A NEW ROLE FOR PVAT: REGULATION OF BLOOD VESSEL FUNCTION THROUGH THE CONTROL OF NOREPINEPHRINE

By

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ABSTRACT

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Perivascular adipose tissue (PVAT), fat that surrounds blood vessels, has an active role in regulating vascular tone. PVAT is important to blood vessel function in many respects by altering the proliferation, migration, and inflammation of vascular smooth muscle, in addition to the modulation of vascular tone through the release of vasoactive molecules. Arteries and veins respond to adrenergic activation followed by changes in blood pressure. Adipose tissue is a complex organ that contains within it cells that have adrenergic components. A comprehensive characterization of an adrenergic system within PVAT has not been performed. An adrenergic system in mesenteric PVAT may mechanistically connect obesity and hypertension. We hypothesized that PVAT can release, metabolize, and take up norepinephrine (NE) to constitute an adrenergic system. We investigated the adrenergic system in PVAT by analyzing PVAT gene expression and protein profiles, performing immunohistochemistry and transport imaging on adipocytes, measuring PVAT catecholamines by HPLC, and by performing functional contractility studies. The research covered in this dissertation provides evidence that PVAT 1) *contains a vasoactive source of NE,* 2) *inactivates NE action on the blood vessel by uptake through molecular transport and that* 3) *NE metabolism reduces contraction of mesenteric arteries to NE.* These findings are an important contribution to the field of PVAT research because they directly connect PVAT to the modulation of blood vessel function through an adrenergic system and set the foundation for the hypothesis that

PVAT's adrenergic system may be dysfunctional in obesity and hypertension to tip the balance of vascular tone toward contraction.

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KEY TO ABBREVIATIONS

5-HT	5-hydroxytryptamine
ACE	angiotensin converting enzyme
AMPT	alpha-methyl-p-tyrosine
ASP^+	4-[4-(dimethylamino)-styrl]-*N*-methylpyridium iodide
BAT	brown adipose tissue
CGx	celiac ganglionectomy
COMT	catechol-O-methyl transferase
Dahl SS	Dahl salt-sensitive
DA	dopamine
DAT	dopamine transporter
DβH	dopamine beta-hydroxylase
DOCA	deoxycorticosterone acetate
E	epinephrine
EFS	electrical field stimulation
EMT	Extraneuronal monoamine transporter
FACS	fluorescence activated cell sorting
HF	high fat
HFD	high fat diet
HPLC	high-performance liquid chromatography
IBAT	interscapular brown adipose tissue

IHC	immunohistochemistry
iNOS	nitric oxide synthase (inducible)
Ki	inhibition constant
KO	Knock-out
MAO	monoamine oxidase
MAO-A/B	monoamine oxidase A/B
MPVAT	mesenteric PVAT
MRV	mesenteric resistance vessels
NC	non-convergent
NE	norepinephrine
NET	norepinephrine transporter
NETO	norepinephrine turnover
OCT3	organic cation transporter 3
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phenylephrine
PKC	protein kinase C
PNMT	phenylethanolamine N-methyltransferase
PNS	parasympathetic nervous system
PSS	physiological saline solution
PVAT	perivascular adipose tissue
RA	rat aorta
RMA	rat mesenteric artery

SERT	serotonin transporter
SGx	sham celiac ganglionectomy
SMA	superior mesentery artery
SNS	sympathetic nervous system
SP	semicarbazide and pargyline
SPC	semicarbazide, pargyline and corticosterone
SPN	semicarbazide, pargyline and nisoxetine
SSAO	semicarbazide sensitive amine oxidase
SVF	stromal vascular fraction
TH	tyrosine hydroxylase
TNF-alpha	tumor necrosis factor alpha
TPH1	tryptophan hydroxylase 1
TTX	tetrodotoxin
UCP-1	uncoupling protein-1
VAP-1	vascular adhesion protein 1
VMAT	vesicular monoamine transporter
WAT	white adipose tissue

CHAPTER 1

Introduction

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New action of an old friend: perivascular adipose tissue's adrenergic mechanisms

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Introduction

Most blood vessels are surrounded by adipose tissue, termed perivascular adipose tissue (PVAT). Other names for PVAT include the fourth layer of the blood vessel, or the "tunica adiposa" (Chaldakov et al., 2012). The revolutionary discovery in 1991 by Soltis and Cassis that PVAT has an anti-contractile effect changed how we thought about PVAT (Soltis and Cassis, 1991). Before Soltis and Cassis decided to closely examine PVAT's role in vascular function, many experiments to study vascular physiology began with the removal of PVAT. Thus, the role of PVAT on the regulation of vascular function was not fully appreciated. It was thought as having a minor role in vascular function and being present only for structural support (Szasz and Webb, 2012). We have since then learned that adipose tissue is a dynamic endocrine organ (Coelho et al., 2013; Trayhurn and Beattie, 2001), and that PVAT specifically, is important in vascular function (Szasz and Webb, 2012). The impetus to study PVAT was precipitated by the need to understand how obesity influences vascular function and it was found that PVAT dysfunction leads to alterations in blood vessel function occurs in obesity (Aghamohammadzadeh and Heagerty, 2012).

PVAT has a unique role in body homeostasis with its involvement in the regulation of blood pressure. Within PVAT resides a complex world of physiology comprised of nerves, immune cells, endothelial cells, pre-adipocytes, mesenchymal cells and adipocytes. PVAT is involved in the recruitment of immune cells to the vasculature, a process implicated in atherosclerosis (Verhagen and Visseren, 2011). We propose that PVAT influences vascular function in another way, through an adrenergic system; defined by having the ability to release, take-up and metabolize norepinephrine (NE).

Components of an adrenergic system exist within the types of cells found in adipose tissue but their function in PVAT specifically has not been studied. Having a complete picture of how the components of an adrenergic system in PVAT work together to affect normal blood vessel function would be informative in understanding PVAT dysfunction in disease. High sympathetic nervous system activity is observed in obesity-associated hypertension (Hall et al., 2010). Thus, an adrenergic system within PVAT may link obesity to the development of hypertension. This chapter will explore what is known about an adrenergic system in adipose tissue and PVAT, and what we still need to know.

Large strides in PVAT research have been made by studying adipose tissue as a whole. Adipose tissue has many roles. It participates in regulating energy balance, energy stores, inflammation, and body temperature. PVAT is an adipose tissue depot distinct from non-PVAT adipose tissues. PVAT differs from non-PVAT depots in adipocyte morphology, inflammatory response and level of differentiation (Guzik et al., 2007b; Rittig et al., 2012). Moreover, the adipocytes found in PVAT differ in phenotype by location. For example, PVAT around the superior mesenteric artery (SMA) and mesenteric artery (MA) branches from rats consists of unilocular adipocytes similar to those found in white adipose tissue (WAT), while aortic PVAT is a mixed PVAT with brown-like adipocytes (Gao, 2007). Mouse thoracic aortic PVAT was described as being similar to interscapular brown adipose tissue (IBAT) in gene expression (including high levels of *Ucp-1,* the gene for uncoupling protein-1) and phenotypically (multilocular mitochondria-rich adipocytes) (Fitzgibbons et al., 2011). Non-PVAT WAT depots have been studied intensely and include the gonadal (epididymal and periovarian), retroperitoneal, perirenal and subcutaneous WAT (Cinti, 2009). IBAT is the most commonly studied brown adipose

tissue (BAT) depot. Research on non-PVAT depots has been informative and serves as a starting point to understanding PVAT. This chapter will discuss non-PVAT adipose tissue as a surrogate for PVAT when information on the adrenergic system in PVAT specifically is not available. Judging from the uniqueness of PVAT, we may expect that what we already know about fat tells part - but not all - of the story of PVAT. This highlights the need for studies directly testing mechanisms in PVAT in locations relevant to blood pressure such as the mesentery resistance artery PVAT.

