terminals and axons, its responses might be less sensitive to any possible disruption of the adventitial neuronal network caused by PVAT removal. In contrast, neurogenic relaxations due to EFS involve action potential generation and require intact neuronal networks, especially for the perfused MABs, and so might be more sensitive to the effects of PVAT removal.

Cumulative concentrations (0.1-30 μM) of capsaicin elicited concentration-dependent vasodilatation of pre-constricted MABs, SMA and 2OMA in both PVAT-intact and PVAT-denuded preparations and there was no significant difference in vasodilator responses between these preparations. According to Hopps et al. (2012), capsaicin at 30 μM influences vascular responses in arteries through inhibition of L-type Ca^{2+} channels and thus sensory-nerve independent effects of capsaicin have to be considered. Therefore, in separate experiments, capsaicin was applied in a dose-response fashion ($1.5x10^{-12}$-$5x10^{-10}$ moles) with the aim of reducing the possibility of capsaicin activating TRPV1-independent mechanisms as well as avoiding capsaicin-induced desensitization. Similar outcomes were obtained with both cumulative capsaicin concentration- and dose-response experiments, where vasodilator responses to capsaicin were comparable between PVAT-intact and PVAT-denuded preparations. These data suggest that sensory nerves in PVAT do not contribute significantly to the capsaicin-evoked vasomotor response in rat mesenteric arteries. The EIA results may provide the explanation for the outcome in the capsaicin experiments. EIA showed that there was no significant difference in the level of capsaicin-evoked CGRP release between PVAT-intact and PVAT-denuded preparations although the concentration of CGRP was consistently higher in all of the PVAT-intact groups compared to PVAT-denuded groups. These data suggest that sensory nerves are expressed in PVAT, however, their density is considerably lower than that of sensory nerves at the adventitia. In addition, a recent study showed that TRPV1 channels are not exclusive to sensory nerves and are expressed in many non-neuronal cells varying from vascular smooth muscle to keratinocytes and endothelium (Fernandes et al., 2012). Kark et al. (2008) demonstrated that TRPV1 channels are distributed on vascular smooth muscle of hind limb, aorta and skeletal muscle in male Wistar rats. These studies add another possibility which is

that capsaicin activated non sensory TRPV1 mechanism. In the present study, the effect of vehicle (ethanol) was not investigated. The concentration of ethanol used in capsaicin experiments was up to 0.03 %. A previous study by our group showed that capsaicin induced concentration-dependent vasorelaxation of the rat artic rings was significantly ($P<0.001$) greater than the small vasorelaxant response caused by the vehicle control (0.15% of final bath volume) alone (Hopps, 2013). Another study demonstrated that the effect of ethanol vehicle in rats small mesenteric arteries (up to 0.6% v/v final concentration in myograph bath) had a negligible relaxant effect (Hoi and Hiley, 2006). Although the concentration of ethanol vehicle was low in the present study, however, future experiment to determine the effect of vehicle in capsaicin-induced vasodilatation is warranted.

The capsaicin data also suggest that the PVAT removal procedures did not damage vascular smooth muscle relaxation in PVAT-denuded preparations. Exogenous CGRP ($1.5x10^{-13}$-$1.5x10^{-11}$ moles) applied as bolus injections, also produced similar relaxation responses in PVAT-intact and PVAT-denuded preparations, also suggesting that the ability of the preparations to relax was unimpaired. The present results are in line with those of Dubrovska et al. (2004), in which there was no difference in vasodilator responses to CGRP in both PVAT-intact and denuded preparations of rat aortas, thus excluding post-junctional CGRP receptors and TRPV1 receptors as the primary mechanism of the PVAT-enhanced EFS responses. There are several CGRP-induced vasodilation mechanisms that have been reported; the majority of these observed that CGRP caused relaxation via a rise in intracellular cAMP, a mechanism which has been demonstrated in rat perfused mesentery, cat cerebral artery and porcine coronary artery (Brain

and Grant, 2004; Edvinsson et al., 1985; Han et al., 1990; Yoshimoto et al., 1998).

4.5 Conclusions

In conclusion, PVAT enhances neurogenic vasodilatation in rat MABs and isolated mesenteric arteries. Cumulative data from this chapter indicate the expression of sensory nerves within PVAT of rat mesenteric arteries. The present study also demonstrated that sensory innervation is greater in rat small mesenteric arteries compared to conduit mesenteric arteries. The interaction between PVAT-derived sensory nerves and PVAT-derived compounds, and their role in physiological and pathological conditions of the cardiovascular system are awaiting investigation.

Chapter 5

Effect of hydrogen sulfide on sensory nerve-mediated responses in the rat mesenteric arterial bed in the absence and presence of PVAT

5.1 Introduction

Hydrogen sulphide (H_2S) is a toxic gas which was first proposed as an endogenous mediator in mammals two decades ago by Abe and Kimura (1996). Since that time, many studies have confirmed the roles of H_2S in biology and in recent years, it has become the subject of intense investigation. H_2S is colourless, water soluble and characterized by a smell of rotten eggs. It is produced endogenously by three enzymes, namely: cystathionine-ß-synthase (CBS); cystathionine-γ-lyase (Bailey-Downs et al.); and the tandem enzymes, cysteine aminotransferase (Moreno-Navarrete et al.) and 3-mercaptopyruvate sulfurtransferase (3-MST). There is growing evidence that H_2S is a vasoregulator and plays a pivotal role in physiology and pathogenesis of the cardiovascular system (Polhemus and Lefer, 2014; Yu et al., 2014).

H_2S has a complex profile in the vasculature. The majority of literature showed that H_2S elicited vasorelaxant responses in a number of different arteries, including rat aorta and portal vein (Hosoki et al., 1997), rat mesenteric arteries (Hedegaard et al., 2016), rat mesenteric arterial bed (Cheng et al., 2004), pig bladder neck arteries (Fernandes et al., 2013), and pig intravesical ureter arteries (Fernandes et al., 2014). In contrast, H_2S also has been reported to induce dose-dependent vasoconstriction in sea lamprey dorsal aorta, marine toad aorta, alligator aorta and pulmonary arteries, duck aorta, and rat thoracic aorta (Dombkowski et al., 2005), biphasic concentration-dependent responses in rat aorta (Ali et al., 2006), and multiphasic contraction-relaxation-contraction in marine toad

pulmonary arteries, duck pulmonary arteries, and rat pulmonary arteries (Dombkowski et al., 2005).

Many experiments support the role of H_2S as a vasodilator (Dunn et al., 2016). The H_2S donor, sodium hydrogen sulfide (NaHS), sodium sulfide (Na_2S) and H_2S slow release agent, GYY4137, have been demonstrated to induce concentration-dependent vasodilatation in a number of blood vessels (Dunn et al., 2016). Moreover, gene therapy to increase synthesis of endogenous H_2S-producing enzymes markedly promotes renovascular relaxation in hyperhomocysteinemia of mice (Sen et al., 2012). It is now becoming clear that H_2S might possess more than one vasodilatation mechanism, depending on the species, tissues and experimental conditions. The most prominent mechanism is activation of ATP-sensitive potassium (K_{ATP}) channels (Cheng et al., 2004; Leffler et al., 2011; Zhao et al., 2001). Other mechanisms include activation of calcium conductance-activated small (SK_{Ca}), intermediate (IK_{Ca}), and large (BK_{Ca}) potassium channels (Jackson-Weaver et al., 2011; Mustafa et al., 2011; Yang et al., 2008), voltage-dependent potassium channels (KCNQ-type K_v) (Schleifenbaum et al., 2010), inhibition of L-type Ca^{2+} channels (Tian et al., 2012), release of neurotransmitter from perivascular nerves (Pozsgai et al., 2012; White et al., 2013) and involvement of nitroxyl (HNO) in nitroxyl-transient receptor potential ankyrin 1-calcitonin gene-related peptide (HNO-TRPA1-CGRP) signalling pathway (Eberhardt et al., 2014). On the other hand, several H_2S-induced vasocontractile mechanisms have also have been reported such as scavenging of nitric oxide (NO), inhibition of adenylate cyclase and modification of vasoactive prostanoid level (Beltowski et al., 2010; d'Emmanuele di Villa Bianca et al., 2011; Liu and Bian, 2010).

Despite the extensive research on H_2S, little is known about the relationship between H_2S and PVAT-derived sensory nerves. H_2S has been recently detected within PVAT and CSE has been shown to be expressed within PVAT and generates endogenous H_2S (Fang et al., 2009). The authors also proposed H_2S as a candidate or modulator of adipocyte-derived relaxing factor (ADRF)-activating K_v channels encoded by KCNQ genes to achieve paracrine control of vascular tone by PVAT (Schleifenbaum et al., 2010). H_2S has been shown to mediate the release of sensory neurotransmitters from perivascular nerves of small mesenteric arteries (White et al., 2013). More recently, CSE was found to be expressed within nerve fibers in pig intravesical ureter and DL-propargylglycine, a CSE inhibitor, was shown to reduce EFS-induced vasorelaxation (Fernandes et al., 2014). In addition, another recent study also showed mammalian neuromuscular synapses endogenously produce H_2S and are capable of evoking quantal transmitter release from motor nerve endings (Gerasimova et al., 2015). However, in all of these studies, the role of PVAT in the interaction between H_2S and sensory nerves was not investigated. In the preceding chapter, we have shown the presence of sensory nerves in PVAT of MABs. Therefore, the present study was undertaken to investigate the effect of H_2S on sensory neurotransmission in the absence and presence of PVAT and to examine the involvement of H_2S-producing enzymes and endogenous H_2S in PVAT-induced vasorelaxation in MABs.

5.2 Materials and methods

5.2.1 Rat perfused mesenteric arterial bed preparation

MABs were obtained and set up as described in Section 2.1.

5.2.2 Experimental protocol

Experiments were carried out in methoxamine (1-10 μM)-pre-constricted (30-70 mmHg above baseline) MABs with and without PVAT in the presence or absence of a sympathetic blocker, guanethidine (5 μM). Once a stable tone had been achieved to methoxamine, a frequency response curve (FRC) to electrical field stimulation (EFS) at 0.5-12 Hz, 60 V, 0.1 ms and 30 s was constructed. This was followed by the addition of sodium sulfide (Na_2S), a hydrogen sulfide (H_2S) donor through a chemical reaction, added cumulatively (1-300 μM) into the perfusate to generate a concentration-dependent relaxation response curve. Each concentration of Na_2S was added when a plateau of response to the previous concentration had been obtained. The pre-constricted tone of the MABs was then re-established and then a second frequency response curve (FRC) to EFS (0.5-12 Hz, 60 V, 0.1 ms and 30 s) was produced in both PVAT-intact and PVAT-denuded preparations. Sensory neuronal responses were generated as described in Section 2.2.2. In separate experiments to assess the modulatory effect of the H_2S donor on sensory neurotransmission, Na_2S (10 μM) was applied after the first FRC and MABs were incubated with it for 30 min before the second FRC was constructed. Na_2S (10 μM) was reapplied 5 min before each electrical stimulation to avoid H_2S loss from the Krebs'-Henseleit solution due to breakdown. In separate experiments

to examine a possible involvement of endogenous H_2S in the response to sensory neurogenic vasorelaxation, MABs were treated with aminooxyacetic acid (AOAA; 100 μM) a CBS inhibitor, DL-propargylglycine (PPAG; 100 μM) , a CSE inhibitor, and aspartic acid (1 mM), a 3-mercaptopyruvate sulfurtransferase (3MST) inhibitor, which were added for 30 min before the second FRC was generated.

In reproducibility assessments, MABs were exposed to cumulative concentrations of Na_2S (1 μM-3 mM) before washing the tissue with fresh Krebs'-Henseleit solution and pre-constricting vessels again with methoxamine. Each concentration was applied for 5 min before the addition of the next concentration. A second concentration response curve to Na_2S was then carried out. Responses to Na_2S were attenuated upon the second exposure. Thus, in a further series of experiments, only single cumulative concentration response curves to Na_2S were constructed. The effects of drugs were tested by adding these at least 30 min before the addition of Na_2S. The drugs used were: HC030031 (100 μM), a selective $TRPA_1$ channel blocker, and capsaicin (10 μM), a TRPV1 channel agonist which also can cause CGRP depletion.

5.2.3 Drugs

Drugs used were as described in Section 2.9. Sodium sulfide was freshly prepared on the day of experiment.

5.2.4 Statistical analysis

Data were analysed as described in Section 2.10.

5.3 Results

5.3.1 The effect of PVAT on H_2S-induced vasodilatation in MABs

In methoxamine pre-constricted MABs, Na_2S produced similar concentration-dependent (1-300 μM) vasodilatation in both PVAT-intact (pEC_{50} = 4.7 ± 0.1, R_{max} = 92 ± 10 %, n = 6) and PVAT-denuded (pEC_{50} = 4.7 ± 0.1, R_{max} = 81 ± 5 %, n = 6) preparations. In the absence of PVAT, Na_2S generated biphasic responses in which Na_2S at 1–10 μM induced vasoconstriction while Na_2S at 30-300 μM caused vasorelaxation (see Figure 5.1). In PVAT-denuded MABs, Na_2S at 30 μM caused a small vasocontractile response that differed to the lack of vasomotor response seen in PVAT-intact preparations ($P<0.05$, unpaired Student's t-test) (Figure 5.1).

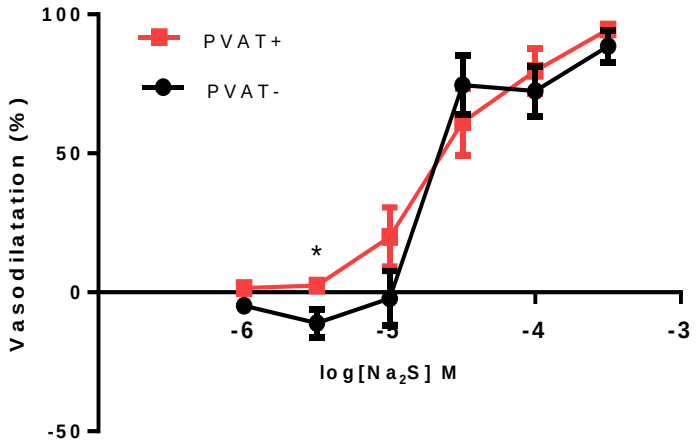

Figure 5.1: Cumulative concentration–response curves to Na_2S in methoxamine (1-5 μM) pre-constricted rat mesenteric arterial beds (MABs) with and without perivascular adipose tissue (PVAT). In both PVAT-intact (PVAT+) and PVAT-denuded (PVAT-) preparations, Na_2S elicited concentration–dependent responses. The contractile response of PVAT-denuded MABs was absent in MABs with PVAT (*P<0.05, unpaired Student's t-test) at 30 μM Na_2S.

5.3.2 Reproducibility of Na_2S-induced vasodilatation in MABs with and without PVAT

In PVAT-intact MABs, Na_2S induced cumulative concentration-dependent vasodilatation which was significantly reduced in the second concentration response curve (RC) at concentrations of 30 and 100 μM (P<0.001 and P<0.01, respectively, unpaired Student's t-test, n=6) (Figure 5.2A). In PVAT-denuded MABs, Na_2S caused biphasic responses for both RC1 (log pEC_{50} = 4.7 ± 0.1, R_{max} = 81 ± 5 %, n = 6) and RC2 (log pEC_{50} = 4.7 ± 0.1, R_{max} = 87 ± 6 %, n=6), with a small vasoconstriction at concentrations below 10 μM Na_2S and vasorelaxation at higher concentrations. Vasodilatation responses to Na_2S were significantly reduced

at 30 μM in the second response curve (P<0.05, unpaired Student's t-test, n=6) (see Figure 5.2B).

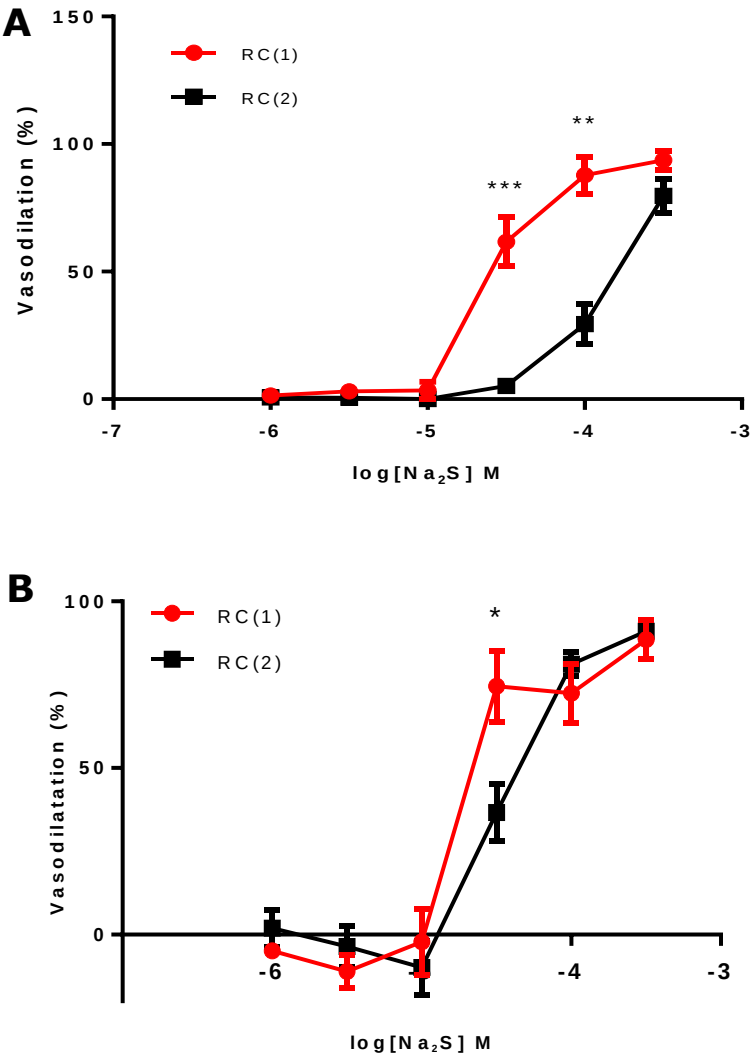

Figure 5.2: Effect of PVAT on reproducibility of Na_2S-induced vasodilatation in MABs. Preparations were submaximally pre-contracted with methoxamine (1-5 μM). (A) In the presence of PVAT, Na_2S caused concentration-dependent vasodilatation responses which were reduced at 30 and 100 μM in the second response curve (RC) (***P<0.001, **P<0.01 respectively, unpaired Student's t-test respectively, n=6). (B) In the absence of PVAT, the Na_2S-induced vasodilatation responses was slightly attenuated (P<0.05, unpaired Student's t-test) at 30 μM in the second RC.