Neural innervation of adipose tissue and PVAT

As functions of PVAT that go beyond structural support and affect vascular homeostasis [including functions that affect the proliferation, differentiation, inflammation, and contractility of the vasculature as reviewed by Szasz and Webb (2012)] were discovered, a neuro-immune-adipose “triactome” was identified that connects adipose tissue, nerves and the immune system to the vasculature in a dynamic physiological system (Chaldakov et al. 2014). Adipose tissue is innervated by sensory nerves (Bartness and Song, 2007) but studies in PVAT to locate sensory nerves have not been done. Little evidence exists for the parasympathetic innervation of adipose tissue (Fliers et al., 2003; Giordano et al., 2006) and this also has not been studied in PVAT. Thus, these two nervous systems will not be discussed further.

Studies by Diculescu and Stoica (1970) carefully investigated the adrenergic innervation in WAT, including fluorochemical staining of adrenergic nerves within rat peri-epididymal WAT and mesenteric WAT. They observed nerves that innervated individual adipocytes and noted that BAT contained more sympathetic nerve fibers than WAT (Diculescu and Stoica, 1970).

Nerves innervating WAT originate in areas of the CNS that are associated with the regulation of energy balance, specifically in the brainstem and forebrain (Shi & Bartness 2001). Contreras et al. (2014) described the importance of sympathetic activation in the maintenance of a brown adipocyte phenotype. Brown adipocytes exist in inguinal adipose tissue from young mice but then turn into white adipocytes without continuous sympathetic stimulation (Contreras et al., 2014). Thus, sympathetic innervation is necessary not only for lipolysis but also for maintaining the thermogenic capacity of brown

adipocytes, and nerve density may affect this. Additional support for the conclusion that nerve density may be important in conferring a brown adipocyte phenotype is provided by the observation that the density of adrenergic nerve fibers (as measured by tyrosine hydroxylase [TH] immunohistochemical staining) correlated with the proportion of brown adipocytes in visceral and subcutaneous WAT from female mice (Murano et al., 2009). Stimulation of sympathetic activity with a 10-day period of cold-acclimation increased the amount of TH positive nerve fibers in those tissues (Murano et al., 2009). This suggests that sympathetic stimulation to WAT is less active than that of BAT. Thus, brown-like PVAT depots may be innervated more densely than WAT-like PVAT depots. The differences in sympathetic innervation and release of NE between specific PVAT depots remains to be studied.

Immunochemical analysis by Ballard et al. (1974) of adipose tissue surrounding canine mesenteric arteries revealed a pool of adipocytes in contact with nerves and another group of adipocytes that were not in contact with nerves. Later, Slavin and Ballard (1978) found that only 2-3% of all adipocytes were in direct connection to adrenergic nerves following careful examination of Sprague-Dawley rat mesenteric adipose tissue by glyoxylic acid/sodium molybdate staining and electron microscopy. Some of the nerves visualized adjacent to adipocytes contained synaptic vesicles. Due to NE having a low affinity for the beta-3 receptor (Lafontan et al., 1995), high concentrations of NE at the nerve-adipocyte junction would be necessary in order to activate lipolysis. Unfortunately, it has not been investigated whether PVAT adipocytes specifically are directly in contact with nerves through varicosities. Focusing on PVAT, Bulloch & Daly (2014) published images of mouse mesenteric artery PVAT stained for the pan-neuronal marker PGP 9.5,

which revealed that nerve fibers traversed through mesenteric PVAT. However, PGP 9.5 staining illuminates all types of nerve fibers. Distinguishing different nerve types in PVAT remains to be done. Part of this dissertation will include experiments to investigate the catecholamine content in PVAT and whether nerves within PVAT contribute to these catecholamines.

Catecholamine release by adipose tissue

In the adipose tissue around canine mesenteric arteries the sympathetic nerves are spread throughout, at times directly innervating adipocytes and at other times passing through adipose tissue to innervate the arteries (Ballard, 1978). Neuronal release of NE and the release of NE from other cell types in adipose tissue has been explored by several groups.

The idea that the adipocyte could contain a system for the handling of vasoactive amines was first presented by Stunes et al. (2011). A biochemical pathway for synthesis and reuptake of serotonin was identified in cultures of primary rat adipocytes isolated from visceral abdominal adipose tissue. Adipocyte culture supernatants contained 5-hydroxytryptamine (5-HT) and adipocytes expressed mRNA for 5-HT2A, 2B, 2C receptors, tryptophan hydroxylase 1 (Tph1) and the serotonin transporter (SERT; Stunes et al. 2011). Synthesis and release of NE from adipocytes has been demonstrated by several studies (Kvetnansky et al., 2012; Vargovic et al., 2011, 2013). A role for the adipocyte in the production of NE in PVAT remains to be investigated.

Other cells in PVAT that may produce NE include those of the stromal vascular fraction (SVF) such as macrophages (Brown et al., 2003) and lymphocytes (Josefsson et al., 1996; Qiu et al., 2004). Dopamine and NE are made in lymphocytes where they have a role in inducing apoptosis (Josefsson et al., 1996). Furthermore, splenic B and T lymphocytes from mice express mRNA for TH and phenylethanolamine N-methyltransferase (PNMT) and splenocytes increase their expression in response to stress by immobilization (Laukova et al., 2013). The role for catecholamine production in lymphocytes present in PVAT and their targets have not been studied. PVAT is a homing

site for immune cells. T cell recruitment into PVAT can lead to vascular dysfunction and hypertension (Guzik et al., 2007a). Adipose tissue macrophage secretion of catecholamines may play a role in adaptive thermogenesis and in stimulating lipolysis (Nguyen et al., 2011). Activating macrophages through the alternative pathway with an injection of IL-4 to male mice increased the level of the catecholamine synthesizing enzymes TH, dopa decarboxylase (DDC) and dopamine beta-hydroxylase (DβH) in peritoneal macrophages. Furthermore, IL-4 increased NE content in WAT of the mice indicating that the alternative activation of macrophages may turn on NE production within adipose tissue (Nguyen et al. 2011).

The finding that macrophages can actively secrete catecholamines is important as macrophage infiltration of tissues commonly occurs in obesity (Weisberg et al., 2003). Superoxide generated from infiltrating macrophages disrupts normal pre-synaptic alpha-2 adrenergic receptor function, increasing the release of NE from the nerves, a mechanism that elevates blood pressure (Thang et al., 2015). Thus, macrophage infiltration in PVAT may influence NE release and blood pressure in disease.

NE released from either neuronal or non-neuronal sources in PVAT can have effects other than directly stimulating vasoconstriction through binding of adrenergic receptors on vascular smooth muscle cells. For example, NE-induced mitochondrial superoxide (O_2^-) production and its rapid dismutation of superoxide into H_2O_2 by manganese-superoxide dismutase in the thoracic aorta is a mechanism by which PVAT exhibits an anti-contractile effect (Costa et al., 2016). Thus, NE released within PVAT may increase vascular tone through direct action on alpha adrenergic receptors on vascular smooth muscle or could tip the balance toward relaxation through H_2O_2

production. We will explore this question by studying the effects of the release of catecholamines from PVAT on arterial contraction.

Catecholamine uptake by PVAT and adipose tissue

Inactivation of catecholamines within an adrenergic system could occur through uptake and metabolism. Adipocytes have a functional uptake system for NE first described by Pizzinat et al. (1999) who identified uptake of [^{3}H]NE into adipocytes isolated from adipose tissue from women that underwent abdominal or mammary dermolipectomies. Evidence also supports catecholamine uptake by PVAT specifically. In the rat thoracic aorta, the presence of PVAT shifts the NE-contraction curve to the right compared to aortae with the PVAT removed. Incubation with the uptake-1 and uptake-2 transporter inhibitors deoxycorticosterone and desipramine removed the shift by PVAT to NE (Soltis and Cassis, 1991). The uptake-1 transporter and uptake-2 transporter are recognized now as the NE transporter (NET) and the organic cation transporter (OCT3), respectively.

The expression of the gene for OCT3 (*Slc22a3*) in PVAT has not been studied. However, it is highly expressed in adipose tissue and less so in other tissues examined (Jacobsson et al., 2007). OCT3 is a high-capacity, low affinity transporter that is able to transport NE (Verhaagh et al., 1999). Thus, OCT3 could be a candidate transporter in PVAT that allows it to act as a NE sink to remove NE from adventitial side of the blood vessel with the effect of reducing vascular contraction.

Uptake of NE has also been examined in IBAT where it was found to be mediated by NET (King et al., 1999). In IBAT, angiotensin II stimulates NE uptake demonstrated by the increase in [^{3}H]nisoxetine binding after an infusion of angiotensin II to rats (King et al., 2013). It is unknown whether angiotensin II stimulates NE uptake in PVAT. However, investigating this is beyond of the scope for this thesis.

The uptake system involved in PVAT's regulation of arterial tone may be an agonist (NE) specific system. It is one mechanism that could contribute to PVAT's anti-contractile effect in addition to the other mechanisms known such as the release of relaxant factor(s) that reduce contraction of the rat aorta to phenylephrine and angiotensin II (Löhn et al., 2002), and the activation of vascular smooth muscle potassium channels (Lynch et al., 2013; Weston et al., 2013). We will carefully assess the capability of PVAT to take up NE, determine which transporter(s) are responsible for NE uptake in PVAT, and assess whether uptake of NE reduces the contractile response of arteries with intact PVAT *vs.* those with PVAT removed.

Catecholamine metabolism by PVAT and adipose tissue

Pizzinat et al. (1999) were the first to identify an active system of uptake and metabolism for amines in adipose tissue. Their study demonstrated that human mammary adipose tissue adipocytes express both monoamine oxidase A (MAO-A) and monoamine oxidase B (MAO-B), with MAO-A being the more active enzyme. Since MAO-A is located intracellularly, amines would have to enter the cell first before being metabolized. The authors also identified that NE uptake occurred through the EMT (extraneuronal monoamine transporter; now named to be OCT3; Pizzinat et al. 1999).

Activity of the semicarbazide sensitive amine oxidase (SSAO) is high in adipose tissue and thus must be considered as a potential amine metabolizer in PVAT. SSAO has roles in adipogenesis (Mercier et al., 2001), cell differentiation (Filip et al., 2016) and glucose transport (El Hadri et al., 2002). Elliott et al. (1989a) investigated amine metabolism in isolated rat mesenteric arteries and found that metabolism of the substrates tyramine and benzylamine was carried out by SSAO and to a lesser extent MAO-A. Inhibition of both MAO-A and SSAO (with clorgyline and MDL 72145, respectively) was required to affect contraction of the arteries to tyramine (Elliott et al., 1989b). Studies to assess this effect in arteries with PVAT are needed in addition to studies investigating whether NE is metabolized by SSAO in PVAT.

Macrophages are another cell type in PVAT that may metabolize NE (Chaitidis et al., 2005). The NE metabolizing enzyme catechol-*O*-methyl transferase (COMT) has been found in dental pulp macrophages (Inoue et al., 1991). Rat peritoneal macrophages were found by Vega et al. (2004) to contain SSAO. However, the presence of COMT or SSAO in adipose tissue macrophages has not yet been reported. *In vitro* experiments

using U937 cells and human peripheral blood macrophages revealed that alternatively activated macrophages express MAO-A (Bhattacharjee et al., 2013; Chaitidis et al., 2005). This information paired with the knowledge that PVAT is pro-inflammatory in nature compared to other types of fats (Chatterjee et al., 2009), further supports the involvement of metabolism in PVAT NE inactivation. Thus, the mechanisms of PVAT NE metabolism and their effect on blood vessel contraction to NE are important to understand and will be explored in this dissertation.

Why study an adrenergic system in PVAT?

Adipose tissue interacts with the adrenergic system through the release of various adipokines. Three important adipokines that have adrenergic actions are leptin, resistin and angiotensin II, all of which are clinically relevant in hypertension and obesity. To understand the complete picture of how adipokines function, it is necessary to know what adrenergic components in PVAT are present that can interact with them especially in diseases like obesity where there is a larger PVAT mass and a dysregulation of adipokine secretion.

Notably, leptin is secreted from adipocytes and is part of a leptin-SNS feedback loop involved in controlling energy balance (Rayner and Trayhurn, 2001). Circulating leptin levels correlate with fat mass (Rayner and Trayhurn, 2001), are increased in obesity (Considine et al., 1996; Couillard et al., 1997) and are higher in rodents fed high fat diets compared to rodents fed non-high fat diets (Bussey et al., 2016; Marques et al., 2015). Evidence for the leptin-SNS feedback loop is supported by a study that found that female C57BL/6J *ob/ob* mice administered leptin have increased sympathetic activity to the IBAT as measured by NE turnover (NETO; Collins et al., 1996). Also, male rats that were administered leptin i.v. over three hours had an increase in sympathetic nerve activity to BAT compared to rats administered vehicle (Haynes et al., 1997). Although SNS activity was increased, arterial pressure and heart rate were not. However, increases in arterial pressure and heart rate were observed in a study where leptin was administered chronically to male Sprague-Dawley rats (Shek et al., 1998). Leptin's ability to increase sympathetic activity is leptin receptor-dependent as leptin did not increase sympathetic activity when administered to Zucker *fa/fa* obese rats that have a mutated leptin receptor

(Haynes et al., 1997). Leptin's effect to increase sympathetic activity are through central mechanisms. Administration of leptin centrally by an intracerebroventricular injection to rhesus macaques increased circulating NE (Tang-Christensen et al., 1999). Interestingly, NE has a role in inhibiting leptin release, suggesting a leptin-SNS feedback loop. Addition of NE or the beta-3 receptor agonist CL316,243 reduced leptin release in rat epididymal WAT adipocytes (Gettys et al., 1996). On the other hand, inhibiting catecholamine synthesis with an i.p. injection of alpha-methyl-p-tyrosine (AMPT) increased circulating leptin and leptin gene expression in the epididymal WAT of male mice (Rayner et al., 1998). In contrast, Evans et al. (1999) found that depletion of catecholamines by reserpine increased *Ob* mRNA expression in BAT, but not WAT, of male mice. Cold exposure reduced leptin expression in fat in a mechanism inhibited by propranolol (beta-1 and beta-2 receptor blocker) and SR 59230A (beta-3 antagonist; Evans et al. 1999), indicating that sympathetic stimulation of beta adrenergic receptors is necessary for stimulating leptin production.

PVAT's production of leptin was reduced in the aortic PVAT of spontaneously hypertensive rats (SHR) compared to their normotensive controls (Galvez-Prieto et al., 2012). By contrast, in the Dahl salt-sensitive (SS) rat, the aortic PVAT has higher leptin content than the aortic PVAT of SS.13^{BN} rats (normotensive, non-salt hypersensitive control for the Dahl SS rat) (Spradley et al., 2016). In high fat fed obese mice, aortic PVAT expression of leptin was markedly higher than that of lean mice (Ketonen et al., 2010). Further investigation of leptin alterations in different rodent models could be especially enlightening when considering obesity and hypertensive status.