5.3.3 The involvement of TRPA1 channels in Na_2S-induced vasodilatation in MABs

Recently, transient receptor potential ankyrin 1 (TRPA1) channels have been identified as being involved in Na_2S-induced vasodilatation responses (Eberhardt et al., 2014; Pozsgai et al., 2012; White et al., 2013). To investigate the involvement of this channel in mediating Na_2S-induced vasodilatation in PVAT-intact and PVAT-denuded preparations, 100 μM of HC030031, a TRPA1 antagonist, was added for a minimum of 30 min before a cumulative concentration (1-300 μM) response curve to Na_2S was conducted. In the presence of PVAT, Na_2S-induced vasodilatation responses were significantly inhibited by HC030031 (100 μM) at all concentrations of Na_2S ($P<0.05$, unpaired Student's t-test, n=4) (Figure 5.3A). In the presence of PVAT and HC030031, a vasocontractile response to low concentrations of Na_2S was observed, producing a biphasic response curve (contraction at concentrations including and below 30 μM) and relaxations at 100 and 300 μM Na_2S (Figure 5.3A). In the absence of PVAT, HC030031 also inhibited the responses to Na_2S and this was significant at 30 μM Na_2S ($P<0.01$, unpaired Student's t-test, n=4) (Figure 5.3B). These data point to an involvement of TRPA1 channels in Na_2S-induced vasorelaxation in MABs with and without PVAT. In addition, a vasocontractile response was revealed in PVAT-intact preparations.

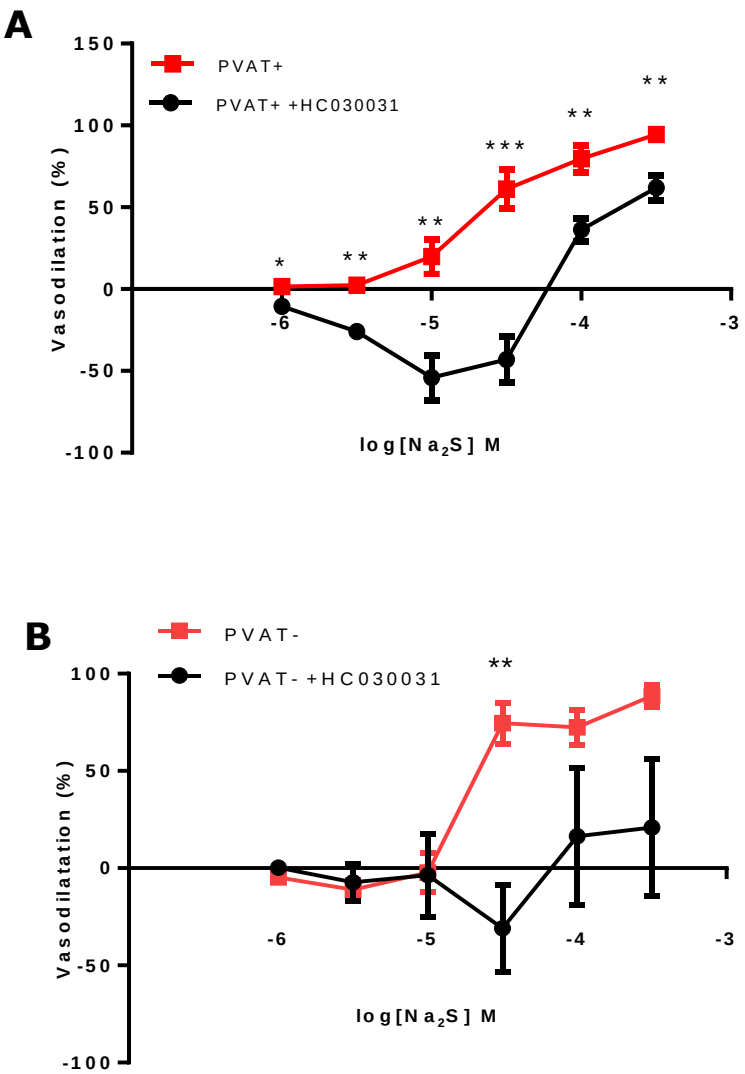

Figure 5.3: The involvement of TRPA1 channels in mediating Na_2S-induced vasodilatation in rat mesenteric arterial beds (MABs). MABs were submaximally pre-contracted with methoxamine (1-5 μM). (A) The presence of HC030031, a TRPA1 inhibitor, decreased Na_2S induced cumulative concentration vasodilatation responses at all concentrations of Na_2S in PVAT-intact preparations (*P<0.05, **P<0.01, ***P<0.001, Student's unpaired t-test, n=4) and (B) reduced responses to Na_2S at 30 μM in PVAT-denuded preparations (**P<0.01, Student's unpaired t-test, n=4).

5.3.4 The effect of capsaicin pre-treatment on Na_2S induced vasodilatation in MABs

The data above with HC030031, a TRPA1 inhibitor, are consistent with a possible involvement of sensory nerves in the relaxation response to Na_2S in MABs. To further investigate a possible involvement of sensory nerves, MABs with and without PVAT were treated with capsaicin (10 μM), added at the beginning of experiments and in contact with the tissue for a minimum time of 30 min before cumulative addition of Na_2S (1-300 μM). Incubation with capsaicin significantly reduced Na_2S-induced concentration-dependent (1-300 μM) relaxation responses in PVAT-intact preparations ($P<0.05$, unpaired Student's t-test, n=5) (Figure 5.4A). In the absence of PVAT, capsaicin also decreased Na_2S-induced concentration-dependent relaxation responses (n=5) as shown in Figure 5.4B. As with HC030031, a contractile response to Na_2S became apparent when relaxation was inhibited (Figure 5.4).

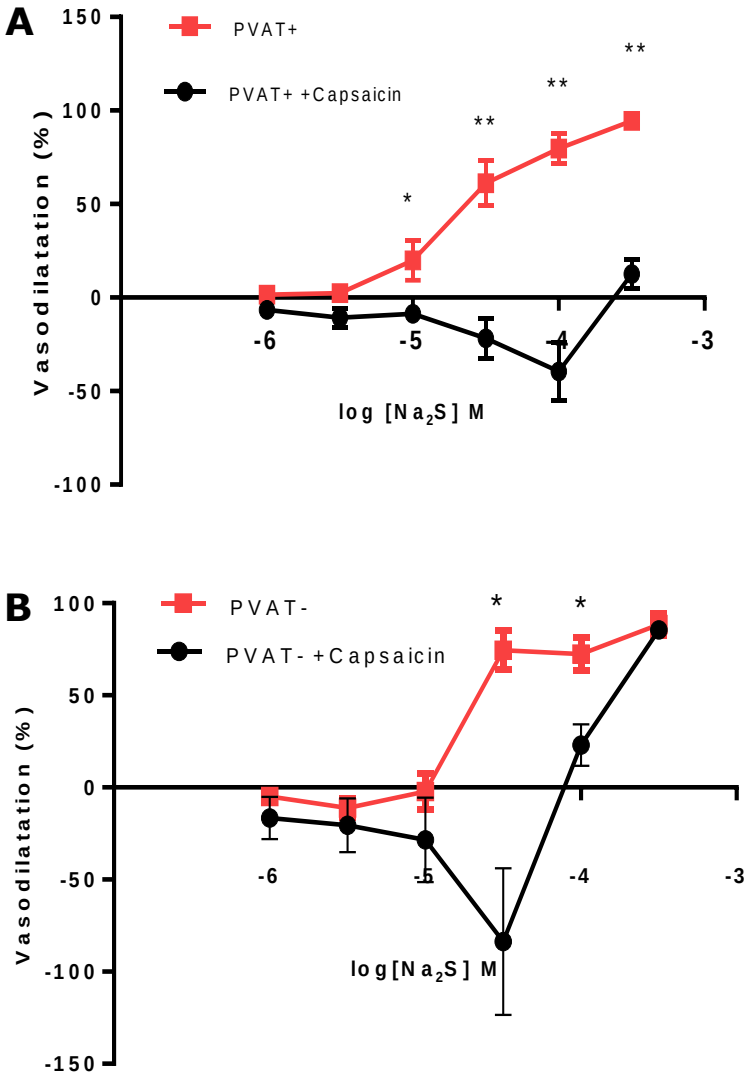

Figure 5.4: The effect of capsaicin (10 μM) treatment on Na_2S-induced concentration-dependent (1-300 μM) vasodilatation in rat mesenteric arterial beds (MABs). MABs were submaximally pre-contracted with methoxamine (1-5 μM). Capsaicin reduced Na_2S-induced concentration-dependent vasodilatation in MABs in both (A) PVAT-intact and (B) PVAT-denuded preparations. *P<0.05, **P<0.01 (unpaired Student's t-test, n=5).

5.3.5 Modulation by H_2S of electrically-evoked vasodilatation in the absence and presence of PVAT

EFS (0.5 -12 Hz, 60 V, 0.1 ms and 30 s) of perfused mesenteric vascular beds in the presence of guanethidine and methoxamine (to block sympathetic neurotransmission and to pre-constrict the preparations respectively), elicited frequency dependent vasodilatation in PVAT-intact but not in PVAT-denuded preparations (Figure 5.5). Previous experiments investigating MABs responses to two consecutive FRCs had shown that these responses are reproducible (see Section 4.3.4). After the end of the first FRC, MABs were incubated with Na_2S (10 μM), which was applied for 30 min before construction of a second FRC. In the present study, 10 μM of Na_2S produces only a very small direct relaxation response in MABs with or without PVAT. A second FRC was then conducted with addition of Na_2S (10 μM), 5 min prior to each stimulation. In the second FRC, in the presence of PVAT, EFS elicited frequency-dependent vasodilatation, however the responses were inhibited significantly in the presence of Na_2S (10 μM) ($P<0.01$, two-way ANOVA with Bonferroni's post-hoc test, n=6) (Figure 5.5A). In contrast, in the absence of PVAT, Na_2S significantly enhanced EFS-elicited vasodilatation responses ($P<0.01$, two-way ANOVA with Bonferroni's post-hoc test, n=5) (see Figure 5.5B).

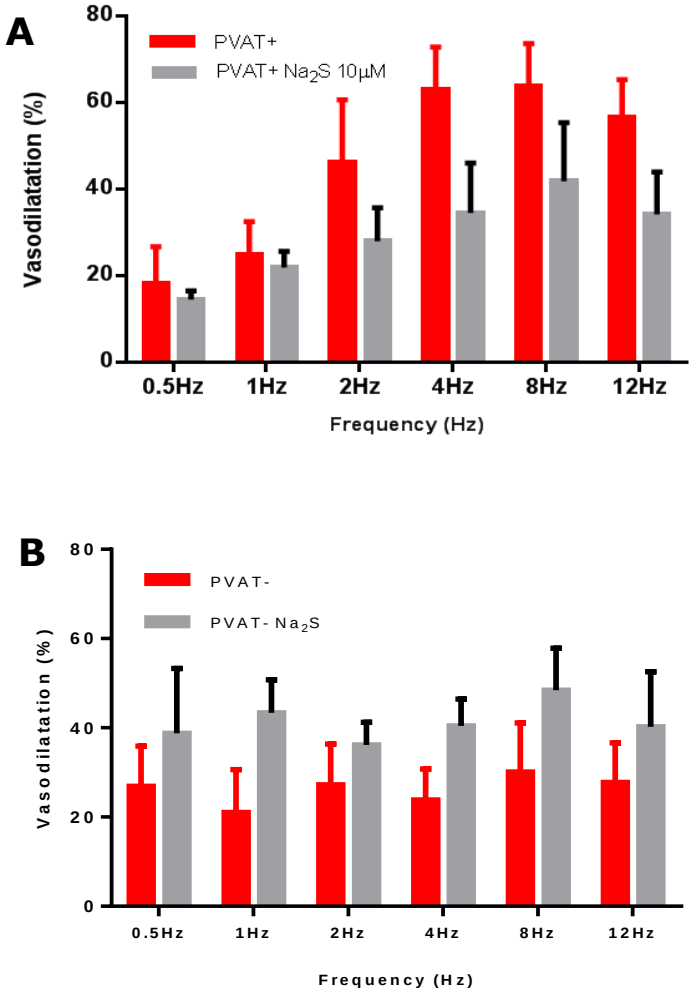

Figure 5.5: Modulation by H_2S of vasorelaxation responses to electrical field stimulation (EFS) of rat mesenteric arterial beds (MABs) (A) with and (B) without perivascular adipose tissue (PVAT). MABs were submaximally pre-contracted with methoxamine and guanethidine (5 µM) was present to block sympathetic neurotransmission. H_2S was applied as Na_2S (10 µM), added 5 min before stimulation at each frequency. (A) In the presence of PVAT, Na_2S decreased EFS-induced (0.5-12 Hz, 60 V, 0.1 ms) neurogenic vasorelaxation ($P<0.01$, two-way ANOVA, n=6) while (B) the responses were enhanced in the presence of Na_2S and absence of PVAT ($P<0.01$, two-way ANOVA, n=5).

5.3.6 The effect of a high concentration of Na_2S on neurogenic vasodilatation in PVAT-intact mesenteric arterial bed preparations

In PVAT-intact methoxamine (1-5 μM) pre-constricted MABs, in the presence of guanethidine (5 μM), to block sympathetic neurotransmission EFS (0.5-12 Hz, 60 V, 0.1 ms) elicited frequency dependent vasodilatation. Na_2S was added cumulatively (1-300 μM) at the end of the first FRC to generate a concentration-response curve during the interval as described in Section 5.3.1. Methoxamine was re-applied to pre-constrict the MABs and a second FRC was carried out. Responses to EFS were reduced in the presence of Na_2S in the second FRC and this effect was significant at frequencies of 1-8 Hz ($P<0.05$, $P<0.01$, $P<0.001$, two-way ANOVA with Bonferroni's post-hoc test, n=6) as shown in Figure 5.6.

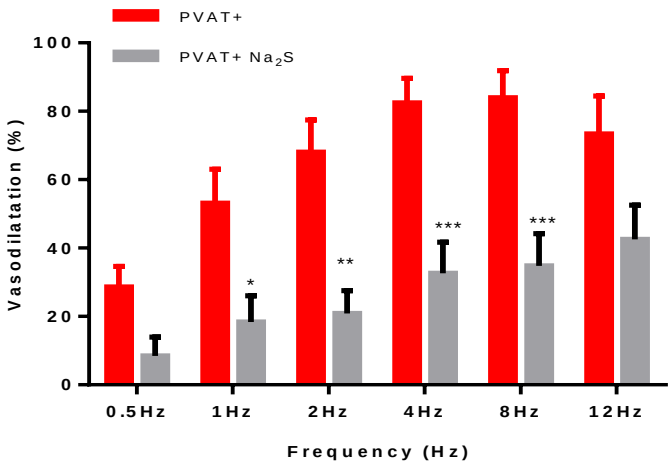

Figure 5.6: Effect of Na_2S on vasorelaxation to electrical field stimulation (EFS) of perivascular adipose tissue (PVAT) intact mesenteric arterial beds. Na_2S, an H_2S donor, applied as cumulative concentrations (1-300 μM, not shown), decreased EFS (0.5 -12 Hz, 60 V, 0.1 ms)-evoked frequency-

dependent vasorelaxation responses (*P<0.05, **P<0.01, ***P<0.001, two-way ANOVA with Bonferroni's post-hoc test, n=6).

5.3.7 The effect of H_2S-producing enzyme inhibition on EFS-induced vasorelaxation in PVAT-intact MABs

The involvement of H_2S-producing enzymes in neurogenic vasodilatation to EFS (0.5 -12 Hz, 60 V, 0.1 ms and 30 s) was examined using enzyme-specific inhibitors in PVAT-intact preparations. DL-propargylglycine (PPAG) (100 μM), amino oxyacetic acid (AOAA) (100 μM), and L-aspartic acid sodium (1 mM), inhibitors of CSE, CBS and 3-MST respectively, were added in separate experiments at the end of the first FRC and MABs were incubated for a minimum of 30 min. There was no difference in sensory neurogenic vasodilatation in the presence of any of the H_2S-producing enzyme inhibitors (P>0.05, unpaired Student's t-test) (n=4-6) as shown in Figure 5.7 (A, B, and C).