Resistin is released by white adipocytes and circulating levels are increased in high fat fed, *ob/ob* and *db/db* mice (Steppan et al., 2001). Patients with essential hypertension have resistin levels that positively associate with inflammatory status and endothelial dysfunction (Fang et al., 2013). Resistin inhibits dopamine and NE release in hypothalamic synaptosomes *in vitro* (Brunetti et al., 2004). In a study of 302 adolescents by Souki et al. (2016), serum resistin concentrations in males were increased with obesity and non-obese females had higher levels of circulating resistin than the males. However, obese females did not have higher resistin levels than non-obese females (Souki et al., 2016). Circulating adiponectin, another adipocyte-derived factor, is decreased in obesity (Weyer et al., 2001). Adiponectin did not affect NE release in hypothalamic synaptosomes (Brunetti et al., 2004). Thus, resistin but not adiponectin may play a role in hypothalamic control of energy balance through NE release.

Angiotensin II from PVAT affects arterial contraction through interaction with the adrenergic system. Soltis and Cassis (1991) found that contraction of the aorta to electric field stimulation (EFS) was reduced in the presence of the alpha-adrenergic receptor antagonist phentolamine and by incubating the tissue with the angiotensin II receptor inhibitor Sar^1, Ile^8-AII. Their findings suggest that endogenous angiotensin II within the PVAT was potentiating the electrically-induced response. The mechanisms by which angiotensin II potentiated contraction to EFS were further explored using male Wistar rats by Lu et al. (2010). The authors found that PVAT around mesenteric arteries expressed angiotensinogen, angiotensin-converting enzyme (ACE) and angiotensin II. Inhibition of ACE by enalaprilat or inhibition of the angiotensin II receptor by candesartan reduced PVAT-dependent EFS-induced contraction of the SMA and *in vivo* administration of the

ACE inhibitor quinapril lowered blood pressure (Lu et al., 2010). Connecting the dots, Lu et al. (2010) proposed that these findings fit in with earlier studies that demonstrated that PVAT potentiated EFS-induced contraction possibly through the production of superoxide, a promoter of contraction through activation of the MAPK/ERK pathway (Gao et al., 2006; Lu et al., 2010) and proposed that PVAT-derived angiotensin II was potentiating EFS-induced contraction through MAPK/ERK activation. However, this hypothesis was not directly tested. A reduction of superoxide production or MAPK/ERK activation followed by EFS after inhibition of angiotensin II production would provide additional support for this hypothesis. Interestingly, experiments performed by Costa et al. (2016) in a different vessel, the aorta with PVAT, found that superoxide promoted relaxation due to its rapid dismutation to H_2O_2. Another mechanism that could explain how angiotensin II could potentiate EFS-induced contraction is through the activation of prejunctional angiotensin (AT) receptors to release NE (Cox et al., 1999) and this remains to be examined in PVAT. Beyond its actions of potentiating EFS-induced contraction, angiotensin II increases NE synthesis and uptake. King et al. demonstrated that angiotensin II facilitates neurotransmission to adipose tissue, which leads to a decrease in body weight (King et al., 2013). Infusion of angiotensin II to rats increased the amount of [^{3}H]nisoxetine binding in IBAT suggesting increased uptake of NE (King et al., 2013). NETO, as a measure of sympathetic activity, was increased during angiotensin II infusion in IBAT, epididymal fat, the left ventricle of the heart and the kidney (King et al., 2013). Increased circulating levels of angiotensin II are observed in obese individuals after beta-adrenergic stimulation with isoprenaline (Goossens et al., 2007). Taken together,

angiotensin II in PVAT may have effects on vascular function by potentiating sympathetically induced contraction.

Another reason why it is important to understand whether PVAT has an adrenergic system is that components of adipose tissue respond to NE through adrenergic receptor activation. Alpha-1, alpha-2, beta-1, beta-2 and beta-3 adrenergic receptors are found on adipocytes (Lafontan and Berlan, 1993). The presence and distribution of adrenergic receptors differ depending on the type of adipocyte and the adipose tissue depot. In white adipocytes, beta adrenergic receptor agonists stimulate lipolysis (beta-1, beta-2, beta-3; which are Gs-coupled), glycogenolysis (beta-1) and inhibit glucose transport (beta-2; Lafontan & Berlan 1993). Alpha-1 adrenergic receptors are coupled to Gq and result in the activation of PKC and thermogenesis in brown adipocytes and glycogenolysis in white adipocytes (Lafontan and Berlan, 1993). Stimulation of alpha-2 adrenergic receptors (Gi-coupled) results in adenylate cyclase inhibition and a resulting decrease in cAMP followed by a decrease in lipolysis (Lafontan and Berlan, 1993). Beyond activation of lipolysis through adrenergic receptors, NE can induce release of fatty acids which can activate thermogenesis through direct activation of UCP-1 in brown adipocytes, as demonstrated in adipocytes isolated from female mice (Matthias et al. 2000).

The adrenergic receptors present within PVAT include alpha and beta adrenergic receptors. Bulloch and Daly (2014) stained mouse mesenteric artery PVAT with fluorescent ligands to alpha and beta adrenergic receptors and found binding for both. However, classification of the receptor subtypes in PVAT was not performed.

Mast cells have been described to have adrenergic receptors and these cells are found in PVAT along adrenergic fibers (Diculescu and Stoica, 1970). *In vitro* stimulation

of beta-2 adrenergic receptors in the human mast cell line (HMC-1) with epinephrine enhanced their IL-1ß-induced production of the pro-inflammatory cytokines IL-6, IL-8 and IL-13 (Chi et al., 2004).

Lymphocytes are another cell type found in PVAT that can be acted upon by NE. Stimulation of beta-2 adrenergic receptors on mice splenic lymphocytes by NE inhibits TNF-α production (Vida et al., 2011). Alpha-1 and alpha-2 adrenergic receptors are expressed on T lymphocytes from rat mesenteric lymph nodes (Bao et al., 2007). NE addition to splenocyte cultures or the stimulation of NE production by lymphocytes following stress immobilization induces apoptosis (Josefsson et al., 1996; Laukova et al., 2013). However, NE responses in adipose tissue lymphocytes have not been examined.

Macrophages are one of most prevalent cell types in PVAT, besides adipocytes. Several groups have identified adrenergic receptors in macrophages. Beta and alpha-2 adrenergic receptors are present in rat peritoneal macrophages (Abrass et al., 1985; Spengler et al., 1990). Chi et al. (2003) found that macrophages can be stimulated by catecholamines to release nitric oxide (NO). To conclude this, the authors cultured RAW264.7 macrophages with lipopolysaccharide (LPS) and either epinephrine, NE or dopamine. LPS enhanced NO production by the macrophage and interestingly, epinephrine and NE enhanced NO production further with an increase in inducible nitric oxide synthase (iNOS; Chi et al., 2003). The implication is that catecholamine-dependent increases in NO production could impact blood vessel function if such a mechanism also is present in PVAT. The affinity of NE at each receptor subtype should be considered when judging whether the presence of adrenergic receptors on each cell type will produce a response. The multiple cell types in PVAT are instruments producing an adrenergic

sonata that can be directed by NE's actions through adrenergic receptors. What remains to be discovered are what mechanisms conduct this comprehensive orchestra in PVAT.

The physiological relevance of an adrenergic system in PVAT

There may be a normal physiological role for an adrenergic system in PVAT. Knowledge on PVAT's influence on vascular function in normal physiology is needed to understand the normal condition of adipose tissue and how PVAT dysfunction alters blood vessel physiology in diseases such as obesity. Worldwide, 1.9 billion people are overweight, classified by having a body mass index (BMI) of ≥25 (WHO, 2014). Moreover, 600 million adults are obese (BMI≥30), a staggering number that represents 13% of the world population. People that are obese are at a higher risk of developing hypertension, metabolic syndrome and cardiovascular disease (WHO, 2014). There is substantial literature supporting increased SNS activity in obesity with hypertension (Hall et al., 2010; Kalil and Haynes, 2012). Obesity-related hypertension responds better to treatment with alpha-adrenergic receptor inhibitors than non-obesity related hypertension, indicating adrenergic activity is an important factor in obesity-related hypertension (Wofford, 2001). Studies have further implicated adipocyte-derived adipokines, such as leptin, as part of the mechanism responsible for the increase in sympathetic activity (Smith and Minson, 2012). Adipocytes enlarge in obesity and size-dependent changes in the adipocyte of obese individuals are associated with the dysregulation of adipokine secretion (Fernandez-Alfonso et al., 2013) and increased release of angiotensin II, a stimulator of NE release (Cassis, 1993). Increased visceral fat mass correlates with increased arterial pressure (Fox et al., 2007). Changes in PVAT SVF resident cell populations occur in obesity (Nishimura et al., 2009) and cells within the SVF can also contribute to the release of NE. Thus, this could be a mechanism that increases NE at the site of alpha-1 adrenergic receptors on arteries to cause contraction.

Antidepressants such as the serotonin-NE reuptake inhibitors (SNRI) venlafaxine increase systolic blood pressure in patients with depression (Licht et al., 2009; Sir et al., 2005; Thase, 1998). Another SNRI, sibutramine (used to treat obesity), had significant effects on blood pressure (Luque and Rey, 1999). The inhibition of NE transporters in PVAT in response to SNRIs could point to another way that an adrenergic system PVAT may be clinically relevant to affect blood pressure. However, the contribution of NE uptake inhibition in PVAT, or adipose tissue in general, leading to increased blood needs to be investigated. This is an important avenue of research as obese individuals are at higher risk of developing high blood pressure (Kotchen, 2010).

We have yet to understand why all overweight and obese people do not develop hypertension. Could a protective mechanism within PVAT exist that is dysfunctional in disease and differs in people? Clearly, we need to closely examine PVAT and the adrenergic components within it. In obesity-associated hypertension, a dysfunction of the normal anti-contractile activities of PVAT is observed in addition to overall changes in adipocyte function (Aghamohammadzadeh and Heagerty, 2012). Whether the PVAT dysfunction extends to its adrenergic system remains to be investigated. A local adrenergic system within PVAT could contribute to the increased SNS activity in obesity. Additionally, alterations in PVAT inflammatory status in obesity could have effects on the underlying blood vessels through catecholamine release from immune cells or the adipocytes. Increased metabolism of catecholamines by amine oxidases also have the potential to affect blood vessel function. Visentin et al. (2005) investigated the effects of mice fed a high fat diet on MAO and SSAO activity in WAT of obesity-prone and obesity-resistant mice. The authors reported increased MAO and SSAO activities in the WAT of

obese mice compared to non-obese mice fed a high fat diet. However, this increase in activity was due to the increase in size of the adipose tissue depot, not the actual activity in the adipocyte (Visentin et al., 2005). Interestingly, while adipocytes contain intracellular MAO-A and SSAO, they also shed a membrane bound form of SSAO into the circulation in a mechanism regulated by TNF-α and insulin (Abella et al., 2004). Because SSAO can be shed from adipocytes, deamination of amines by SSAO may occur in the circulation at a distance away from the tissue depot from which it came. Metabolism of adrenergic agonists may reduce the amount of constrictor exposure to the underlying vessels, decreasing vascular tone. The reaction catalyzed by the amine oxidases releases H_2O_2 and aldehydes as products, which could also affect vascular tone. H_2O_2 can act as a vasodilator or vasoconstrictor depending on the vascular bed (Rubanyi, 1988) and can affect signaling in vascular smooth muscle. While aldehydes have the capability to form advanced glycation end products and contribute to atherosclerosis (Vasdev et al., 2007). MAO-A attenuates endothelium-dependent relaxation in the aorta through H_2O_2 production, which limits endothelial NO formation (Sturza et al., 2013). H_2O_2 production through SSAO activity may also have this effect. Thus, the release of H_2O_2 and aldehydes as products of amine metabolism could have multiple effects on vasomotor function.

Conclusion

PVAT makes up a neuro-immune-adipose “triactome” that surrounds the vasculature (Chaldakov et al., 2014). This makes PVAT an integral partner in obesity and hypertension. In both of these diseases, PVAT undergoes numerous changes that alter blood vessel function and response to agonists (Aghamohammadzadeh and Heagerty, 2012; Galvez et al., 2006). Factors that contribute to increases in blood pressure in hypertension include inflammation, endothelial dysfunction, atherosclerosis, adipokine secretion and reactive oxygen species generation, and each of these are affected by obesity in some way. Considering that PVAT is an essential part of the blood vessel environment and that it may form a dynamic adrenergic system directly outside of most blood vessels, PVAT should be included as a focus in understanding obesity-associated hypertension.

This leads us to study the presence of an adrenergic system in PVAT- one that can release, metabolize and take up catecholamines. The focus is on PVAT handling of the catecholamine NE, a potent vasoconstrictor. We will examine whether an adrenergic system (release, uptake, and metabolism of NE) exists in PVAT and identify the critical components that affect vascular contraction. The following hypotheses were designed to gather information on the mechanistic properties of what constitutes this adrenergic system in normal PVAT (not from a disease model) and to determine what are its functional consequences on blood vessel contractility. A diagram of the project’s aims is shown in Figure 1. This dissertation is a starting point from which we can begin to probe adrenergic alterations in disease to reveal new therapeutic targets to alleviate vascular dysfunction in obesity and obesity-associated hypertension.

Hypothesis

PVAT contains an adrenergic system that can release, metabolize, take up extracellular NE, and alter vascular contraction.

Specific aims

Aim 1: To test the hypothesis that PVAT releases NE and causes arterial contraction.

Aim 2: To test the hypothesis that PVAT inactivates NE action on the blood vessel by uptake through molecular transporters thereby, decreasing arterial contraction.

Aim 3: To test the hypothesis that PVAT NE metabolism reduces contraction of mesenteric arteries to NE.

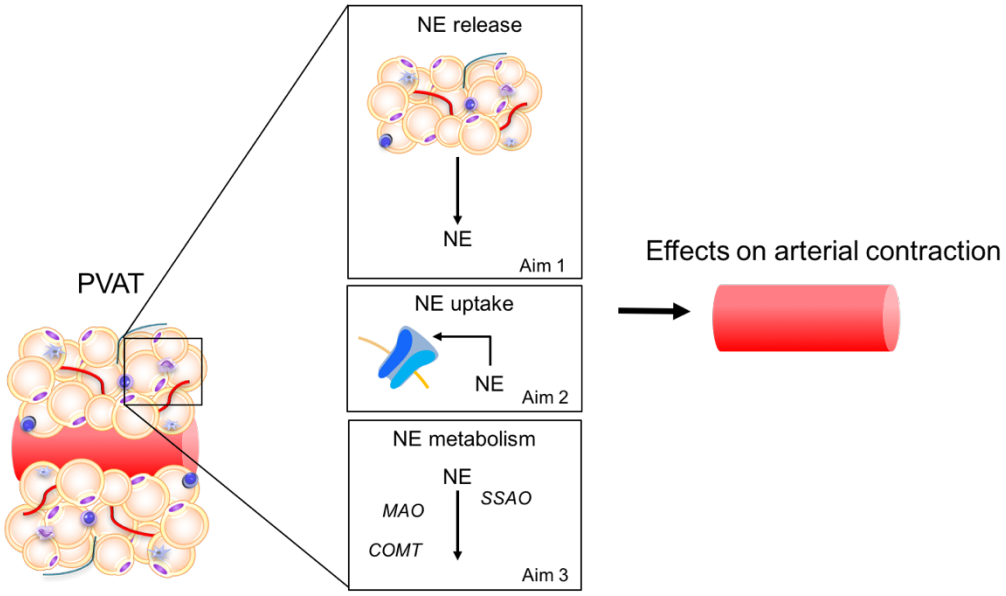

Figure 1. Diagram of specific aims.