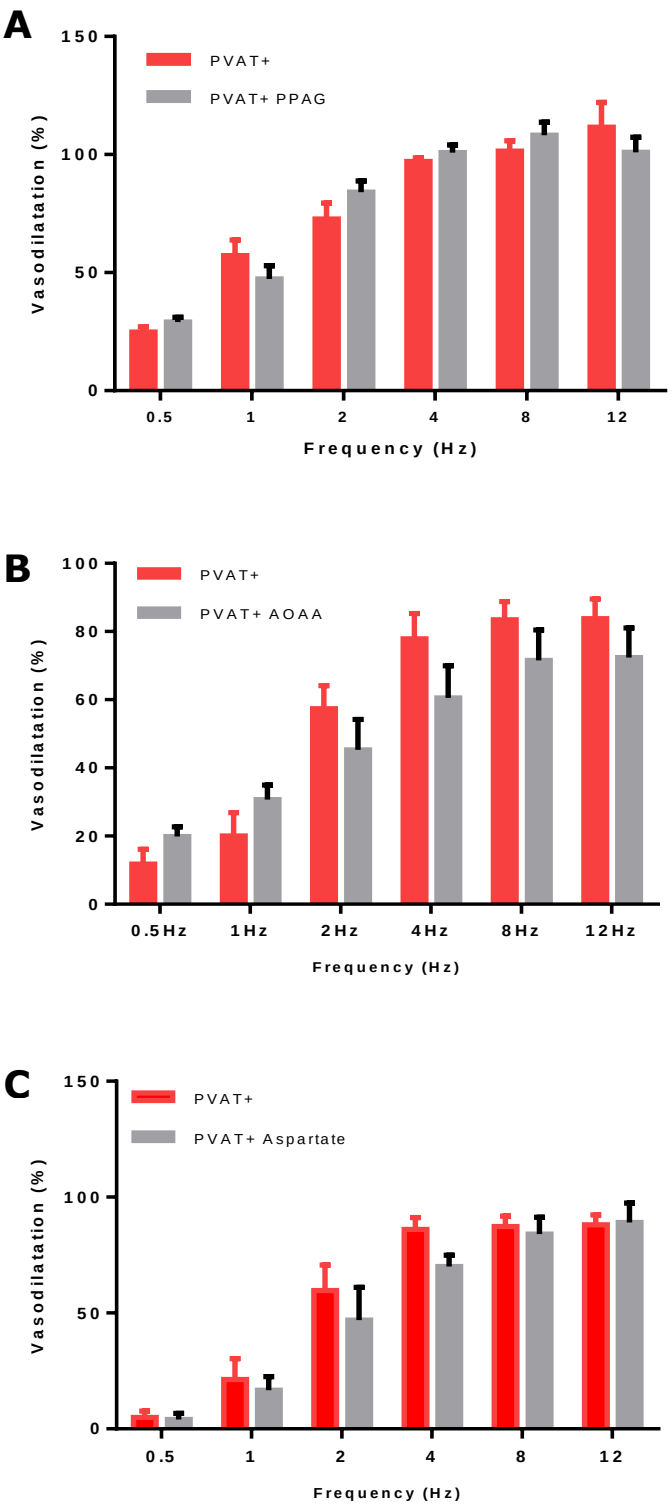

Figure 5.7: The effect of H_2S-producing enzyme inhibition on electrical field stimulation (EFS)-induced vasorelaxation responses in perivascular adipose tissue (PVAT)-intact mesenteric arterial beds (MABs). Preparations were submaximally pre-contracted with methoxamine and guanethidine (5 μM) was added to block sympathetic neurotransmission and EFS (0.5-12

Hz, 60 V, 0.1 ms and 30 s) was applied in two consecutive frequency response curves at an interval of 30 min. The presence of inhibitors of H_2S synthesis: (A) DL-propargylglycine (PPAG) (100 μM), a cystathionine-γ-lyase (Bailey-Downs et al.) inhibitor (100 μM), (B) amino oxyacetic acid (AOAA) (100 μM), a cystathionine-ß-synthase (CBS) inhibitor, and (C) L-aspartic acid sodium (1mM), a 3-mercaptopyruvate sulfurtransferase (3-MST) inhibitor, had no effect on sensory neurogenic vasodilatation ($P>0.05$, Student's unpaired t-test, n=4-6).

5.4 Discussion

In the preceding chapter, I demonstrated the role of PVAT on sensory neurotransmission and existence of sensory nerves within PVAT of MABs. In this chapter, the effect of H_2S on sensory nerve-mediated vasodilatation responses in the presence and absence of PVAT was investigated. The main findings in the present chapter are:

(1) in the presence of PVAT, a H_2S donor, Na_2S, caused concentration-dependent vasodilatation while in the absence of PVAT, Na_2S generated biphasic responses. Na_2S responses were not reproducible in preparations with and without PVAT, and thus suggests involvement of a desensitization mechanism. These vasodilatation responses were inhibited by a TRPA1 inhibitor, which suggests the involvement of TRPA1 channels in Na_2S-evoked relaxation. Since pre-treatment with capsaicin markedly reduced Na_2S induced vasorelaxation in both preparations, these data

suggest that H_2S induces vasodilatation in MABs with and without PVAT through the release of CGRP from sensory nerves.

(2) Na_2S can act as an inhibitory modulator of sensory neurotransmission in the MABs.

(3) H_2S-producing enzymes, cystathionine-ß-synthase (CBS), cystathionine-γ-lyase and the tandem enzymes cysteine aminotransferase and 3-mercaptopyruvate sulfurtransferase (3-MST), are not involved in EFS-evoked neurogenic vasodilator responses under the conditions of the present study.

5.4.1 H_2S regulates MABs tone by activation of sensory nerves-TRPA1-CGRP signalling pathway

In the rat mesenteric arterial bed, a preparation which has abundant PVAT and sensory neurotransmission, Na_2S induced concentration-dependent (1-300 μM) vasodilatation in PVAT-intact preparations. This observation is consistent with a study by Cheng et al. (2004), which showed that both NaHS, a H_2S donor, and H_2S-saturated solution, caused concentration-dependent (1 μM-10 mM) vasorelaxation in MABs. Their study also used PVAT-intact MABs, but in the presence and absence of endothelium, and concluded that the H_2S-induced relaxation in the MABs was partially due to activation of K_{ATP} channels and was mediated by the endothelium via an endothelium-derived hyperpolarizing factor (EDHF)-related mechanism (Cheng et al., 2004). In the present study, the endothelium was left intact; the aim of the study was to determine the role of PVAT in H_2S-induced vasorelaxation in MABs.

There was a slight difference in Na_2S-induced concentration-dependent (1-300 μM) responses between PVAT-intact and PVAT-denuded preparations; at a concentration of 30 μM, Na_2S caused a small vasocontractile response in PVAT-denuded preparations that differed to the lack of vasomotor response in PVAT-intact preparations (Figure 5.1). In the absence of PVAT, Na_2S generated biphasic responses in which Na_2S at 1–10 μM induced vasoconstriction while Na_2S at 30-300 μM caused vasorelaxation. A similar observation also has been demonstrated in rat aorta in which lower concentrations (50-100 μM) of NaHS or H_2S gas in solution resulted in vasoconstriction while high concentrations (200-1600 μM) of NaHS, or H_2S gas in solution, caused vasorelaxation (Ali et al., 2006). That study also showed that the NaHS responses were inhibited by glibenclamide, a K_{ATP} channel inhibitor, at high but not at low concentrations of NaHS (Ali et al., 2006), thus indicating the potential involvement of different signalling mechanisms at different concentrations of H_2S.

In the present study, responses to Na_2S were not reproducible in both PVAT-intact and PVAT-denuded preparations. It is apparent that most studies seem to report examination of the effects of inhibitors on an unpaired basis. However, there are a few reports which showed that NaHS-induced vasorelaxation responses were not reproducible in several elasmobranch species and small mesenteric arteries (Dombkowski et al., 2005; White et al., 2013). The observation suggests that the desensitization took place as a consequence of a non-specific metabolic inhibition via suppression of oxidative phosphorylation produced by high concentration (>100 μM) of NaHS (Kiss et al., 2008). However, White et al. (2013) showed in rat small mesenteric arteries that the desensitization also occurred at low concentration of NaHS (30 μM). Desensitization is a complex process that can involve receptor phosphorylation, downregulation

and internalisation (Akopian et al., 2007). Desensitization is a dose-dependent phenomenon, single lower dose of an agonist can cause activation, while repeated application of low doses or a single high dose can lead to immediate desensitization (Kennedy et al., 2010; LaMotte et al., 1991; Simone et al., 1989). In the context of the present study, possible mechanisms are desensitization of TRPV1, and/or TRPA1 channels and also depletion of sensory neurotransmitters from nerves. The molecular mechanisms underlying agonist-induced TRPV1 desensitization and/or tachyphylaxis are still not well understood (Sanz-Salvador et al., 2012). Persistent exposure of TRPV1 to agonists induces rapid receptor endocytosis and lysosomal degradation in both sensory neurons and recombinant systems (Sanz-Salvador et al., 2012). For instance, capsaicin, a putative TRPV1 receptor agonist which has been widely used to study TRPV1 desensitization, has been reported to desensitize sensory neurons as well as to induce degeneration of the DRG sensory afferents when it is administered in supratherapeutic doses to neonatal rodents (Jancso et al., 1977). Similarly, the underlying mechanism/s for functional TRPA1 channel desensitization is still unknown (Akopian et al., 2007). However, it is proposed that the TRPA1 channel desensitization mechanisms involve multiple cellular pathways that are agonist dependent and modulated by TRPV1 (Akopian et al., 2007). The same group demonstrated that capsaicin-induced pharmacological desensitization of TRPA1 channels is Ca^{2+}-dependent, with involvement of phosphatidylinositol (4,5)-bisphosphate (PIP2) depletion through activation of phospholipase C (Akopian et al., 2007).

Previous studies have shown that CGRP is the neuropeptide that is involved in H_2S-induced vasorelaxation (Eberhardt et al., 2014; Pozsgai et al., 2012; White et al., 2013), consistent with the important role of this

neuropeptide as a vasodilator neurotransmitter in sensory nerves. To determine whether sensory nerves were involved in Na_2S-induced vasorelaxation, MABs were pre-treated with capsaicin (10 μM) for 30 mins. Similar to previous studies (Eberhardt et al., 2014; Pozsgai et al., 2012; White et al., 2013), responses to NaHS were sensitive to capsaicin pre-treatment, which desensitizes TRPV1 or/and TRPA1 channels on sensory nerves and lead to the release and depletion of CGRP. Na_2S-induced vasorelaxation was attenuated due to capsaicin treatment in both PVAT-intact and denuded preparations. In PVAT-intact preparations, Na_2S-induced vasorelaxation was reduced at high concentrations (up to and including 300 μM) while in the absence of PVAT, the response to 300 μM Na_2S was insensitive to capsaicin pre-treatment. One possible explanation is that the effective concentration of Na_2S is greater in preparations without PVAT (due to less absorption and/or breakdown) leading to capsaicin-resistant non-specific effects. However, the overall conclusion from these data is that, in MABs, pre-treatment with a relatively high concentration of capsaicin activates TRPV1 and/or TRPA1 channels on sensory nerves and causes CGRP depletion, which then leads to inhibition of vasodilatation due to Na_2S.

It has been demonstrated that H_2S activates TRPV1 channels on sensory nerves and consequently releases neuropeptides in rat bladder (Trevisani et al., 2005), however, more recent evidence showed that TRPV1 channel blockers, capsazepine and BCTC, did not inhibit NaHS-induced vasorelaxation in rat small arteries (Pozsgai et al., 2012; White et al., 2013). Furthermore, Pozsgai et al. (2012) showed that genetic deletion of TRPV1 in trachea of knockout mice had no effect on vasorelaxant responses to NaHS and to allyisothiocyanate (Marti et al.), a TRPA1 and TRPV1 activator, compared to control mice (C57BL/mice). More recently,

an involvement of TRPA1 channels in relaxation to H_2S has been shown in rat mesenteric small arteries, rat tracheae and the mouse ear vasculature (Pozsgai et al., 2012; White et al., 2013). In the present study, Na_2S-induced vasorelaxation was markedly reduced in the presence of HC030031, a TRPA1 channel blocker, in both PVAT-intact and PVAT-denuded preparations, in line with the observations of White et al. (2013) in rat mesenteric small arteries. This finding is consistent with previous studies on the involvement of sensory nerves-TRPA1 channel-CGRP signalling pathway in rat small mesenteric arteries, rat tracheae and mouse ear (Pozsgai et al., 2012; White et al., 2013). The current study did not investigate the effect of vehicle dimethyl sulphoxide (DMSO). The concentration of DMSO used in the methyl palmitate experiments was 0.1 % v/v. Previous studies demonstrated that DMSO at the final concentration (0.1 % v/v) did not affect electrically-evoked contractions of rat small mesenteric arteries (Kun et al., 2014). Based on the previous data, although it is less likely that the concentration of DMSO used in the current study affected the present data, however, future study to investigate the DMSO vehicle effect is warranted.

Other mechanisms, in addition to sensory nerves, may be involved in the relaxation to Na_2S in the rat mesenteric arterial bed. White et al. (2013) showed in rat small mesenteric arteries that the NaHS-induced vasorelaxation response at 30 μM was attenuated by high extracellular potassium concentration treatment, but was resistant to glibenclamide, tetraethylammonium ion (a BK_{ca} channel inhibitor), and XE991 (a K_v channel inhibitor). However, an alternative explanation for the effect of high extracellular potassium on NaHS-induced vasorelaxation is that depolarization stimulates sensory nerves and releases sensory neuropeptides (White et al., 2013). Previous studies have shown that H_2S-

induced vasorelaxation was sensitive to 4,4′ diisothiocyanatostilbene-2,2′-disulfonic acid (DIDs), a chloride channel blocker (Kiss et al., 2008; White et al., 2013). However, it was found that neither of the non-selective chloride channel blockers, NPPB or A9C, affected the responses to NaHS, thus excluding the involvement of gating of chloride channels in the rat mesenteric arteries (White et al., 2013). A possible explanation for this observation is that intracellular acidification activated the $Cl^-/HCO3^-$ exchange protein during the transduction process and this observation has been reported in both rat aorta (Lee et al., 2007) and cultured glial cells (Lu et al., 2010b). It has been shown that intracellular acidification with pH values of 5.5-6 stimulates CGRP secretion (Vause et al., 2007).

5.4.2 Modulation of H_2S on electrically-evoked neurogenic vasodilatation in MABs

The present study was conducted to investigate the modulation by H_2S of electrically-evoked neurogenic vasodilation in MABs in the absence and presence of PVAT. Two different experimental approaches were used; Na_2S was added cumulatively (1-300 μM) or a single concentration (10 μM) of Na_2S was added before each electrical stimulation. In PVAT-intact preparations, the presence of Na_2S at 10 μM resulted in a significant reduction in EFS-elicited frequency-dependent vasorelaxation and addition of a high concentration of Na_2S in a cumulative concentration fashion abolished EFS-evoked neurogenic vasorelaxation in MABs, which resembled a capsaicin-like action. In PVAT-denuded preparations, the presence of Na_2S enhanced electrically-evoked vasorelaxation, however these responses were not frequency-dependent and thus may indicate that these responses were not neurogenic. Modulation by H_2S of electrically-evoked

neurogenic vasodilatation in MABs has not previously been reported. The endogenous source of the H_2S is unclear and from the studies using the H_2S synthesizing enzyme inhibitors, we found that it did not appear to be produced by the PVAT or vasculature, at least under the conditions of the present study (discussed below in Section 5.4.3). However, the involvement of endogenous H_2S mediated inhibitory neurotransmission in pig bladder neck (Fernandes et al., 2013) and pig intravesical ureter have been recently reported (Fernandes et al., 2014). These studies suggest that H_2S, synthesized by CSE, acts as a potent inhibitory neurotransmitter to the pig intravesical ureter through a NO independent mechanism, producing smooth muscle relaxation via K_{ATP} channel activation. H_2S also promotes the release of pituitary adenylate cyclase-activating polypeptide (PACAP) 38 and CGRP from capsaicin-sensitive primary afferents through activation of TRPA1, TRPV1 and/or related ion channels in the sensory nerves (Fernandes et al., 2014). The explanation for the discrepancy with the present study is likely to be related to the difference between the biological tissues.

5.4.3 The effect of H_2S-producing enzyme inhibition on EFS-induced vasorelaxation in PVAT-intact MABs

In order to investigate the crosstalk between PVAT and endogenous H_2S, intact PVAT preparations were treated with specific H_2S-producing enzymes antagonists; DL-propargylglycine (PPAG), amino oxyacetic acid (AOAA), and L-aspartic acid sodium, inhibitors of CSE, CBS and 3-MST, respectively. None of these inhibitors reduced sensory neurogenic vasodilatation, which indicates that H_2S-producing enzymes were not involved in sensory neurogenic vasodilatation in the present study. In contrast, a previous

study proposed a significant involvement of CSE in the activation of inhibitory neurotransmission in pig intravesical ureter, which led the authors to concluded that it is H_2S which activates sensory nerves and causes relaxation in intravesical ureter (Fernandes et al., 2014). Species, tissues and experimental procedure differences probably are the reasons for this discrepancy. To the best of my knowledge, this is the first study that has investigated the effect of H_2S on electrically-evoked sensory neurotransmission in the vasculature, and the involvement of H_2S producing enzymes in EFS-induced neurogenic vasodilation responses in the presence of PVAT.

5.4.4 Conclusion

In conclusion, H_2S causes vasodilatation of MABs by activating sensory nerves through the TRPA1-CGRP signalling pathway, and subsequently impairs sensory nerve function, demonstrating a capsaicin-like action. The present study also suggests that H_2S-producing enzymes and endogenous H_2S are not involved in EFS-evoked neurogenic vasodilator responses under the conditions of the present study. This is the first study that demonstrates that H_2S inhibits electrically-evoked sensory nerve actions using the perfused mesenteric vascular bed, a preparation with abundant PVAT. The present study is also the first to investigate the effect of H_2S on sensory neurotransmission in the presence of PVAT, and this study also indicates the activation of the sensory nerve-TRPA1-CGRP signalling pathway as one of H_2S vasorelaxant mechanisms in in rat MAB PVAT.

Chapter 6

The interactions between sympathetic and sensory nerves and PVAT-derived mediators

6.1 Introduction

PVAT is receiving increasing attention due to the recognition that it is a source of a number of vasoactive compounds and has a potential role in physiological and pathological conditions; it therefore holds promise as a novel therapeutic target. Despite this, information on the link between PVAT-derived mediators and the neurovascular system is sparse.

The interactions between sympathetic nerves and the most studied adipokine, leptin, have been reviewed (Hall et al., 2010; Rayner and Trayhurn, 2001). Recently, it has become apparent that the sympathetic system is a key regulator of leptin production in white adipose tissue (WAT) (Rayner and Trayhurn, 2001). Sympathomimetic amines and cold exposure or fasting (which results in sympathetic stimulation in WAT), decrease leptin gene expression in the tissue and thus leptin production (Rayner and Trayhurn, 2001). Conversely, sympathetic blockade often increases circulating leptin and leptin gene expression, and it has been postulated that the sympathetic system might contribute to support an inhibitory action on leptin synthesis (Rayner and Trayhurn, 2001). In obesity, it has been shown that cytokines derived from adipocytes such as leptin, tumor necrosis factor-α, and interleukin-6 mediate sympathetic activation (Hall et al., 2010).

The relationship between sensory nerves and adipokines is less studied and very little is known about it. A recent publication provides evidence for an interaction between leptin and sensory nerves in WAT, in which endogenous leptin secreted from white adipocytes acts by paracrine signalling to activate spinal sensory nerves innervating the tissue (Murphy et al., 2013). The authors also demonstrated the existence of the long form

of the leptin receptor (Ob-Rb) on dorsal root ganglion (DRG) pseudounipolar neurons immunohistochemically labelled after injections of fluorogold, a retrograde tract tracer, into inguinal WAT (Murphy et al., 2013). Another study suggests that interleukin-6, a cytokine, is essential to modulate sensory functions in inflammatory and immune reactions *in vivo* (Zhong et al., 1999). Very little is known about the interactions between sensory nerves and PVAT-derived mediators in the vasculature.