COMT= catechol-o-methyltransferase, MAO= monoamine oxidase, NE= norepinephrine, SSAO= semicarbazide sensitive amine oxidase.

REFERENCES

REFERENCES

Abella, A., Garcia-Vicente, S., Viguerie, N., Ros-Baro, A., Camps, M., Palacin, M., et al. (2004). Adipocytes release a soluble form of VAP-1/SSAO by a metalloprotease-dependent process and in a regulated manner. *Diabetologia* 47, 429–438.

Abrass, C. K., O'Connor, S. W., Scarpace, P. J., and Abrass, I. B. (1985). Characterization of the beta-adrenergic receptor of the rat peritoneal macrophage. *J Immunol* 135, 1338–1341.

Aghamohammadzadeh, R., and Heagerty, A. M. (2012). Obesity-related hypertension: epidemiology, pathophysiology, treatments, and the contribution of perivascular adipose tissue. *Ann Med* 44 Suppl 1, S74-84.

Ballard, K., Malmfors, T., and Rosell, S. (1974). Adrenergic innervation and vascular patterns in canine adipose tissue. *Microvasc Res* 8, 164–171.

Ballard, K. W. (1978). Functional characteristics of the microcirculation in white adipose tissue. *Microvasc Res* 16, 1–18.

Bao, J.-Y., Huang, Y., Wang, F., Peng, Y.-P., and Qiu, Y.-H. (2007). Expression of alpha-AR subtypes in T lymphocytes and role of the alpha-ARs in mediating modulation of T cell function. *Neuroimmunomodulation* 14, 344–353. doi:10.1159/000129670.

Bartness, T., and Song, C. (2007). Sympathetic and sensory innervation of white adipose tissue. 48, 1655–1672. doi:10.1194/jlr.R700006-JLR200.

Bhattacharjee, A., Shukla, M., Yakubenko, V. P., Mulya, A., Kundu, S., and Cathcart, M. K. (2013). IL-4 and IL-13 employ discrete signaling pathways for target gene expression in alternatively activated monocytes/macrophages. *Free Radic Biol Med* 54, 1–16. doi:10.1016/j.freeradbiomed.2012.10.553.

Brown, S. W., Meyers, R. T., Brennan, K. M., Rumble, J. M., Narasimhachari, N., Perozzi, E. F., et al. (2003). Catecholamines in a macrophage cell line. *J Neuroimmunol* 135, 47–55. doi:10.1016/S0165-5728(02)00435-6.

Brunetti, L., Orlando, G., Recinella, L., Michelotto, B., Ferrante, C., and Vacca, M. (2004). Resistin, but not adiponectin, inhibits dopamine and norepinephrine release in the hypothalamus. *Eur J Pharmacol* 493, 41–44. doi:10.1016/j.ejphar.2004.04.020.

Bulloch, J. M., and Daly, C. J. (2014). Autonomic nerves and perivascular fat: interactive mechanisms. *Pharmacol Ther* 143, 61–73. doi:10.1016/j.pharmthera.2014.02.005.

Bussey, C. E., Withers, S. B., Aldous, R. G., Edwards, G., and Heagerty, A. M. (2016). Obesity-Related Perivascular Adipose Tissue Damage Is Reversed by Sustained Weight Loss in the Rat. *Arterioscler Thromb Vasc Biol*.

doi:10.1161/ATVBAHA.116.307210.

Cassis, L. A. (1993). Role of angiotensin II in brown adipose thermogenesis during cold acclimation. *Am J Physiol* 265, E860-5.

Chaitidis, P., Billett, E., Kuban, R. J., Ungethuem, U., and Kuhn, H. (2005). Expression regulation of MAO isoforms in monocytic cells in response to Th2 cytokines. *Med Sci Monit* 11, BR259-65.

Chaldakov, G. N., Beltowsky, J., Ghenev, P. I., Fiore, M., Panayotov, P., Rančič, G., et al. (2012). Adipoparacrinology--vascular periadventitial adipose tissue (tunica adiposa) as an example. *Cell Biol Int* 36, 327–330. doi:10.1042/CBI20110422.

Chaldakov, G. N., Fiore, M., Ghenev, P. I., Beltowski, J., Rancic, G., Tuncel, N., et al. (2014). Triactome: neuro-immune-adipose interactions. Implication in vascular biology. *Front Immunol* 5, 130. doi:10.3389/fimmu.2014.00130.

Chatterjee, T. K., Stoll, L. L., Denning, G. M., Harrelson, A., Blomkalns, A. L., Idelman, G., et al. (2009). Proinflammatory phenotype of perivascular adipocytes: influence of high-fat feeding. *Circ Res* 104, 541–549. doi:10.1161/CIRCRESAHA.108.182998.

Chi, D. S., Fitzgerald, S. M., Pitts, S., Cantor, K., King, E., Lee, S. A., et al. (2004). MAPK-dependent regulation of IL-1- and beta-adrenoreceptor-induced inflammatory cytokine production from mast cells: implications for the stress response. *BMC Immunol* 5, 22. doi:10.1186/1471-2172-5-22.

Chi, D. S., Qui, M., Krishnaswamy, G., Li, C., and Stone, W. (2003). Regulation of nitric oxide production from macrophages by lipopolysaccharide and catecholamines. *Nitric Oxide* 8, 127–132. doi:10.1016/S1089-8603(02)00148-9.

Cinti, S. (2009). Transdifferentiation properties of adipocytes in the adipose organ. *AJP Endocrinol Metab* 297, E977–E986. doi:10.1152/ajpendo.00183.2009.

Coelho, M., Oliveira, T., and Fernandes, R. (2013). Biochemistry of adipose tissue: An endocrine organ. *Arch Med Sci* 9, 191–200. doi:10.5114/aoms.2013.33181.

Collins, S., Kuhn, C. M., Petro, A. E., Swick, A. G., Chrunyk, B. A., and Surwit, R. S. (1996). Role of leptin in fat regulation. *Nature* 380, 677. doi:10.1038/380677a0.

Considine, R. V, Sinha, M. K., Heiman, M. L., Kriauciunas, A., Stephens, T. W., Nyce, M. R., et al. (1996). Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 334, 292–5. doi:10.1056/NEJM199602013340503.

Contreras, G. A., Lee, Y., Mottillo, E. P., and Granneman, J. G. (2014). Inducible brown adipocytes in subcutaneous inguinal white fat : the role of continuous sympathetic stimulation. *AJP Endocrinol Metab* 307, E793–E799. doi:10.1152/ajpendo.00033.2014.