Based on the limited existing literature, it can be seen that there are complex interactions between sympathetic and sensory nerves with adipokines. These nerves play an important role in mediating adipokines release, and vice versa. The present study was carried out to investigate the effect of electrically-evoked sympathetic and sensory activation on the release of PVAT-derived mediators, namely adiponectin, leptin, interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor alpha (TNF-α), interleukin beta (IL-β) and total plasminogen activator inhibitor-1 (PAI-1), under standard experimental oxygen (95 % oxygen (O_2) and 5 % carbon dioxide (CO_2)) and low oxygen (95 % nitrogen (N_2) and 5 % CO_2) conditions. There is accumulating evidence that indicates that the anti-contractile function of PVAT is reduced under pathological states (Szasz and Webb, 2012) and one of the explanations is a possible alteration of relaxation factor(s) secreted from PVAT (Chatterjee et al., 2009; Lohmann et al., 2009; Szasz and Webb, 2012). Previous studies have shown that hypoxia lessens the anti-contractile effect of PVAT in mesenteric arteries (Greenstein et al., 2009) but enhances the vasorelaxing effect of PVAT in aorta (Maenhaut et al., 2010). The present study investigated the effect of decreasing the perfusate oxygen tension on the release of PVAT-derived mediators in MABs. Altogether, the results from the present study can contribute to understanding of the interaction

between PVAT and nerves controlling the vasculature which is poorly recognized and extremely important for normal function of blood vessels as well as therapeutic intervention.

6.2 Material and methods

6.2.1 Rat perfused mesenteric arterial bed preparation

MABs were obtained and set up as described in Section 2.1. The flow rate was reduced to 3 ml min^{-1} in these experiments (compared to 5 ml min^{-1}) with the aim of concentrating analytes released from the MABs into the collected perfusate (refer to Figure 2.2). In the same preparations (either with or without PVAT), perfusate was collected at basal tone conditions (control) and during the electrical field stimulation (EFS) evoked vasocontractile response at 24 Hz, 90 V, 1 ms, 30 s for measuring adipokines release during sympathetic activation. Fractions were collected at basal tone for sympathetic control (5 min before EFS), and during EFS-evoked sympathetic activation, for 1 min. In separate experiments, the sensory responses were conducted in preparations in the presence of guanethidine (5 μM) and methoxamine (1-5 μM), to inhibit sympathetic neurotransmission and pre-contract the preparations respectively. EFS at 8 Hz, 60 V, 0.1 ms, 30 s was carried out to evoke a neurogenic vasodilatation (sensory) response. Fractions were collected at raised tone for sensory control (5 min before EFS), and during EFS-evoked sensory stimulation for 1 min.

Under standard oxygen conditions, preparations were perfused and gassed with 95 % O_2 and 5 % CO_2. After EFS and collection of fractions under

standard oxygen conditions, the low oxygen pressure/hypoxic condition was introduced by gassing the Krebs'-Henseleit buffer with 95 % nitrogen (N_2) and 5 % CO_2, and the preparations were then perfused with this for 1.5 hours. Fractions were then immediately stored in a -80ºC freezer for 24 hours and then lyophilised using a freeze dryer (Edwards Modulyo, U.S.A) at -80^{0}C for 48 hours. Prior to assay, these samples were reconstituted with 50 µl of distilled water per tube, in order to concentrate the samples.

6.2.2 Rat adipocyte immunoassay/Multiplex assay procedure

Samples of MAB perfusate were collected and prepared as described in Section 2.6.2. EFS was generated as described in Section 2.2.1 and 2.2.2. Multiplex assay was conducted and the multiplex plate was analysed as described in Section 2.6.2 and 2.6.3 Seven types of antibody-immobilized (adiponectin, leptin, MCP-1, IL-1β, IL-6, TNF-α and total PAI-1) beads were prepared by sonicating the individual vials of beads for 30 seconds and then vortexing them for 1 minute. 150 µl from each antibody-beads vial was added into a mixing bottle and finally, 1.95 ml assay buffer was added to the mixing bottle.

6.2.3 Statistical analysis

Data were analysed as described in Section 2.10.

6.3 Results

6.3.1.1 The effect of standard and low oxygen supply on neurogenic vasoconstriction of perfused MABs

At a flow rate of 3 ml min^{-1}, there was no difference in basal perfusion pressure between MABs at standard oxygen (51 ± 4 mmHg) and low oxygen (49 ± 4 mmHg) groups (P>0.05, paired Student's t-test, n=10) as shown in Figure 6.1A. EFS at 24 Hz, 90 V, 1 ms, 30 s of perfused mesenteric vascular beds at resting tone produced a vasocontractile response, which was not different between standard oxygen (115 ± 7 mmHg) and low oxygen (123 ± 9 mmHg) groups (P>0.05, paired Student's t-test, n=7) (Figure 6.1B).

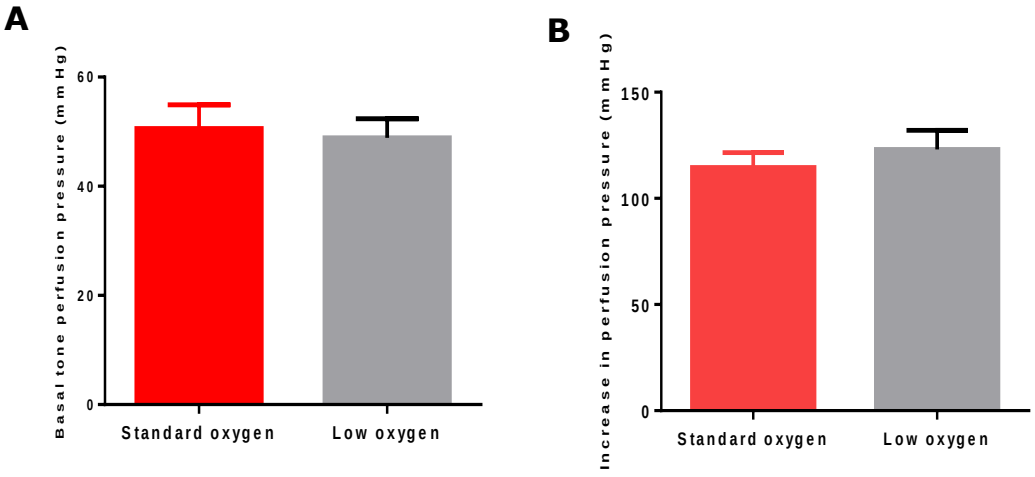

Figure 6.1: **(A)** Basal perfusion tone of rat perfused mesenteric vascular beds between standard oxygen and low oxygen groups. The basal tone was not different between standard oxygen and low oxygen groups (P>0.05, paired Student's t-test, n=10). **(B)** EFS at 24 Hz, 90 V, 1 ms, 30 s of perfused mesenteric vascular beds generated vasoconstriction in both standard oxygen and low oxygen groups, however, there was no difference between them (P>0.05, paired Student's t-test, n=7).

6.3.1.2 The effect of standard and low oxygen supply on neurogenic vasorelaxation of perfused MABs

In MABs pre-contracted with methoxamine (1-5 μM) and in the presence of guanethidine (5 μM), at a flow rate of 3 ml min^{-1}, raised tone in the standard oxygen (48 ± 9 mmHg) preparations was significantly decreased in preparations with a low oxygen supply (14 ± 7 mmHg) ($P<0.05$, paired Student's t-test, n=8) as shown in Figure 6.2A. EFS at 8 Hz, 60 V, 0.1 ms, 30 s of the perfused mesenteric vascular beds under raised tone produced a vasorelaxant response, which was not different between the standard oxygen (73 ± 5 %) and low oxygen (67 ± 18 %) groups ($P>0.05$, paired Student's t-test, n=7) (see Figure 6.2B).

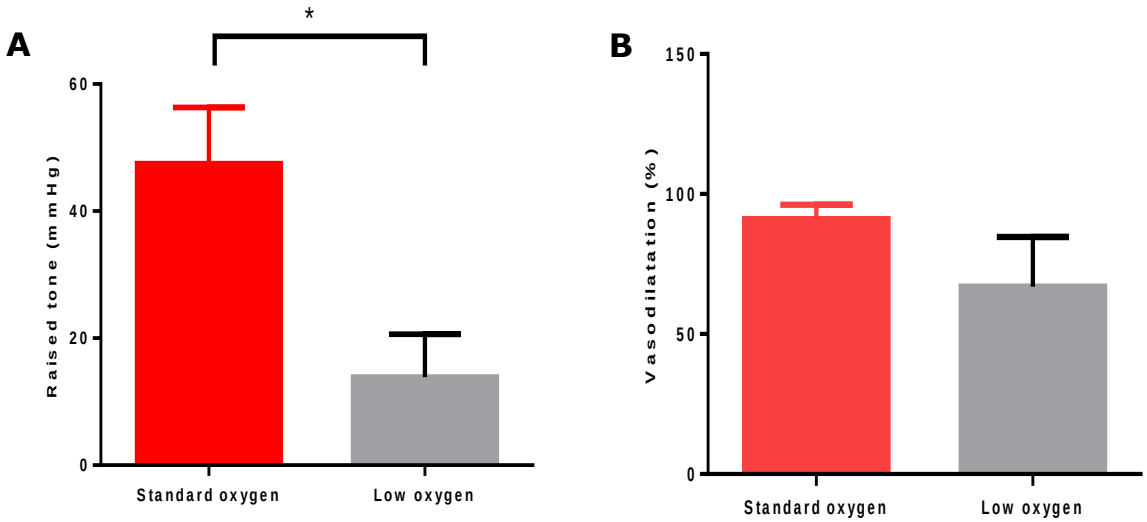

Figure 6.2: Effect of standard and low oxygenation of the Krebs'-Henseleit solution on the level of methoxamine-induced tone and neurogenic vasodilatation in perfused rat mesenteric arterial beds. Preparations were submaximally pre-contracted with methoxamine (1-5 μM) and guanethidine (5 μM) was present to block sympathetic neurotransmission. (A) Raised perfusion tone in standard oxygen and low oxygen groups. The raised tone in standard oxygen preparations was attenuated in the low oxygen group (*P<0.05, paired Student's t-test, n=8). (B) Electrical field stimulation (EFS) at 8 Hz, 60 V, 0.1 ms, 30 s of perfused mesenteric vascular beds generated vasorelaxation in both standard oxygen and low oxygen groups, which were not different (paired Student's t-test, n=5).

6.3.2 Adiponectin

6.3.2.1 The effect of PVAT on adiponectin release during EFS-induced neurogenic vasocontractile responses

In perfused MABs, under basal tone conditions (control), the adiponectin level detected in the perfusate was not significantly different than PVAT-denuded MABs preparations ($P>0.05$, one-way ANOVA with Bonferroni's post-hoc test, n=5-9) as shown in Figure 6.3. There was no difference in the level of adiponectin in the perfusate collected during EFS-evoked neurogenic vasocontractile responses compared to basal tone in either PVAT-intact or PVAT-denuded preparations ($P>0.05$, one-way ANOVA with Bonferroni's post-hoc test, n=5-10) (Figure 6.3).

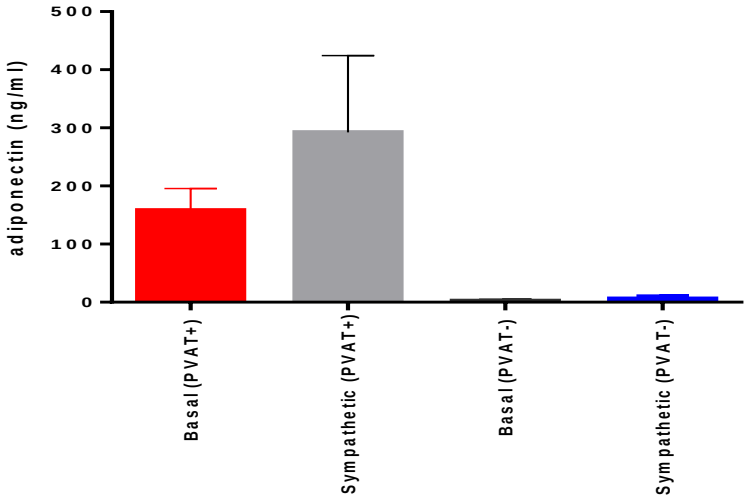

Figure 6.3: The effect of perivascular adipose tissue (PVAT) on adiponectin release at basal tone conditions and during electrically-evoked (EFS; 24 Hz, 90 V, 1 ms, 30 s) sympathetic neurogenic vasocontractile responses. There was no difference in adiponectin release between PVAT-intact (PVAT+) and PVAT-denuded (PVAT-) preparations. $P>0.05$ (one-way ANOVA with Bonferroni's post-hoc test, n=5-10).

6.3.2.2 The effect of PVAT on adiponectin release during EFS-induced neurogenic vasorelaxation responses

In MABs pre-contracted with methoxamine (1-5 μM) and in the presence of guanethidine (5 μM), it was found that PVAT-intact preparations released a similar amount of adiponectin into the perfusate as at basal tone indicating good reproducibility of the assay (compare control PVAT+ in Figure 6.3 and Figure 6.4). At raised tone conditions PVAT-intact MABs released a significantly greater amount of adiponectin into the perfusate than PVAT-denuded preparations ($P<0.01$, one-way ANOVA with Bonferroni's post-hoc test, n=5-11) (Figure 6.4). At raised tone, adiponectin release was similar with electrically-evoked (EFS: 8 Hz, 60 V, 0.1 ms, 30 s) neurogenic vasorelaxation ($P>0.05$, one-way ANOVA, n=5-11) (Figure 6.4).

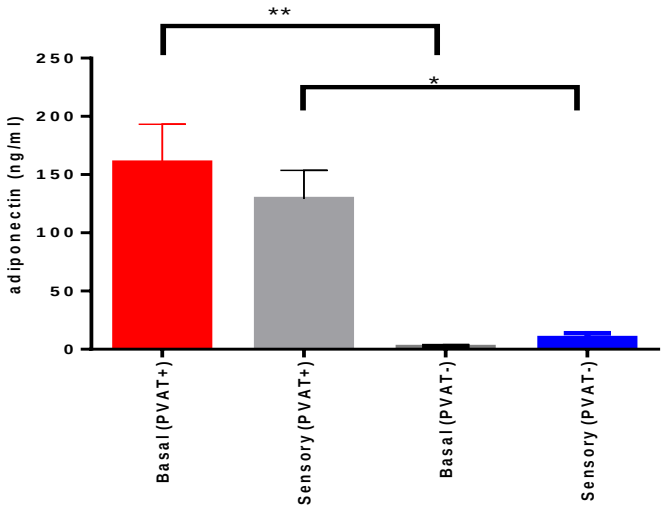

Figure 6.4: The effect of perivascular adipose tissue (PVAT) on adiponectin release under raised tone and during electrically-evoked (EFS; 8 Hz, 60 V, 0.1 ms, 30 s) neurogenic vasorelaxation of pre-constricted mesenteric arterial beds (MABs). Tone of the preparations was raised with methoxamine, and guanethidine (5 μM) was present to block sympathetic constriction. The absence of PVAT (PVAT-) abolished the adiponectin level

collected in the perfusate in both basal conditions and during EFS (sensory). *<0.05, **P<0.01 (one-way ANOVA with Bonferroni's post-hoc test, n=5-11).

6.3.2.3 The effect of standard and low oxygen level on adiponectin release: effect of PVAT and EFS-induced neurogenic vasoconstriction

In the same preparations (with PVAT), the effect of reduced oxygenation on adiponectin release was investigated and it was found that reducing the level of oxygen had no significant effect on adiponectin levels of the perfusate in PVAT-intact MABs (Figure 6.5). The level of adiponectin in the perfusate collected during EFS (24 Hz, 90 V, 1 ms, 30 s) was also not significantly different to that in the controls in the absence of EFS, during either under standard oxygen or low oxygen (P>0.05, one-way ANOVA with Bonferroni's post-hoc test, n=5-9) (Figure 6.5).

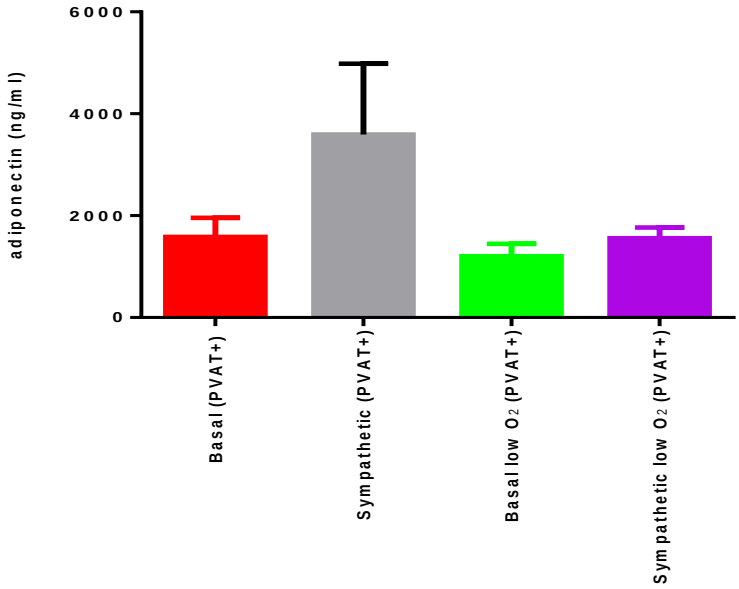

Figure 6.5: The effect of oxygen level on adiponectin release at basal tone and during electrically-evoked (EFS; 24 Hz, 90 V, 1 ms, 30 s) neurogenic vasocontractile responses of mesenteric arterial beds with intact perivascular adipose tissue (PVAT+). There was no difference in adiponectin release under conditions of standard and low oxygen supply ($P>0.05$, one-way ANOVA with Bonferroni's post-hoc test, n=5-9).

6.3.2.4 The effect of standard and low oxygen on adiponectin release: effect of EFS-induced neurogenic vasorelaxation

In both standard and low oxygen supply, adiponectin was detected in the perfusate of PVAT-intact MABs pre-contracted with methoxamine (1-5 μM), in the presence of guanethidine (5 μM) to block sympathetic neurotransmission (Figure 6.6). Low oxygenation had no effect on the level of adiponectin collected in the perfusate at basal conditions and during electrically-evoked (EFS; 8 Hz, 60 V, 0.1 ms, 30 s) neurogenic vasorelaxation under raised tone conditions ($P>0.05$, one-way ANOVA with Bonferroni's post-hoc test, n=8-12) (Figure 6.6).

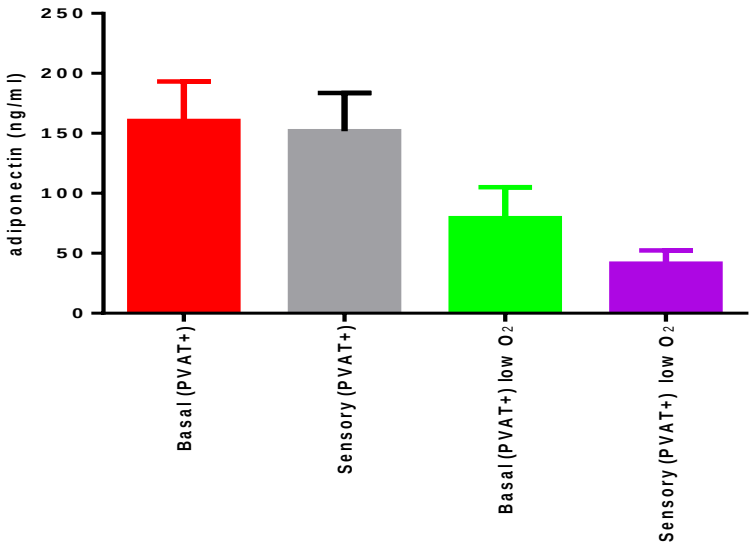

Figure 6.6: The effect of oxygen level on adiponectin release during electrically-evoked neurogenic vasorelaxation in pre-contracted mesenteric arterial beds (MABs) with perivascular adipose tissue (PVAT+). Preparations were submaximally pre-contracted with methoxamine and guanethidine (5 μM) was present to block sympathetic neurotransmission. Electrical field stimulation (EFS; 8 Hz, 60 V, 0.1 ms, 30 s) was applied. There was no difference in adiponectin release under conditions of standard and low oxygen supply ($P>0.05$, one-way ANOVA with Bonferroni's post-hoc test, n=8-12).

6.3.3 Leptin

6.3.3.1 The effect of PVAT on leptin release during EFS-induced neurogenic vasocontractile responses

Under basal tone (control), PVAT-intact vessels released a significantly larger amount of leptin than PVAT-denuded MABs ($P<0.05$, one-way ANOVA with Bonferroni's post-hoc test, n=6-8). In the presence of PVAT, the level of leptin collected in the perfusate during electrically-evoked

(EFS; 24 Hz, 90 V, 1 ms, 30 s) neurogenic vasocontraction was comparable to that of the control, but significantly enhanced compared to PVAT-denuded preparations (P>0.05, P<0.001 respectively, one-way ANOVA with Bonferroni's post-hoc test, n=6-8) (Figure 6.7).

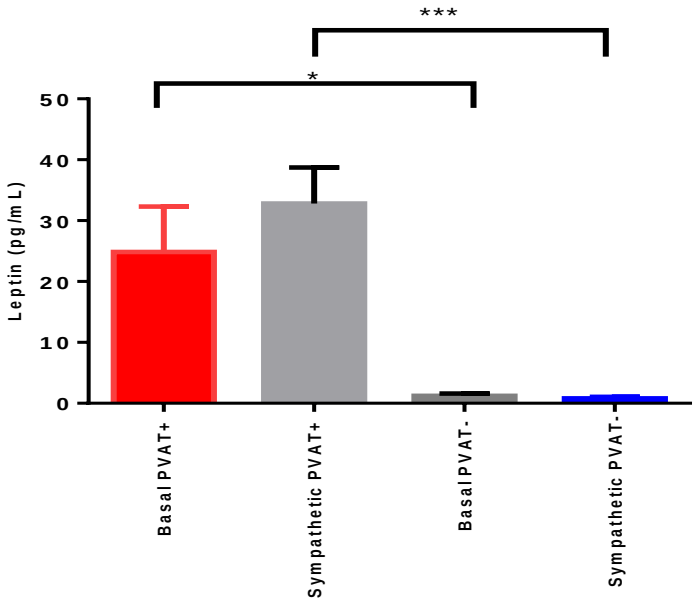

Figure 6.7: The effect of perivascular adipose tissue (PVAT) on leptin release at basal tone and during electrically-evoked (EFS; 24 Hz, 90 V, 1 ms, 30 s) neurogenic vasocontraction in mesenteric arterial beds (MABs). MABs were prepared with intact PVAT (PVAT+) and without PVAT (PVAT-). Leptin level collected in perfusate at basal conditions (control), and during EFS was significantly enhanced in PVAT+ compared to PVAT- preparations. There was no effect of EFS on the leptin level compared to the control. *P<0.05, ***P<0.001 (one-way ANOVA with Bonferroni's post-hoc test, n=6-8).

6.3.3.2 The effect of PVAT on leptin release: effect of EFS-induced neurogenic vasorelaxation

In MABs pre-contracted with methoxamine (1-5 μM) and in the presence of guanethidine (5 μM) (control), levels of leptin collected in the perfusate under basal conditions were significantly greater in PVAT-intact preparations than in PVAT-denuded preparations ($P<0.05$, one-way ANOVA with Bonferroni's post-hoc test, n=6-10) (Figure 6.8). In the presence of PVAT, leptin release during electrically-evoked (EFS; 8 Hz, 60 V, 0.1 ms, 30 s) neurogenic vasorelaxation at raised tone was significantly enhanced ($P<0.05$, one-way ANOVA with Bonferroni's post-hoc test, n=10) (see Figure 6.8).

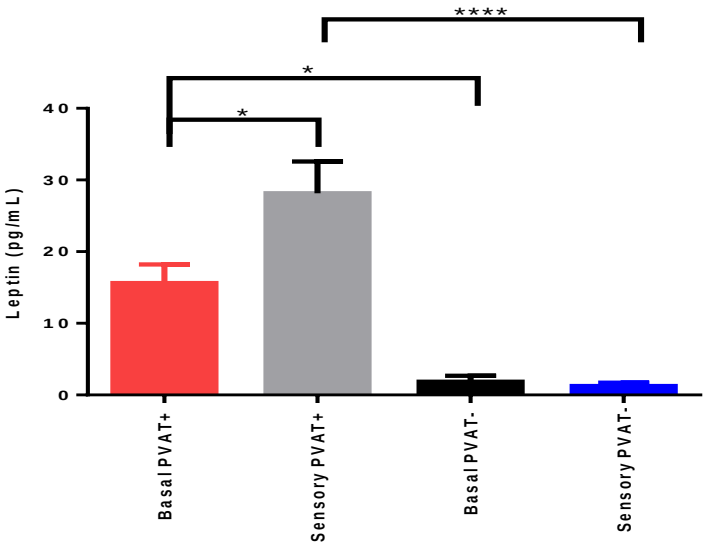

Figure 6.8: The effect of perivascular adipose tissue (PVAT) on leptin release under raised tone and during electrically-evoked neurogenic vasorelaxation responses in pre-contracted mesenteric arterial beds (MABs). MABs were pre-contracted with methoxamine in the presence of guanethidine (5 μM) and electrical field stimulation applied (EFS; 8 Hz, 60 V, 0.1 ms, 30 s). EFS enhanced leptin release in preparations with PVAT (PVAT+). In the presence of PVAT (PVAT+) leptin release was greater in both under basal (control) conditions and during EFS than in the absence of

PVAT (PVAT-). *P<0.05, ****P<0.0001 (one-way ANOVA with Bonferroni's post-hoc test, n=6-10).

6.3.3.3 The effect of standard and low oxygen on leptin release: effect of EFS-induced neurogenic vasoconstriction

In PVAT-intact MABs gassed with a standard level of oxygen, the level of leptin release in the perfusate was not significantly altered by EFS (24 Hz, 90 V, 1 ms, 30 s) (P>0.05, one-way ANOVA with Bonferroni's post-hoc test, n=4-6) (Figure 6.9). Decreasing the oxygenation had no effect on the level of leptin in the control groups (P>0.05, one-way ANOVA with Bonferroni's post-hoc test, n=6-8) (Figure 6.9). Under these conditions of reduced oxygenation the amount of leptin released during EFS was significantly increased compared to the control (P<0.05, one-way ANOVA with Bonferroni's post-hoc test, n=8).

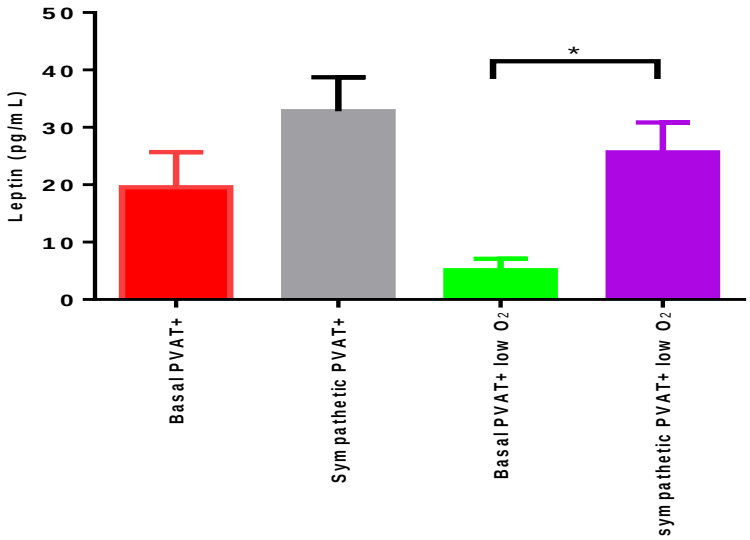

Figure 6.9: The effect of oxygen level on leptin release into the perfusate at basal tone and during electrically-evoked (EFS; 24 Hz, 90 V, 1 ms, 30 s) neurogenic vasocontraction in perivascular adipose tissue (PVAT) intact mesenteric arterial beds (MABs). With a standard level of oxygen, there was no effect of EFS on leptin release into the perfusate of the MABs. Under conditions of low oxygen the level of leptin during EFS was significantly greater than in the control. *P<0.05 (one-way ANOVA with Bonferroni's post-hoc test, n=6-8).

6.3.3.4 The effect of standard and low oxygen on leptin release: effect of EFS-induced neurogenic vasorelaxation

In PVAT-intact perfused MABs pre-contracted with methoxamine (1-5 μM), in the presence of guanethidine (5 μM), the level of leptin in the perfusate was significantly reduced in the presence of reduced oxygen during electrically-evoked (EFS; 8 Hz, 60 V, 0.1 ms, 30 s) neurogenic vasorelaxation at raised tone (P<0.0001, one-way ANOVA with Bonferroni's post-hoc test, n=7-10) as shown in Figure 6.10. EFS had no significant effect on leptin release compared to basal conditions in low oxygenation

(P>0.05, one-way ANOVA with Bonferroni's post-hoc test, n=7-8) (Figure 6.10).

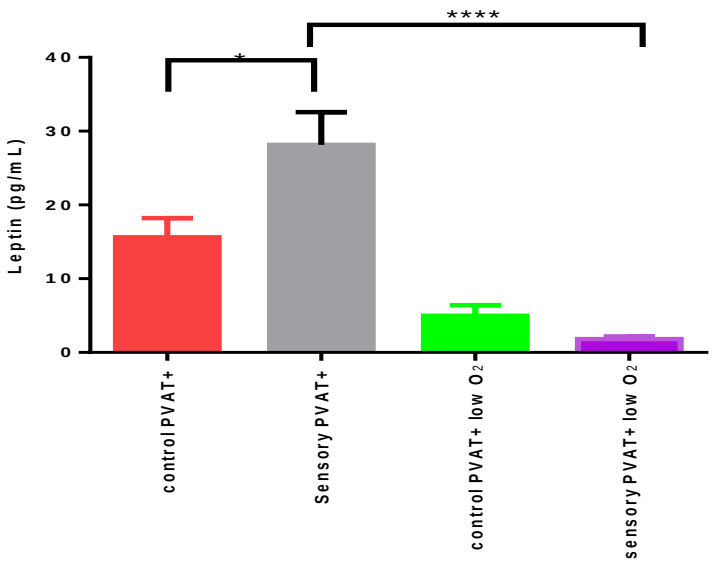

Figure 6.10: The effect of oxygen level on leptin release in pre-contracted mesenteric arterial beds (MABs) with perivascular adipose tissue (PVAT+) and during electrically-evoked (EFS; 8 Hz, 60 V, 0.1 ms, 30 s) neurogenic vasorelaxation. There was no difference between the control and EFS groups in low level of oxygen, but not in standard oxygenation. The leptin level was significantly reduced during EFS under low oxygen level compared to standard oxygen levels. *P<0.05, ****P<0.0001 (one-way ANOVA with Bonferroni's post-hoc test, n=7-10).

6.3.4 Interleukin-6 (IL-6)

6.3.4.1 The effect of PVAT on IL-6 release during EFS-induced neurogenic vasocontraction

In perfused MABs at basal tone (control) and during electrically-evoked (EFS; 24 Hz, 90 V, 1 ms, 30 s) neurogenic vasocontraction, interleukin-6

(IL-6) was not detected in either PVAT-intact (Figure 6.11) or PVAT-denuded (data not shown) preparations (n=5).

6.3.4.2 The effect of PVAT on IL-6 release during EFS-induced neurogenic vasorelaxation

In MABs pre-contracted with methoxamine (1-5 μM) and in the presence of guanethidine (5 μM) (control), or during electrically-evoked (EFS; 8 Hz, 60 V, 0.1 ms, 30 s) neurogenic vasorelaxation under raised tone conditions, relatively, interleukin-6 (IL-6) was not detected in either PVAT-intact (Figure 6.12) or PVAT-denuded preparations (data not shown)(n=4-6).

6.3.4.3 The effect of standard and low oxygen supply on IL-6 release: effect of EFS-induced neurogenic vasoconstriction

In PVAT-intact MABs perfused with Krebs'-Henseleit solution gassed with a standard level of oxygen, no IL-6 was detected during baseline conditions or during EFS (24 Hz, 90 V, 1 ms, 30 s) (n=5) (Figure 6.11). In contrast, in a low level of oxygen, the amount of IL-6 released was significantly increased and was detected under both control conditions and during EFS ($P<0.01$, one-way ANOVA with Bonferroni's post-hoc test, n=4-5). In PVAT-intact MABs gassed with the lower level of oxygen, there was no difference between control and EFS with regard to the amount of IL-6 released ($P>0.05$, one-way ANOVA with Bonferroni's post-hoc test, n=4-5) (see Figure 6.11).

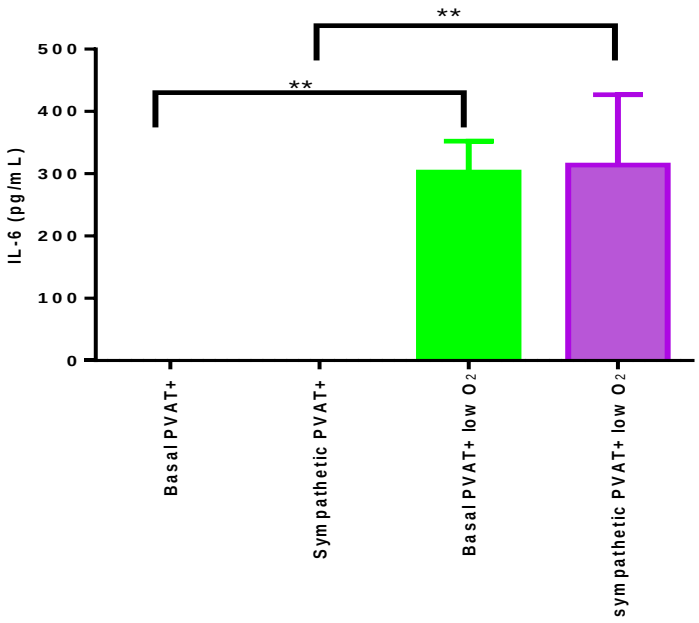

Figure 6.11: The effect of oxygen level on interleukin-6 (IL-6) release at basal tone and during electrically-evoked (EFS; 24Hz, 90V, 1ms, 30s) neurogenic vasocontractile responses in perivascular adipose tissue (PVAT) intact mesenteric arterial beds (MABs). In MABs gassed with a standard level of oxygen, IL-6 was not detected in both control and EFS (24 Hz, 90 V, 1 ms, 30 s) conditions but was increased during gassing with a lower oxygen level. **P<0.01 (one-way ANOVA with Bonferroni's post-hoc test, n=4-5).

6.3.4.4 The effect of standard and low oxygen supply on IL-6 release: effect of EFS-induced neurogenic vasorelaxation

In PVAT-intact perfused MABs pre-contracted with methoxamine (1-5 μM), with the presence of guanethidine (5 μM), the amount of IL-6 in the perfusate under basal conditions and during electrically-evoked (EFS; 8 Hz, 60 V, 0.1 ms, 30 s) neurogenic vasorelaxation at raised tone significantly increased when the oxygen level was reduced (P<0.01, one-way ANOVA with Bonferroni's post-hoc test) (n=3-7) (Figure 6.12). EFS had no

significant effect, in either low or standard oxygen supply, on the amount of IL-6 released (P>0.05, one-way ANOVA with Bonferroni's post-hoc test, n=3-7) (Figure 6.12).