Costa, R. M., Filgueira, F. P., Tostes, R. C., Carvalho, M. H. C., Akamine, E. H., and

Lobato, N. S. (2016). H2O2 generated from mitochondrial electron transport chain in thoracic perivascular adipose tissue is crucial for modulation of vascular smooth muscle contraction. *Vascul Pharmacol*. doi:10.1016/j.vph.2016.05.008.

Couillard, C., Mauriège, P., Prud'homme, D., Nadeau, A., Tremblay, A., Bouchard, C., et al. (1997). Plasma leptin concentrations: gender differences and associations with metabolic risk factors for cardiovascular disease. *Diabetologia* 40, 1178–84. doi:10.1007/s001250050804.

Cox, S. L., Trendelenburg, A. U., and Starke, K. (1999). Prejunctional angiotensin receptors involved in the facilitation of noradrenaline release in mouse tissues. *Br J Pharmacol* 127, 1256–62. doi:10.1038/sj.bjp.0702652.

Diculescu, I., and Stoica, M. (1970). Fluorescence histochemical investigation on the adrenergic innervation of the white adipose tissue in the rat. *J Neurovisc Relat* 32, 25–36.

Elliott, J., Callingham, B. A., and Sharman, D. F. (1989a). Metabolism of amines in the isolated perfused mesenteric arterial bed of the rat. *Br J Pharmacol* 98, 507–14.

Elliott, J., Callingham, B. A., and Sharman, D. F. (1989b). The influence of amine metabolizing enzymes on the pharmacology of tyramine in the isolated perfused mesenteric arterial bed of the rat. *Br J Pharmacol* 98, 515–22.

Evans, B. A., Agar, L., and Summers, R. J. (1999). The role of the sympathetic nervous system in the regulation of leptin synthesis in C57BL/6 mice. *FEBS Lett* 444, 149–154. doi:10.1016/S0014-5793(99)00049-6.

Fang, C., Lei, J., Zhou, S., Zhang, Y., Yuan, G., and Wang, J. (2013). Association of higher resistin levels with inflammatory activation and endothelial dysfunction in patients with essential hypertension. *Chin Med J (Engl)* 126, 646–9.

Fernandez-Alfonso, M. S., Gil-Ortega, M., Garcia-Prieto, C. F., Aranguez, I., Ruiz-Gayo, M., and Somoza, B. (2013). Mechanisms of perivascular adipose tissue dysfunction in obesity. *Int J Endocrinol* 2013, 402053. doi:10.1155/2013/402053.

Filip, A., Pinzano, A., Bianchi, A., Fève, B., Jalkanen, S., Gillet, P., et al. (2016). Expression of the semicarbazide-sensitive amine oxidase in articular cartilage: its role in terminal differentiation of chondrocytes in rat and human. *Osteoarthr Cartil* 24, 1223–1234. doi:10.1016/j.joca.2016.01.340.

Fitzgibbons, T. P., Kogan, S., Aouadi, M., Hendricks, G. M., Straubhaar, J., Czech, M. M. P., et al. (2011). Similarity of mouse perivascular and brown adipose tissues and their resistance to diet-induced inflammation. *Am J Physiol Hear Circ Physiol* 301, H1425-37. doi:10.1152/ajpheart.00376.2011.

Fliers, E., Kreier, F., Voshol, P. J., Havekes, L. M., Sauerwein, H. P., Kalsbeek, A., et al. (2003). White adipose tissue: Getting nervous. *J Neuroendocrinol* 15, 1005–1010.

doi:10.1046/j.1365-2826.2003.01096.x.

Fox, C. S., Massaro, J. M., Hoffmann, U., Pou, K. M., Maurovich-Horvat, P., Liu, C. Y., et al. (2007). Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study. *Circulation* 116, 39–48. doi:10.1161/CIRCULATIONAHA.106.675355.

Galvez-Prieto, B., Somoza, B., Gil-Ortega, M., Garcia-Prieto, C. F., de Las Heras, A. I., Gonzalez, M. C., et al. (2012). Anticontractile Effect of Perivascular Adipose Tissue and Leptin are Reduced in Hypertension. *Front Pharmacol* 3, 103. doi:10.3389/fphar.2012.00103.

Galvez, B., De Castro, J., Herold, D., Dubrovska, G., Arribas, S., Gonzonlez, M. C., et al. (2006). Perivascular adipose tissue and mesenteric vascular function in spontaneously hypertensive rats. *Arter Thromb Vasc Biol* 26, 1297–1302. doi:10.1161/01.ATV.0000220381.40739.dd.

Gao, Y.-J. J. (2007). Dual modulation of vascular function by perivascular adipose tissue and its potential correlation with adiposity/lipoatrophy-related vascular dysfunction. *Curr Pharm Des* 13, 2185–2192. doi:10.2174/138161207781039634.

Gao, Y.-J. J., Takemori, K., Su, L.-Y. Y., An, W.-S. S., Lu, C., Sharma, A. M., et al. (2006). Perivascular adipose tissue promotes vasoconstriction: the role of superoxide anion. *Cardiovasc Res* 71, 363–373. doi:10.1016/j.cardiores.2006.03.013.

Gettys, T. W., Harkness, P. J., and Watson, P. M. (1996). The beta 3-adrenergic receptor inhibits insulin-stimulated leptin secretion from isolated rat adipocytes. *Endocrinology* 137, 4054–7. doi:10.1210/endo.137.9.8756584.

Giordano, A., Song, C. K., Bowers, R. R., Ehlen, J. C., Frontini, A., Cinti, S., et al. (2006). White adipose tissue lacks significant vagal innervation and immunohistochemical evidence of parasympathetic innervation. *Am J Physiol Regul Integr Comp Physiol* 291, R1243-55. doi:10.1152/ajpregu.00679.2005.

Goossens, G. H., Jocken, J. W. E., Blaak, E. E., Schiffers, P. M., Saris, W. H. M., and van Baak, M. A. (2007). Endocrine role of the renin-angiotensin system in human adipose tissue and muscle: effect of beta-adrenergic stimulation. *Hypertension* 49, 542–7. doi:10.1161/01.HYP.0000256091.55393.92.

Guzik, T. J., Hoch, N. E., Brown, K. A., McCann, L. A., Rahman, A., Dikalov, S., et al. (2007a). Role of the T cell in the genesis of angiotensin II induced hypertension and vascular dysfunction. *J Exp Med* 204, 2449–2460. doi:10.1084/jem.20070657.

Guzik, T. J., Marvar, P. J., Czesnikiewicz-Guzik, M., and Korbut, R. (2007b). Perivascular adipose tissue as a messenger of the brain-vessel axis: Role in vascular inflammation and dysfunction. *J Physiol Pharmacol* 58, 591–610. doi:10.1111/j.1742-6723.2011.01449.x.

El Hadri, K., Moldes, M., Mercier, N., Andreani, M., Pairault, J., and Feve, B. (2002). Semicarbazide-sensitive amine oxidase in vascular smooth muscle cells: differentiation-dependent expression and role in glucose uptake. *Arterioscler Thromb Vasc Biol* 22, 89–94.

Hall, J. E., da Silva, A. A., do Carmo, J. M., Dubinion, J., Hamza, S., Munusamy, S., et al. (2010). Obesity-induced hypertension: role of sympathetic nervous system, leptin, and melanocortins. *J Biol Chem* 285, 17271–6. doi:10.1074/jbc.R110.113175.

Haynes, W. G., Morgan, D. A., Walsh, S. A., Mark, A. L., and Sivitz, W. I. (1997). Receptor-mediated regional sympathetic nerve activation by leptin. *J Clin Invest* 100, 270–278. doi:10.1172/JCI119532.