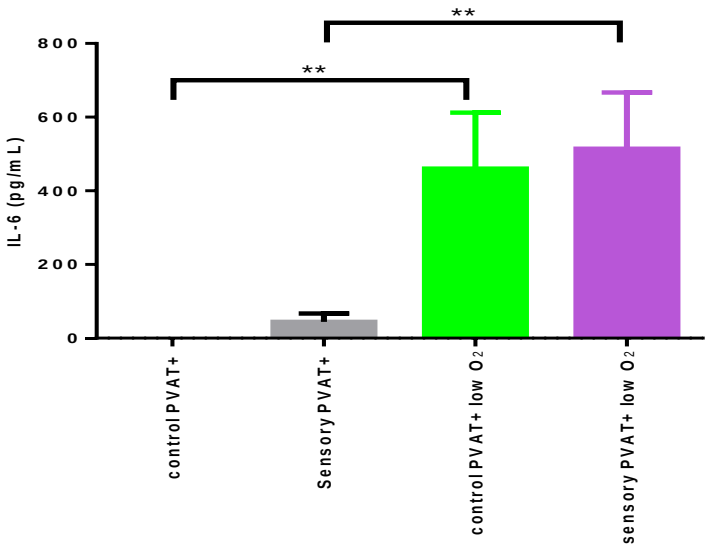

Figure 6.12: The effect of oxygen level on interleukin-6 (IL-6) release in pre-contracted mesenteric arterial beds (MABs) with intact perivascular adipose tissue (PVAT) and during electrically-evoked neurogenic vasorelaxation. Preparations were pre-contracted with methoxamine (1-5 μM), guanethidine (5 μM) was added to block sympathetic neurotransmission and electrical field stimulation applied to evoke neurogenic vasodilatation (EFS; 8 Hz, 60 V, 0.1 ms, 30 s). The IL-6 level was significantly increased in a reduced oxygen level compared to a standard oxygen level in both basal conditions and during EFS. **P<0.01 (one-way ANOVA with Bonferroni's post-hoc test, n=3-7).

6.3.5 Monocyte Chemoattractant Protein-1 (MCP-1)

6.3.5.1 The effect of PVAT on MCP-1 release during EFS-induced neurogenic vasocontraction

In perfused MABs, under basal tone, the level of MCP-1 in the perfusate in both control and electrically-evoked (EFS; 24 Hz, 90 V, 1 ms, 30 s) neurogenic vasocontractile responses in PVAT-intact preparations were not different to those in PVAT-denuded preparations (P>0.05, one-way ANOVA with Bonferroni's post-hoc test, n=3-6) (see Figure 6.13).

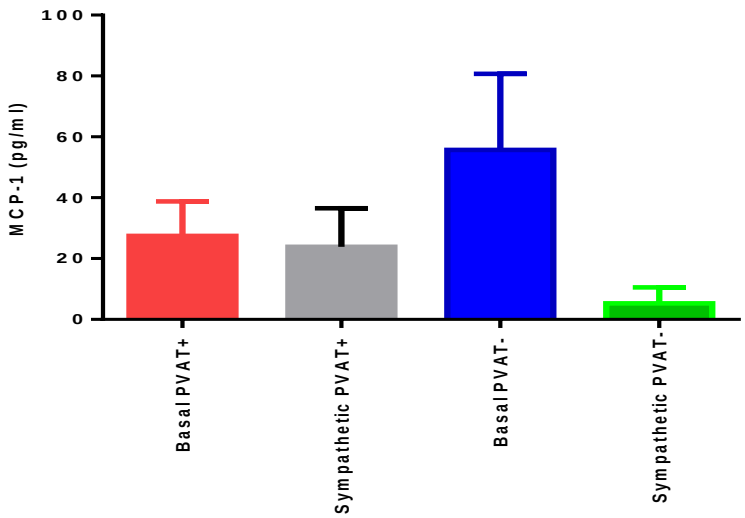

Figure 6.13: The effect of perivascular adipose tissue (PVAT) on monocyte chemoattractant protein-1 (MCP-1) release at basal tone conditions and during electrically-evoked (EFS; 24 Hz, 90 V, 1 ms, 30 s) neurogenic vasocontraction (sympathetic). There was no difference in the release of MCP-1 between the groups (one-way ANOVA with Bonferroni's post-hoc test, n=3-6).

6.3.5.2 The effect of PVAT on MCP-1 release: effect of EFS-induced neurogenic vasorelaxation

In MABs pre-contracted with methoxamine (1-5 μM) and with the presence of guanethidine (5 μM) (control), the presence of PVAT did not alter MCP-1 level ($P>0.05$, one-way ANOVA with Bonferroni's post-hoc test, n=3-7). MCP-1 level collected into the perfusate during electrically-evoked (EFS; 8 Hz, 60 V, 0.1 ms, 30 s) neurogenic vasorelaxation responses under raised tone conditions was comparable with the absence of PVAT ($P>0.05$, one-way ANOVA, n=3-7) as shown in Figure 6.14.

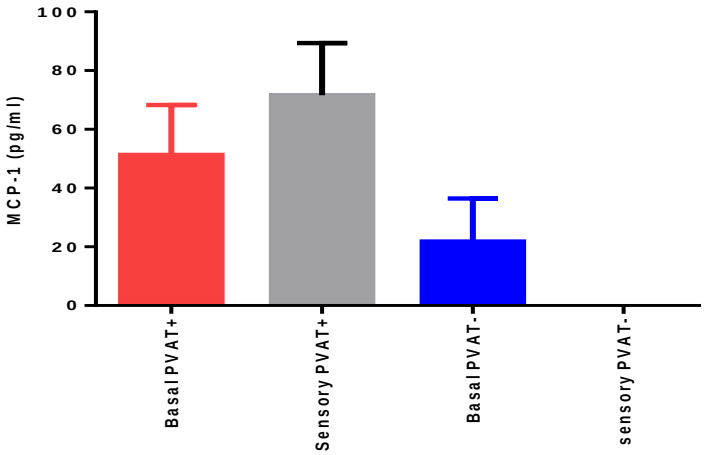

Figure 6.14: The effect of perivascular adipose tissue (PVAT) on monocyte chemoattractant protein-1 (MCP-1) release under raised tone and during electrically-evoked neurogenic vasorelaxation responses in pre-contracted mesenteric arterial beds (MABs). Preparations were pre-contracted with methoxamine (1-5 μM), guanethidine (5 μM) was added to block sympathetic neurotransmission and electrical field stimulation (EFS; 8 Hz, 60 V, 0.1 ms, 30 s) applied to evoke neurogenic vasodilatation. At raised tone and during EFS, PVAT-intact (PVAT+) preparations released comparable amount of MCP-1 to PVAT-denuded (PVAT-) preparations. $P>0.05$ (one-way ANOVA, n=3-7).

6.3.5.3 The effect of standard and low oxygen supply on MCP-1 release during EFS-induced neurogenic vasoconstriction responses

In PVAT-intact MABs gassed with standard level of oxygen, all groups released comparable amount of MCP-1 in the perfusate under standard and low oxygen conditions. There was no difference between control and EFS groups which received similar amounts of oxygen (P>0.05, one-way ANOVA with Bonferroni's post-hoc test, n=6-9) (P>0.05, one-way ANOVA with Bonferroni's post-hoc test, n=6-9) (see Figure 6.15).

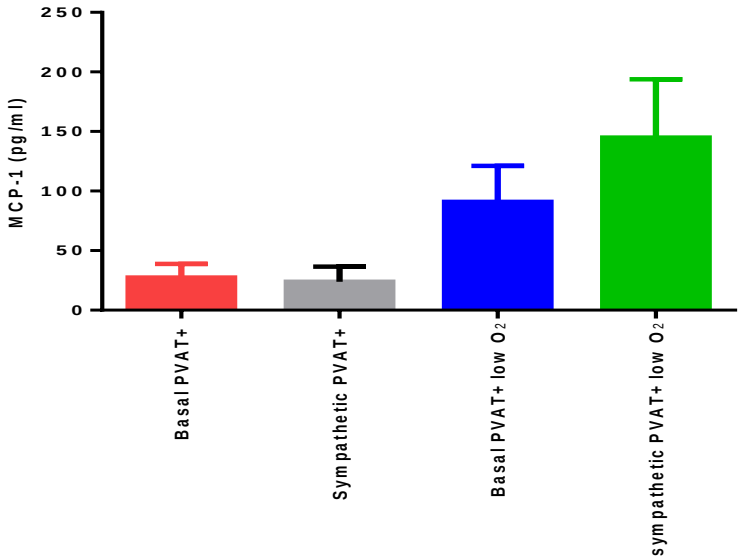

Figure 6.15: The effect of oxygen level on monocyte chemoattractant protein-1 (MCP-1) release at basal tone and during electrically-evoked (EFS; 24 Hz, 90 V, 1 ms, 30 s) neurogenic vasocontractile responses in perivascular adipose tissue intact (PVAT+) mesenteric arterial beds (MABs). Reduced oxygen level had no significant effect on the release of MCP-1 between all groups. P>0.05 (one-way ANOVA with Bonferroni's post-hoc test, n=6-9).

6.3.5.4 The effect of standard and low oxygen supply on MCP-1 release during EFS-induced neurogenic vasorelaxation

In PVAT-intact perfused MABs pre-contracted with methoxamine (1-5 μM), with the presence of guanethidine (5 μM), the amount of MCP-1 was similar between the control group with the lower oxygen supply and standard oxygen level (P>0.05, one-way ANOVA with Bonferroni's post-hoc test, n=4-5). In either low or standard oxygen supply, there was no difference in the amount of MCP-1 release between control and EFS groups (P>0.05, one-way ANOVA with Bonferroni's post-hoc test) (n=3-7) (see Figure 6.16).

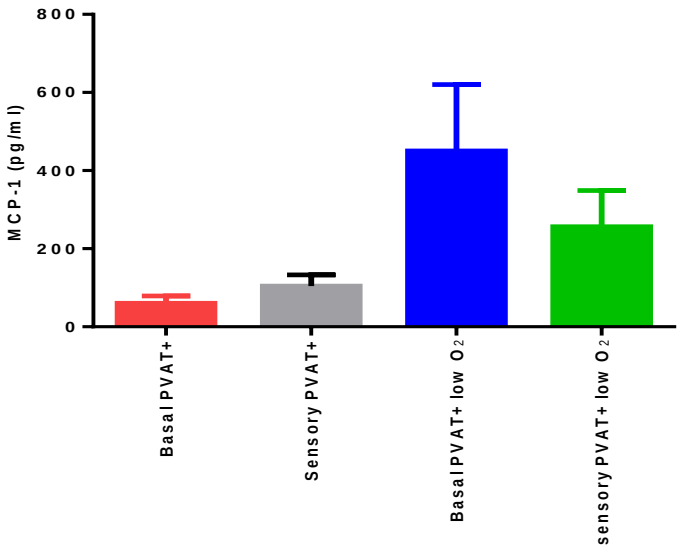

Figure 6.16: The effect of oxygen level on MCP-1 release at the pre-contracted mesenteric arterial beds (MABs) and during electrically-evoked neurogenic vasorelaxation under raised tone in the presence of perivascular adipose tissue (PVAT). Preparations were pre-contracted with methoxamine, guanethidine was added to block sympathetic neurotransmission and electrical field stimulation (EFS; 8 Hz, 60 V, 0.1 ms, 30 s) applied to evoke neurogenic vasodilatation. There was no difference between basal and EFS groups which received similar amounts of oxygen

(P>0.05, one-way ANOVA with Bonferroni's post-hoc test, n=3-7). Reduced oxygen level had no effect on the release of MCP-1 under basal and during EFS. P>0.05 (one-way ANOVA, n=3-7).

6.3.6 Others

In the present study, seven compounds were investigated for possible changes in their expression during sympathetic and sensory neurogenic activation. Of these compounds, four, namely adiponectin, leptin, IL-6 and MCP-1 were detected within samples of mesenteric arterial bed perfusate while the rest, tumor necrosis factor alpha (TNF-α), interleukin beta (IL-β) and total plasminogen activator inhibitor-1 (PAI-1), were below the detectable range. Data of the present experiments are summarized in Table 6.1.

Conditions / Adipokines	Sympathetic						Sensory					
	PVAT+		PVAT-		PVAT+ Low $[O_2]$		PVAT+		PVAT-		PVAT+ Low $[O_2]$	
	Control	EFS	Control	EFS	Control	EFS	Control	EFS	Control	EFS	Control	EFS
Adiponectin	D	D	D	D	ND	ND	D	D	↓↓	↓	D	D
Leptin	D	D	↓	↓↓↓	D	↑	D	↑	↓	↓↓↓↓	D	↓↓↓↓
IL-6	BD	BD	BD	BD	↑↑	↑↑	BD	BD	BD	BD	↑↑	↑↑
MCP-1	D	D	ND	ND	D	D	D	D	ND	D	D	ND

Table 6.1: Summary of the effects of PVAT and level of oxygen on adipokines release during sympathetic and sensory activation. D = detected, ND = not detected, BD = below detection, ↓ = significantly reduced P<0.05, ↓↓ =significantly reduced P<0.01, ↓↓↓ = significantly reduced P<0.001, ↓↓↓↓ = significantly reduced P<0.0001, ↑ = significantly enhanced P<0.05, ↑↑ = significantly enhanced P<0.01, ↑↑↑ = significantly enhanced P<0.001. Black arrow/s is/are based on comparison of adipokine release with perivascular adipose tissues intact preparations (PVAT+), red arrow/s is/are based on comparison of adipokine release between basal (control) and electrical field stimulation (EFS) in a same preparation.

6.4 Discussion

The present study investigated the effect of sympathetic and sensory activation on mediator release in the absence and presence of PVAT under conditions of standard or low oxygen level. The main novel findings in the present study are (1) sensory nerve activation stimulates leptin release in the presence of PVAT, (Straub et al.) under low oxygen, sympathetic activation enhances leptin release in the presence of PVAT. To our knowledge, this is the first study to examine the direct effect of sympathetic and sensory nerve stimulation on the level of PVAT-derived mediator release.

There is call to elucidate the link between autonomic fibres and PVAT (Bulloch and Daly, 2014). In addition, anti-contractile properties of PVAT have been reported to be diminished in pathophysiological states (Aghamohammadzadeh et al., 2012; Szasz and Webb, 2012). The present study was conducted in order to investigate the link between PVAT and the nervous system controlling blood vessels under standard and low oxygen conditions. Previous studies have used gassing with 95 % N_2 and 5 % CO_2 for 2.5 hours to induce hypoxia in isolated mesenteric arteries (Agabiti-Rosei et al., 2014; Withers et al., 2011). Using an oxygen sensing electrode, 30 min exposure to 95 % N_2 and 5 % CO_2 markedly reduced the partial oxygen level (PaO_2) of the Krebs'-Henseleit solution from 619 ± 17 mmHg in standard oxygen (95 % O_2, 5 % CO_2) to 33.6 ± 6 mmHg (95 % N_2 and 5 % CO_2) in studies of isolated rat mesenteric arteries in organ baths (White, 2012). In the present study, I made an attempt to induce hypoxia of the MABs by changing the gassing of the Krebs'-Henseleit

solution reservoir from 95 % O_2 and 5 % CO_2, to 95 % N_2 and 5 % CO_2, but there is a significant delay between the perfusate reaching the MABs as it passes through the plastic tubing, and the preparations themselves are in a chamber that is open to O_2. Thus, the composition of the gas that the MABs see is unclear and attempts that we made to measure this were unsuccessful. However, the significantly impaired contractile response to methoxamine in preparations after 1.5 hours exposure to the hypoxic solution suggests a possible effect of low oxygen. Time control experiments are needed to determine the real influence of oxygen level on sympathetic or sensory nerve-evoked mediator release.

The present study utilized a high-throughput rat adipocycte magnetic beads panel for detection of adiponectin, leptin, interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor alpha (TNF-α), interleukin beta (IL-β) and total plasminogen activator inhibitor-1 (PAI-1). Due to quick neuronal activation (30 s) for both sympathetic and sensory nerves in the present study, it is postulated that possibly the time course of EFS on PVAT-derived mediators reflects the release of pre-formed mediators, but not on their synthesis. Some of these mediators were below detectable levels in normoxic and low oxygen conditions, and some of them only increased under a low level of oxygen (see below); further experiments with more concentrated samples are necessary to reveal the effects of sympathetic and sensory activation on these PVAT-derived mediators release. The data from the present study add new insight on the relationship between PVAT-derived mediators and the sympathetic and sensory nervous systems.

6.4.1 The effect of standard and low oxygen supply on basal tone and neurogenic vasoconstriction of perfused MABs in the absence and presence of PVAT

In the present study, under basal tone, the oxygen level had no effect either on basal tone or on EFS-evoked vasoconstriction of perfused MABs. It has been reported that decreasing bath PaO_2 to 40 %, 20 %, and 12 % under resting tone elicited sustained increases in basal tension, ranging between 10 % and 20 % of the contraction induced by 40 mM potassium chloride in porcine coronary arteries (Rubanyi and Paul, 1985). A further decrease in PaO_2 to near zero (anoxia) resulted in relaxation to baseline (Rubanyi and Paul, 1985). With regards to neurogenic transmission, it has been demonstrated that hypoxia inhibited EFS-evoked sympathetic responses in rabbit pulmonary arteries (MacLean et al., 1993). In the present study, although a change in tone might not be seen under basal tone conditions, if hypoxia was causing relaxation of the MABs, it might be expected to attenuate contractile responses; this was observed for methoxamine-induced contractions, but not for sympathetic neurogenic contractions (see Figure 6.1 and Figure 6.2).

Under standard oxygenation, EFS-induced neurogenic vasoconstriction did not alter adiponectin release in both PVAT-intact and PVAT-denuded preparations. The release of adiponectin was also not different between PVAT-intact preparations under basal conditions, and during EFS activation with PVAT-denuded preparations. This finding is not consistent with previous studies that PVAT contains adiponectin (Fesus et al., 2007; Szasz and Webb, 2012). However, there was a trend that PVAT-intact preparations released higher level of adiponectin compared to PVAT-denuded preparations. Therefore, further experiment to increase 'n

number' is needed to evaluate the effect of sympathetic neurotransmission on PVAT-derived adiponectin release. Imai et al. (2006) demonstrated that the sympathetic nervous system physiologically regulates serum adiponectin levels and adiponectin synthesis in WAT *in vivo*, and the regulation differs among WAT depots of mice. In their study, it was shown that cold exposure suppresses serum adiponectin levels through sympathetic nerve activation (Imai et al., 2006). In the present study, we stimulated sympathetic nerves directly using EFS and our data are not in agreement with those of Imai et al. (2006) as different protocols were employed. Under low oxygen levels in preparations with PVAT, there was no change in the basal level of adiponectin and EFS-induced neurogenic vasoconstriction did not change adiponectin release. This is important in the context of the other mediators, some of which were found to increase or decrease under low oxygen conditions. A previous study has shown that local inflammation and hypoxia of obese patient led to suppression of exogenous adiponectin vasodilator effect (Greenstein et al., 2009).

Under standard oxygenation, EFS-induced neurogenic vasoconstriction did not alter leptin release in both PVAT-intact and PVAT-denuded preparations. However, the release of leptin was significantly greater in PVAT-intact preparations under basal condition and during EFS activation than in PVAT-denuded preparations. Leptin has been detected in PVAT of canine mesenteric arteries (Mohammed et al., 2007), rat aorta (Sahin and Bariskaner, 2007) and human forearm (Nakagawa et al., 2002). Low oxygen reduced leptin release in two separate experiments (Figure 6.7 and Figure 6.9). Sierra-Johnson et al. (2008) also demonstrated that leptin level was reduced in healthy males under acute hypoxic conditions. Interestingly, the release of leptin in the MABs was augmented during EFS-induced vasoconstriction under low oxygen. The link between leptin and

sympathetic nerves has been reviewed (Hall et al., 2010; Rayner and Trayhurn, 2001). Leptin released has been shown to activate sympathetic activity (Hall et al., 2010). Sympathomimetic amines and cold exposure or fasting activates sympathetic nerves in white fat tissue, decreases leptin gene expression in the tissue and leptin production (Rayner and Trayhurn, 2001). Furthermore, it has been shown that the beta 3-adrenoceptor is involved in inhibiting insulin-stimulated leptin secretion from isolated rat adipocytes (Gettys et al., 1996). Nonetheless, there is evidence that indicates that acute hypoxia, caused by decreasing oxygen saturation to 75% for 30 min, does not affect plasma leptin in healthy men (Schmoller et al., 2009). In the present study, direct activation of sympathetic nerves *in vitro* under standard oxygenation did not alter the leptin release probably due to different species and protocols used. In contrast, the augmentation by EFS of leptin levels under low oxygenation suggests that a possible connection between sympathetic innervation and leptin becomes more evident in low oxygen level conditions.

Under standard oxygenation, IL-6 release was not detected in both PVAT-intact and PVAT-denuded preparations. This observation was consistent in experiments on MABs carried out both at basal tone (to investigate sympathetic neurotransmission), and at raised tone (to investigate sensory neurotransmission). IL-6 was only detected under low oxygen level in preparations with PVAT, and there was no additional effect on IL-6 levels of EFS at basal tone. The present data suggests that IL-6 release can only be detected under low oxygen conditions and sympathetic activation had no effect on IL-6 release. IL-6 and interleukin-6 receptors (IL-6R) are present in sympathetic ganglia and neurons (Gadient and Otten, 1996). IL-6 is a proinflammatory cytokine shown to be secreted in higher concentrations from PVAT compared with other fat depots, and potentially has a role in

promoting arterial stiffness (Chatterjee et al., 2009; Fleenor et al., 2014). Levels of IL-6 have been reported to increase during hypoxia due to high altitude exercise in human (Boos et al., 2015; Goods et al., 2015), which may explain why IL-6 release only was detected under low PaO_2 in the present study. The present study suggests that sympathetic activation has no effect on IL-6 release under low oxygenation. An *in vivo* study by De Luigi et al. (1998) showed that sympathectomy increased lipopolysaccharide-induced interleukin-1beta (IL-1β) and IL-6 messenger RNA in rat adrenals and spleen, and concluded that sympathetic nervous system denervation enhances the synthesis and production of peripheral IL-1β and IL-6 by central lipopolysaccharide. Sympathetic neurons have also been shown to stimulate production of IL-6 *in vitro* using cell culture (Marz et al., 1998).

Levels of another PVAT-derived cytokine, MCP-1, were not different under low PaO_2 compared to standard oxygenation, although there was a trend that MCP-1 release were increased under low PaO_2. Therefore, further experiment to increase 'n number' is needed to evaluate the effect of sympathetic neurotransmission on PVAT-derived MCP-1 release under low PaO_2. EFS stimulation had no effect on MCP-1 release compared to control in both low and standard oxygenation. It has been reported that MCP-1 is rapidly expressed by sympathetic ganglion neurons following axonal injury (Schreiber et al., 2001), consistent with the role of MCP-1 as a pro-inflammatory modulator.

6.4.2 The effect of standard and low oxygen supply on basal tone and neurogenic vasorelaxation of perfused MABs in the absence and presence of PVAT

In the present study, in pre-constricted MABs, switching gassing conditions from 95 % O_2 and 5 % CO_2 (standard oxygen) to 95 % N_2 and 5 % CO_2 (low oxygen) attenuated the methoxamine raised tone. Decreased tone in rat pre-constricted MABs due to hypoxia has been reported by Otter and Austin (1999). The present data shows that low oxygen level in pre-constricted MABs had no effect on neurogenic relaxation responses due to EFS.

Under standard oxygenation, EFS-induced neurogenic vasorelaxation did not alter adiponectin release in either PVAT-intact or PVAT-denuded preparations. However, the release of adiponectin was markedly increased in PVAT-intact preparations compared to PVAT-denuded preparations. Adiponectin release under standard oxygenation in both control conditions and during EFS was not different than under lower oxygen (although a trend for a reduction was observed). Therefore, further experiment to increase 'n number' to assess the effect of sensory neurotransmission on PVAT-derived adiponectin release under low PaO_2 is needed.

Under standard oxygenation, interestingly, EFS-induced neurogenic vasorelaxation corresponded with an enhanced leptin release in PVAT-intact preparations, which suggests that there is a link between endogenous leptin and sensory nerves. Presumably, CGRP and/or other neurotransmitter(s) released from sensory nerves during EFS act at receptors on the adipocytes in PVAT to cause a release of leptin. The release of leptin was virtually abolished by both PVAT removal and under

conditions of low oxygen. Leptin levels were similarly abolished by removal of PVAT and under low oxygen conditions in separate experiments focusing on sympathetic neurotransmission, giving robustness to these findings (Figures 6.7 and 6.9). Recently, the role of leptin secreted from WAT as a paracrine factor to activate spinal sensory nerves has been shown in Siberian hamsters (Murphy et al., 2013). A more recent publication shows that leptin receptors *LepR* are expressed on sensory neurons, and the deletion of leptin signalling in vagal afferent neurons results in hyperphagia and obesity (de Lartigue et al., 2014). Hence, the present data adds a new insight on the relationship between leptin and sensory nerves.

Under standard oxygenation, IL-6 release was not detected in both PVAT-intact and PVAT-denuded preparations but it was significantly elevated under low oxygen conditions. This was observed in 2 separate experiments adding robustness to the finding (Figures 6.11 and 6.12). The expression of IL-6 in sensory ganglia and neurons has been reported by Gadient and Otten (1996). It has been demonstrated that in IL-6 knockout mice, the IL-6 deficiency reduced sensory nerves action potential (Zhong et al., 1999).

In the presence of PVAT, both control and EFS of sensory nerves were not significantly different than in the absence of PVAT under standard oxygenation condition. Under low oxygen conditions, the MCP-1 level was not increased compared to the standard oxygen group. However, there was a trend that the MCP-1 release was increased under low oxygen conditions and this was observed in separate experiments carried out in preparations at both basal and raised tone (Figures 6.15 and 6.16), demonstrating the reproducibility of the finding. MCP-1 is secreted by DRG neurons and appears that MCP-1 can be stored in the same vesicles as CGRP (Jung et

al., 2008). MCP-1 has been shown to be upregulated in sensory neurons following peripheral nerve injury (Jung et al., 2008).

6.5 Conclusion

The present chapter has provided clear evidence that leptin release is enhanced by EFS of sensory nerves under standard oxygen level and by EFS of sympathetic nerves under low oxygen level in the presence of PVAT. Our data appear to indicate that there are interactions between sympathetic and sensory nerves with PVAT-derived leptin and that the activation of these nerves modulates the release of PVAT-derived leptin. Under the conditions of the present study, I demonstrate that leptin release is greater in MABs with PVAT than in those without PVAT. Under raised tone, but not under basal condition, adiponectin release is greater in MABs with PVAT than in those without PVAT. The present study also demonstrates that the level of oxygen plays a role in mediating the release of some PVAT-mediators. Low oxygen levels were shown to enhance inflammatory mediator, IL-6 release at both basal and raised tone, and reduced leptin levels under raised tone condition. The current data indicate the existence of interactions between PVAT-derived nerves and PVAT-mediators, although the relationship between them is yet to be fully elucidated.

Chapter 7

General discussion

General discussion

Despite various efforts and extensive research, cardiovascular diseases remain the leading cause of mortality worldwide. Perivascular adipose tissue (PVAT), which was completely ignored previously, is now recognized to hold a potential role in physiological and pathological conditions of the cardiovascular system (Aghamohammadzadeh et al., 2012). In spite of this, the link between PVAT and nerves controlling the vasculature remains unclear. The present study investigated the effects of PVAT on sympathetic and sensory neurotransmission and the interaction between these nerves with selected PVAT-derived vasoactive compounds.

There is a growing body of evidence which suggests that there are regional phenotypic and functional differences among PVAT depots, depending on the specific vascular bed or different regions in the vascular bed (Gil-Ortega et al., 2015). The current study employed tissues from different species (rat and porcine) and vascular regions (rat MABs, rat AAs, PSA and PCA), with rat MAB as the main tissue as this tissue is well-known to receive substantial sensory and sympathetic innervation. Furthermore, MABs consist of both small and large arteries, which is useful in comparative studies of the influence of vessel size. Although histological study of the PVAT was not carried out in the present study, there are some descriptions of the PVAT of some of the arteries used. PVAT of rodent mesenteric arteries is white, with larger unilocular adipocytes that lack

UCP-1 and less vascularized than brown aortic PVAT (Szasz et al., 2013). Rat AA has been reported to be surrounded by mixture of cells with unilocular white adipocytes (Padilla et al., 2013; Police et al., 2009), although rat thoracic aorta is surrounded by multilocular brown adipocytes (Padilla et al., 2013). Regional differences of PVAT contribute to the physiological regulation, as well as possibly contributing to the regional susceptibility of arteries and veins to vascular disease, and hence represent a novel target for pharmacological intervention (Gil-Ortega et al., 2015). Therefore, further study could be conducted to investigate the histological and physiological differences of PVAT from different regions.

Effects of perivascular adipose tissue on sympathetic neurotransmission

The innervation of PVAT was largely disregarded until recently. Only a few publications demonstrated the existence of sympathetic nerves in PVAT using immunofluorescence or immunochemistry techniques (Bulloch and Daly, 2014; Dashwood and Loesch, 2011; Diculescu and Stoica, 1970). Although a few reports investigated the expression of sympathetic nerves in PVAT in rat and mice MABs (Bulloch and Daly, 2014; Diculescu and Stoica, 1970), there is no report of the sympathetic innervation of PVAT of different orders/parts of MABs. The present study clearly showed the presence of sympathetic nerves within PVAT of different orders of MA (SMA, 1OMA, 2OMA and 3OMA). Double immunostaining in the present study revealed the expression of sympathetic nerves in PVAT of mesenteric arteries. To further confirm this observation, treatment with a sympathetic neurotoxin (such as 6-hydroxydopamine, a selective catecholamine inhibitor) in dissected PVAT is required. It has been reported that

treatment with this inhibitor can diminish sympathetic innervation (Morris et al., 1986) in rat mesenteric arteries and the effects of the treatment can be observed using confocal scanning. Immunofluorescence study demonstrated that sympathetic nerves appeared to be more extensive in small arteries in both PVAT-intact and PVAT-denuded preparations, compared to conduit arteries. However, further work to confirm this observation such as quantification of nerves using advance confocal technique, western blotting or assay to detect and quantify TH need to be carried out. In the present study, although confocal analysis in both dimensions (longitudinal and cross sections) suggests that PVAT-derived sympathetic nerves were independent of sympathetic nerves at the adventitia, further experiments including the application of viral tract tracing methodology is necessary to determine the source of PVAT-derived nerves and more importantly, to investigate potential physiological connections between these nerves, if any. In Chapter 3, replacing dissected PVAT on PVAT-denuded preparations abolished EFS-evoked vasoconstriction. One of the possible explanations is that the signalling connection between sympathetic nerves at the adventitia and sympathetic nerves within PVAT was modified and resulted in a reduced sympathetic response in PVAT-denuded preparations. Another possibility is there was an underlying interaction/s between PVAT-derived sympathetic nerves and PVAT-derived mediators, which contributed significantly to the EFS outcomes. The current study provides some evidence on their interactions (see Chapter 6 and below for further discussion).

There is currently a lack of information about the role of sympathetic nerves in PVAT, although potentiation of sympathetic vasocontractile responses in isolated rat aortas and mesenteric arteries due to electrical field stimulation (EFS) in the present of PVAT has been reported (Gao et

al., 2006; Lu et al., 2010a; Soltis and Cassis, 1991). The present study employed a tissue perfusion technique and used MABs which consist of conduit and resistant arteries. Similar to previous studies, the current study showed that neurogenic vasocontractile responses were significantly enhanced in preparations with PVAT present and these responses were abolished by guanethidine, indicating an involvement of sympathetic nerves (McGregor, 1965; Shatarat et al., 2014). PVAT-potentiated neurogenic contractile responses were attenuated with the treatment with candesartan, but not losartan, angiotensin II antagonists. There are a few clinical reports which suggest that candesartan is more potent than losartan (Bakris et al., 2001; Gradman et al., 1999; Grosso et al., 2011). Candesartan treatment had no effect on EFS-evoked neurogenic vasocontractile responses in the absence of PVAT, thus further supporting the notion that PVAT-derived angiotensin II is involved in mediating neuronal vasoconstriction in MABs.

Consistent with the EFS data in MABs, electrically-evoked neurogenic vasocontractile responses in PSA were significantly attenuated in PVAT-denuded preparations, thus supporting the concept of a role of PVAT in potentiating neurogenic vasocontractile responses. The present study investigated modulation by exogenous methyl palmitate and apelin-13, both of which can be produced by PVAT, of electrically-evoked neurogenic vasocontractile responses in PSA. It showed that exogenous methyl palmitate had no effect on sympathetic neurogenic contractions in PSAs in the presence and absence of PVAT, although there was a trend for the contractile responses to be reduced with increasing concentrations of methyl palmitate. Therefore, similar experiments to increase n number (from n=6-8) are needed to assess the effect of methyl palmitate on sympathetic neurotransmission with the presence of PVAT. The present

study provides the first data on the effect of methyl palmitate on sympathetic neurotransmission in the vasculature. Apelin-13 has been shown to activate brown adipose tissue sympathetic nerve activity in the paraventricular nucleus (Masaki et al., 2012) and caused hypertension via sympathetic activation and arginine vasopressin release in SHR (Zhang et al., 2014). In the present study, apelin-13 reduced sympathetic contractile responses in both PVAT-intact and PVAT-denuded PSAs. However, it is unclear whether this involves a pre-and/or post-junctional mechanism. Functional studies (such as EFS experiments) in which involve pharmacological intervention (with the presence of agonists and/or antagonists) are useful to understand the underlying mechanism/s of both methyl palmitate and apelin-13. Studies to evaluate the possible direct effects of both methyl palmitate and apelin-13 on the vasculature are also warranted.

Expression and vasomotor role of sensory nerves in PVAT

Although PVAT has been recognized to play a key role in regulating vascular tone, the possibility of sensory nerve innervation of PVAT has been largely ignored with relatively no existing literature on this subject. Sensory nerves have been demonstrated to be expressed in both BAT and WAT (Bartness and Bamshad, 1998; Bartness and Song, 2007; Giordano et al., 1996; Norman et al., 1988) but the existence of these nerves in PVAT is unclear. Therefore, the aims of the present study were to investigate the existence of sensory nerves within PVAT of mesenteric arteries and to examine the effect of PVAT on sensory neurotransmission in resistance and conduit arteries (2OMA and SMA, respectively).

Double immunostaining in the present study revealed the expression of sensory nerves in PVAT of mesenteric arteries. To further confirm this observation, treatment with a sensory nerve depletor (such as capsaicin at high concentration), in dissected PVAT is required. It has been reported that treatment with these inhibitors can diminish sensory innervation in rat mesenteric arteries (Kawasaki et al., 1988) and the effects of the treatment can be observed using confocal scanning. Similar to previous findings (Kawasaki et al., 2009; Kawasaki et al., 1988; Kawasaki et al., 2011), immunofluorescence staining of rat mesenteric arteries (SMA, 1OMA, 2OMA and 3OMA, in PVAT-denuded segments), showed the presence of dense innervation with perivascular nerves where fibres immunoreactive to CGRP were identified. CGRP-immunoreactivity was also visualized within PVAT in PVAT-intact mesenteric artery segments, which indicates the presence of sensory nerves within PVAT of MABs. This is the first report to show the existence of sensory nerves within PVAT. The EIA study further supports the notion of the existence of sensory nerves in PVAT of mesenteric arteries. The CGRP release in the EIA study was observed to be larger in all PVAT-intact artery segments compared to denuded segments, although the difference was not significant. Furthermore, the CGRP level in the Krebs'-Henseleit solution bathing dissected PVAT from 2OMA, but not SMA, was significantly greater with capsaicin treatment than in the absence of capsaicin, thus suggesting that sensory nerves are distributed in PVAT and the density of these nerves is greater in small arteries. The information on the density of sensory nerves within PVAT is very useful in understanding their roles in physiological and pathological control of the cardiovascular conditions. I attempted to compare the density of sensory nerves at the adventitia and within PVAT, in conduit and resistant vessels, using EIA and normalized the CGRP

release per weight. However, differences in the mass of vascular smooth muscle and PVAT complicated interpretation. The best approach probably is confocal scanning analysis which can directly count the distribution of nerves in PVAT or at the adventitia.

In isometric tension experiments, in methoxamine-pre-constricted SMA and 2OMA with the presence of guanethidine, EFS elicited frequency-dependent vasodilatation responses. Removal of PVAT in both SMA and 2OMA segments reduced EFS-evoked vasodilatation responses. Consistent with EIA data in the current study, relaxation in 2OMA was found to be greater than that in the SMA, suggesting that neurogenic vasorelaxation is dominant in smaller arteries compared to conduit arteries. Consistent with the findings in the isometric tension experiments, electrically-evoked neurogenic vasodilatation responses in perfused rat MABs were also markedly attenuated in PVAT-denuded preparations. These data are important because they may explain why there are so few reports of NANC relaxation in isolated vessel rings, which are routinely studied after PVAT removal. These outcomes highlights the essential role of PVAT in modulating sensory-evoked vasodilatation and indicate that sensory nerves innervate mesenteric PVAT, and that these nerves are recruited during EFS, accounting for the larger sensory neurogenic relaxant responses in the presence of PVAT. As both EIA and immunofluorescence studies revealed PVAT has sparser sensory innervation compared to adventitia, it is intriguing why removal of PVAT abolished vasodilatation in PVAT-denuded preparations while there were still substantial amount of CGRP-containing nerves remaining in the adventitia. One of the possible explanations is that the signalling connection between sensory nerves at the adventitia and sensory nerves within PVAT was altered in PVAT-denuded preparations and resulted in an abolished sensory response in PVAT-denuded preparations.

Another possibility is there was an underlying interaction/s between PVAT-derived sensory nerves and PVAT-derived mediators, which contributed significantly to the EFS outcomes. The current study provides some evidences on their interactions (see Chapter 6 and below for further discussion). Although the current study is the first study that demonstrated the role of PVAT in modulating sensory-induced vasodilatation responses, however, the underlying mechanism is still unknown. The hypothesis that vanilloid receptor subtype 1 (TRPV1) channels were critically involved can be ruled out (see explanation below).

Capsaicin, an agonist at TRPV1 channels, was used to examine the integrity of perivascular sensory nerves. Cumulative concentrations of capsaicin elicited concentration-dependent vasodilatation of pre-constricted MABs, and in SMA and 2OMA, in both PVAT-intact and PVAT-denuded preparations and there was no significant difference in vasodilator responses between these preparations. Addition of capsaicin as bolus injections, elicited dose-dependent vasodilatation of pre-constricted MABs, and this also was not different between PVAT-intact and denuded preparations. Similarly, exogenous CGRP applied as bolus injections, also produced similar relaxation responses in PVAT-intact and PVAT-denuded preparations. These data suggest that smooth muscle layer was intact and the ability of the preparations to relax was unimpaired. Hence, post-junctional CGRP receptors and TRPV1 receptors as the primary mechanism of the PVAT enhanced EFS responses can be ruled out. Therefore, future experiments including applications of pharmacological intervention, in the presence or absence of EFS to elucidate the underlying PVAT-induced sensory-evoked vasorelaxation mechanism is warranted.

Effect of hydrogen sulphide on sensory nerve-mediated vasodilatation responses in the rat mesenteric arterial bed in the absence and presence of PVAT

H_2S has now has become the subject of intense investigation as it holds promise in cardiovascular therapeutic strategies. This 'gasotransmitter' has recently been detected in PVAT and CSE, a H_2S-synthesizing enzyme, has also been shown to be expressed within PVAT and to generate endogenous H_2S (Fang et al., 2009). The effect of H_2S on sensory-nerve mediated vasodilatation responses is unclear. In Chapter 4, I demonstrated the expression of sensory nerves within PVAT of MABs and the role of PVAT on sensory neurotransmission, and in Chapter 5, I investigated the effects of H_2S on sensory neurotransmission.

In Chapter 5, I showed that H_2S regulates MABs tone by activation of sensory nerves-TRPA1-CGRP signalling pathway. This finding was achieved as in the presence of PVAT, Na_2S, a H_2S donor, caused concentration dependent vasodilatation. Na_2S responses were not reproducible in preparations with and without PVAT (the reduction in responses was more pronounced in PVAT-intact preparations), and thus suggests the involvement of a desensitization mechanism(s). These vasodilatation responses were inhibited by a TRPA1 inhibitor, which suggests the involvement of TRPA1 channels in Na_2S-evoked relaxation. Since pre-treatment with capsaicin markedly reduced Na_2S-induced vasorelaxation in both preparations, these data suggest that H_2S induces vasodilatation in MABs with and without PVAT through the release of CGRP from sensory

nerves. This proposed mechanism is similar to that of suggested by White et al. (2013) in PVAT-denuded rat small mesenteric arteries and Pozsgai et al. (2012) in rat trachea and ear blood vessels of TRPA1 and TRPV1 receptor gene knockout mice. Although the present study has shown potential H_2S-induced vasodilatation in rat MABs, further experiment with the presence of specific CGRP antagonists, such as olcegepant and/or $CGRP_{8-37}$ is still warranted to support the present data. H_2S has been reported to possess more than one vasodilatation mechanism, depending on the species, tissues and experimental conditions with the most prominent mechanism being activation of ATP sensitive potassium (K_{ATP}) channels (Cheng et al., 2004; Leffler et al., 2011; Zhao et al., 2001).

Another novel finding in Chapter 5 is that exogenous H_2S can modulate electrically-evoked neurogenic vasodilatation in MABs. In PVAT-intact preparations, the presence of Na_2S at a concentration (10 μM) which had no direct vasomotor effect, resulted in a significant reduction in EFS-elicited frequency-dependent vasorelaxation, and addition of higher concentrations of Na_2S abolished EFS-evoked neurogenic vasorelaxation in MABs, demonstrating a capsaicin-like action. Conversely, in PVAT-denuded preparations, the presence of Na_2S enhanced electrically-evoked vasorelaxation, however these responses were not frequency-dependent thus may indicate that these responses were not neurogenic. In the present study, none of H_2S-producing enzyme inhibitors affected sensory neurogenic vasodilatation, which indicates that H_2S-producing enzymes were not involved in sensory neurogenic vasodilatation in the present study. In contrast, a previous study proposed a significant involvement of CSE in the activation of inhibitory neurotransmission in pig intravesical ureter, which led the authors to conclude that it is H_2S which activates sensory nerves and causes relaxation in intravesical ureter (Fernandes et

al., 2014). Species, tissues and experimental procedure differences may account for this discrepancy. Western blotting can be carried out to detect the presence of H_2S-producing enzyme in MABs, in the presence and absence of PVAT to compare the expression of these enzymes in PVAT and vessels.

The interactions between sympathetic and sensory innervations with PVAT-derived mediators

Despite PVAT receiving increasing attention due to the recognition that it is a source of a number of vasoactive compounds, little is known about the interactions between sympathetic and sensory nerves with PVAT-derived mediators in the vasculature. Few publications have described the interactions between PVAT-derived vasoactive compounds and sympathetic and/or sensory nerves (Hall et al., 2010; Murphy et al., 2013; Rayner and Trayhurn, 2001; Zhong et al., 1999). However, none of the publications showed the effects of direct sympathetic or sensory stimulation on PVAT mediators. Therefore, the present study was conducted with the aim of investigating the effect of electrical stimulation of sympathetic and sensory nerves on PVAT-mediators release. The present study was carried out under normal and low oxygenation to assess the effect of low oxygen on the nerve-evoked PVAT mediator release.

Chapter 6 investigated the effect of PVAT on several identified PVAT-derived adipokines and cytokines (adiponectin, leptin, MCP-1, IL-1β, IL-6, TNF-α and total PAI-1) release and also interactions between

sympathetic/sensory nerves, under normal and low oxygen levels. It is possible that there were some other mediators which were released under basal or pre-constricted tone, and during EFS-evoked sympathetic or sensory activation, but the mediators were not measured using multiplex assay in the present study. Therefore, chromatography and mass spectrometry could be employed to elucidate other mediators released from PVAT. The oxygen level should be determined, using methods including dissolved oxygen probe, to verify oxygen concentration in Krebs'-Henseleit solution, although it has been shown that in previous myography studies gassing with 95 % N_2 and 5 % CO_2 for 30 min markedly reduced the partial oxygen level (PaO_2) (White, 2012) and gassing for 2.5 hours induced hypoxia (Agabiti-Rosei et al., 2014; White, 2012; Withers et al., 2011).

The most interesting finding was that EFS-evoked sympathetic nerve activation enhanced leptin release under low oxygen levels in the presence of PVAT, and thus suggests that the interaction between sympathetic innervation and leptin becomes more evident in low oxygen level conditions. It has been reported that sympathetic stimulation in WAT, decreases leptin gene expression in the tissue and thus leptin production while sympathetic blockade often increases circulating leptin and leptin gene expression (Rayner and Trayhurn, 2001). A more recent study by Wang et al. (2008) showed contradictory effect on leptin gene expression in human preadipocyte cells, in which both leptin mRNA and immunoreactive leptin was essentially undetectable in preadipocytes incubated under normoxia (21 % O_2), but exposure to 1 % O_2 for 4 or 24 hours, resulted in an induction of leptin gene expression. Previous findings were in accordance with my findings in the current study. Little is known about the interactions between sensory nerves and PVAT-derived

mediators in the vasculature. A very recent report indicates that endogenous leptin secreted from white adipocytes acts by paracrine signalling to activate spinal sensory nerves innervating the tissue (Murphy et al., 2013). However, the role of sensory nerves on adipokines regulation remains unclear. The current study provides evidence on the direct control of leptin by sensory nerves. EFS-evoked sensory activation enhanced leptin release under normal oxygenation and this effect was virtually abolished by both PVAT removal and under conditions of low oxygen. These data indicate sympathetic and sensory nerves can directly control leptin release and, therefore, holds promise in pharmacological intervention (as discussed under Future directions).

Under the conditions of the present study, I demonstrate that leptin release is greater in MABs with PVAT than in those without PVAT. Under raised tone, but not under basal condition, adiponectin release is greater in MABs with PVAT than in those without PVAT. The present study also demonstrates that the level of oxygen plays a role in mediating the release of some PVAT-mediators. Low oxygen levels were shown to enhance inflammatory mediator, IL-6 release at both basal and raised tone, and reduced leptin levels under raised tone condition. Future experiments that could investigate the relationship between perivascular nerves and PVAT-derived mediators such as application of sympathectomy (guanethidine) or CGRP depletion (capsaicin pre-treatment) can be carried out to investigate the effect of denervation on mediator release. The current data indicate the existence of interactions between PVAT-derived nerves and PVAT-mediators, and hence may represent a potential strategy in the treatment of cardiovascular diseases.

Future directions

White adipose tissue is a primary site for leptin production (Zhang et al., 1994). Leptin has a significant role in the cardiovascular system, as a direct vasodilator (Mohammed et al., 2007; Nakagawa et al., 2002; Sahin and Bariskaner, 2007) and indirect vasoconstrictor in pathological states such as hypertension (Chatterjee et al., 2009; Rodriguez et al., 2007). Leptin is being studied extensively as it holds promise in obesity treatment. Obesity is closely related to hyperleptinemia and most obese humans are found to be resistant or tolerant to leptin (Dardeno et al., 2010). Increased leptin also leads to elevated haemostasis imbalance, vascular inflammation and insulin resistance (Wannamethee et al., 2007). In fact, chronic intravenous infusion of leptin increases heart rate and mean arterial blood pressure via sympathetic nervous system activation and increased release of catecholamines (Satoh et al., 1999; Shek et al., 1998; Shirasaka et al., 2003). The present study provides clear evidence that activation of sympathetic and sensory nerves in the presence of PVAT can directly modify leptin release in rat MABs. Therefore, based on the present data, further research should be carried out to investigate whether pharmacological modification of the nervous systems can alleviate leptin-related pathological conditions such as obesity, diabetes mellitus and hypertension. For instance, sympathetic stimulation in WAT has been shown to decrease leptin gene expression in the tissue and leptin production (Rayner and Trayhurn, 2001). Hence, it is possible that modification of sympathetic nervous system pharmacologically can control leptin release and this alteration may be useful in some pathological conditions including obesity. Furthermore, it is essential to identify the underlying mechanism/s, whether it involves a pre and/or post-junctional

mechanism. Further experiments, with the presence of exogenous leptin and NA, with or without EFS are required to investigate the underlying mechanism/s. Leptin is a hypoxia-sensitive adipocyte protein (Trayhurn et al., 2008); its expression increases in adipocytes under low PaO_2 tension (Grosfeld et al., 2002; Polotsky et al., 2004), and it is proposed to be involved in the development of the inflammatory response in adipose tissue, which occurs in the obese state (Trayhurn and Wood, 2004). The current study indicates that sympathetic activation in the presence of PVAT stimulated leptin release under low oxygen condition. Whether sympathetic nervous system modification using pharmacological approaches can improve the inflammatory response in hypoxia, is awaiting investigation.

Chapter 6 reveals the role of PVAT-derived sensory nerves in controlling leptin release, which holds promise in leptin deficiency and/or leptin dysfunction intervention. Leptin deficiency can lead to severe metabolic, endocrine, insulin resistance and immunological consequences (Bluher et al., 2009; German et al., 2010). Furthermore, leptin is also an important adipokine in the vasculature. Therefore, further research to investigate the interaction between nerves and leptin and application of this interaction is required as it has potential as therapeutic target. Chapter 4 indicates that PVAT is critically important for sensory-induced vasorelaxation. Further investigation to determine whether the neurogenic relaxation involves adipokine/s (especially leptin) is needed.

In addition, sympathetic perivascular neurotransmission is known to increase in hypertension (Grisk and Rettig, 2004; Lambert et al., 2010), and sensory neurotransmission is known to decrease in obesity, ageing and hypertension (Haddock and Hill, 2011; Watson et al., 2002; Westcott and Segal, 2013a). Whether similar changes occur for sympathetic and sensory

neurotransmission within PVAT, and the effect of this on control of vascular tone and PVAT-derived mediator release, remains to be explored. Furthermore, PVAT also has been recognized to contribute significantly in diabetes mellitus and metabolic syndromes (Yudkin et al., 2005), hypertension (Galvez-Prieto et al., 2008; Galvez et al., 2006) and atherosclerosis (Verhagen et al., 2012; Verhagen and Visseren, 2011). As the present study revealed the presence of sympathetic and sensory nerves in both large and small arteries of MABs PVAT, hence, the PVAT-derived nerves are potentially involve in pathophysiology of some diseases, therefore represent a potential strategy in the treatment for cardiovascular diseases and metabolic disorders through pharmacological intervention.

Conclusion

The present study demonstrated clear effects of PVAT on sympathetic and sensory neurotransmission. Chapter 3 characterized the expression of sympathetic nerves in PVAT of MABs and also indicates that PVAT-derived sympathetic nerves potentiate neurogenic vasocontractile responses. The present study also showed the involvement of angiotensin II in mediating enhanced sympathetic activity. The effect of replacing dissected PVAT on PVAT-denuded preparations also was examined. It was shown that PVAT removal attenuated EFS-evoked vasoconstriction, suggesting a release of transferrable vasorelaxing/inhibitory factors. This chapter also investigated modulation by exogenous PVAT-derived compounds, methyl palmitate and apelin-13. Exogenous methyl palmitate had no effect on sympathetic neurogenic contractions in PSAs in the presence and absence of PVAT while apelin-13 reduced sympathetic responses in both PVAT-intact and PVAT-denuded PSAs. Chapter 4 revealed the expression of sensory nerves in

PVAT of mesenteric arteries and clearly showed that removal of PVAT abolished EFS-evoked sensory responses. This chapter also suggested that rat small mesenteric arteries receive greater innervation than large arteries. EFS-evoked sensory vasodilatation measured in SMA under isometric tension indicates that sensory nerves have little or no role in regulating vasodilatation in large arteries. Chapter 5 demonstrated that H_2S causes vasodilatation of MABs by activating sensory nerves through the TRPA1-CGRP signalling pathway. This chapter also demonstrates that H_2S inhibits electrically-evoked sensory nerve actions, demonstrating a capsaicin-like action in the perfused mesenteric vascular bed, a preparation with abundant PVAT. However, my investigation showed that none of the H_2S-producing enzymes are involved in mediating this effect under the conditions of the present study. Chapter 6 revealed that leptin release is enhanced by EFS of sensory nerves under normal oxygen level and by EFS of sympathetic nerves under low oxygen level in the presence of PVAT. Leptin release was enhanced in MABs with PVAT compared to those without PVAT. The present study also showed that the level of PVAT-derived cytokine, IL-6 was elevated under low oxygen levels, thus indicating that the level of oxygen plays a role in mediating the release of some PVAT-mediators. Collectively, the findings in the present study clearly demonstrate the role of PVAT in modulating sympathetic and sensory neurotransmission and the information on this relatively unexplored link holds potential as a therapeutic strategy for cardiovascular diseases.

Appendix

Appendix 1: A 96-well multiplex assay plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A		S4	QC1									
B		S4										
C	S1	S5	QC2									
D	S1	S5										
E	S2	S6										
F	S2	S6										
G	S3	S7										
H	S3	S7										

	0 pg/mL background
	Standard 1-7
	QC-1 control
	QC-2 control
	Sympathetic basal (PVAT+): Krebs'-Henseleit solution
	Sympathetic EFS (PVAT+): 24 Hz, 90 V, 1 ms, 30 s + Krebs'-Henseleit solution
	Sensory basal (PVAT+): Krebs'-Henseleit solution + guanethidine (5 µM) + methoxamine (5 µM)
	Sensory EFS (PVAT+): 8 Hz, 60 V, 0.1 ms, 30 s + guanethidine 5 µM + methoxamine (5 µM) + Krebs'-Henseleit solution
	Sympathetic basal (PVAT-): Krebs'-Henseleit solution
	Sympathetic EFS (PVAT-): 24 Hz, 90 V, 1 ms, 30 s + Krebs'-Henseleit solution
	Sensory basal (PVAT-): Krebs'-Henseleit solution + guanethidine (5 µM) + methoxamine (5 µM)
	Sensory EFS (PVAT-): 8 Hz, 60 V, 0.1 ms, 30 s + guanethidine (5 µM) + methoxamine (5 µM) + Krebs'-Henseleit solution
	Sympathetic basal (PVAT+): Krebs'-Henseleit solution + 5 % $C0_2$+ 95 % N_2
	Sympathetic EFS (PVAT+): 24 Hz, 90 V, 1 ms, 30 s, 5 % $C0_2$+ 95 % N_2
	Sensory basal (PVAT+): Krebs'-Henseleit solution + guanethidine (5 µM) + methoxamine (5 µM) +5 % $C0_2$+ 95 % N_2
	Sensory EFS (PVAT+): 8 Hz, 60 V, 0.1 ms, 30 s + guanethidine (5 µM) + methoxamine (5 µM) + 5 % $C0_2$+ 95 % N_2

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