Inoue, K., Nishino, T., and Creveling, C. R. (1991). Immunocytochemical evidence for the site of O-methylation in rat dental pulp. *J Dent Res* 70, 966–9.

Jacobsson, J. A., Haitina, T., Lindblom, J., and Fredriksson, R. (2007). Identification of six putative human transporters with structural similarity to the drug transporter SLC22 family. *Genomics* 90, 595–609. doi:10.1016/j.ygeno.2007.03.017.

Josefsson, E., Bergquist, J., Ekman, R., and Tarkowski, A. (1996). Catecholamines are synthesized by mouse lymphocytes and regulate function of these cells by induction of apoptosis. *Immunology* 88, 140–6.

Kalil, G. Z., and Haynes, W. G. (2012). Sympathetic nervous system in obesity-related hypertension: mechanisms and clinical implications. *Hypertens Res* 35, 4–16. doi:10.1038/hr.2011.173.

Ketonen, J., Shi, J., Martonen, E., and Mervaala, E. (2010). Periadventitial adipose tissue promotes endothelial dysfunction via oxidative stress in diet-induced obese C57Bl/6 mice. *Circ J* 74, 1479–1487.

King, V. L., Dwoskin, L. P., and Cassis, L. A. (1999). Cold exposure regulates the norepinephrine uptake transporter in rat brown adipose tissue. *Am J Physiol* 276, R143-51.

King, V. L., English, V. L., Bharadwaj, K., and Cassis, L. a (2013). Angiotensin II Stimulates Sympathetic Neurotransmission to Adipose Tissue. *Physiol Rep* 1, 1–12. doi:10.1002/phy2.14.

Kotchen, T. A. (2010). Obesity-Related Hypertension: Epidemiology, Pathophysiology, and Clinical Management. *Am J Hypertens* 23, 1170–1178. doi:10.1038/ajh.2010.172.

Kvetnansky, R., Ukropec, J., Laukova, M., Manz, B., Pacak, K., and Vargovic, P. (2012). Stress stimulates production of catecholamines in rat adipocytes. *Cell Mol Neurobiol* 32, 801–813. doi:10.1007/s10571-012-9822-6.

Lafontan, M., and Berlan, M. (1993). Fat cell adrenergic receptors and the control of

white and brown fat cell function. *J Lipid Res* 34, 1057–91.

Lafontan, M., Bousquet-Melou, A., Galitzky, J., Barbe, P., Carpéné, C., Langin, D., et al. (1995). Adrenergic receptors and fat cells: differential recruitment by physiological amines and homologous regulation. *Obes Res* 3 Suppl 4, 507S–514S.

Laukova, M., Vargovic, P., Vlcek, M., Lejavova, K., Hudecova, S., Krizanova, O., et al. (2013). Catecholamine production is differently regulated in splenic T- and B-cells following stress exposure. *Immunobiology* 218, 780–789. doi:10.1016/j.imbio.2012.08.279.

Licht, C. M. M., de Geus, E. J. C., Seldenrijk, A., van Hout, H. P. J., Zitman, F. G., van Dyck, R., et al. (2009). Depression is associated with decreased blood pressure, but antidepressant use increases the risk for hypertension. *Hypertens (Dallas, Tex 1979)* 53, 631–8. doi:10.1161/HYPERTENSIONAHA.108.126698.

Löhn, M., Dubrovska, G., Lauterbach, B., Luft, F. C., Gollasch, M., Sharma, A. M., et al. (2002). Periadventitial fat releases a vascular relaxing factor. *FASEB J* 16, 1057–1063. doi:10.1096/fj.02-0024com.

Lu, C., Su, L. Y., Lee, R. M., and Gao, Y. J. (2010). Mechanisms for perivascular adipose tissue-mediated potentiation of vascular contraction to perivascular neuronal stimulation: the role of adipocyte-derived angiotensin II. *Eur J Pharmacol* 634, 107–112. doi:10.1016/j.ejphar.2010.02.006.

Luque, C. A., and Rey, J. A. (1999). Sibutramine: a serotonin-norepinephrine reuptake-inhibitor for the treatment of obesity. *Ann Pharmacother* 33, 968–78.

Lynch, F. M., Withers, S. B., Yao, Z., Werner, M. E., Edwards, G., Weston, A. H., et al. (2013). Perivascular adipose tissue-derived adiponectin activates BK(Ca) channels to induce anticontractile responses. *Am J Physiol Hear Circ Physiol* 304, H786-95. doi:10.1152/ajpheart.00697.2012.

Marques, C., Meireles, M., Norberto, S., Leite, J., Freitas, J., Pestana, D., et al. (2015). High-fat diet-induced obesity Rat model: a comparison between Wistar and Sprague-Dawley Rat. *Adipocyte* 5, 11–21. doi:10.1080/21623945.2015.1061723.

Matthias, A., Ohlson, K. B. E., Fredriksson, J. M., Jacobsson, A., Nedergaard, J., and Cannon, B. (2000). Thermogenic responses in brown fat cells are fully UCP1-dependent. UCP2 or UCP3 do not substitute for UCP1 in adrenergically or fatty acid-induced thermogenesis. *J Biol Chem* 275, 25073–25081. doi:10.1074/jbc.M000547200.

Mercier, N., Moldes, M., El Hadri, K., and Fève, B. (2001). Semicarbazide-sensitive amine oxidase activation promotes adipose conversion of 3T3-L1 cells. *Biochem J* 358, 335–342.

Murano, I., Barbatelli, G., Giordano, A., and Cinti, S. (2009). Noradrenergic parenchymal nerve fiber branching after cold acclimatisation correlates with brown

adipocyte density in mouse adipose organ. *J Anat* 214, 171–8. doi:10.1111/j.1469-7580.2008.01001.x.

Nguyen, K. D., Qiu, Y., Cui, X., Goh, Y. P. S., Mwangi, J., David, T., et al. (2011). Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis. *Nature* 480, 104–108. doi:10.1038/nature10653.

Nishimura, S., Manabe, I., Nagasaki, M., Eto, K., Yamashita, H., Ohsugi, M., et al. (2009). CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat Med* 15, 914–920. doi:10.1038/nm.1964.

Obesity and overweight (Fact Sheet 311) (2014). 2015. Available at: http://www.who.int/mediacentre/factsheets/fs311/en/.

Pizzinat, N., Marti, L., Remaury, A., Leger, F., Langin, D., Lafontan, M., et al. (1999). High expression of monoamine oxidases in human white adipose tissue: evidence for their involvement in noradrenaline clearance. *Biochem Pharmacol* 58, 1735–1742.

Qiu, Y. H., Peng, Y. P., Jiang, J. M., and Wang, J. J. (2004). Expression of Tyrosine Hydroxylase in Lymphocytes and Effect of Endogenous Catecholamines on Lymphocyte Function. *Neuroimmunomodulation* 11, 75–83. doi:10.1159/000075316.

Rayner, D. V., Simón, E., Duncan, J. S., and Trayhurn, P. (1998). Hyperleptinaemia in mice induced by administration of the tyrosine hydroxylase inhibitor α-methyl-p-tyrosine. *FEBS Lett* 429, 395–398. doi:10.1016/S0014-5793(98)00642-5.

Rayner, D. V., and Trayhurn, P. (2001). Regulation of leptin production: Sympathetic nervous system interactions. *J Mol Med* 79, 8–20. doi:10.1007/s001090100198.

Rittig, K., Dolderer, J. H., Balletshofer, B., Machann, J., Schick, F., Meile, T., et al. (2012). The secretion pattern of perivascular fat cells is different from that of subcutaneous and visceral fat cells. *Diabetologia* 55, 1514–1525. doi:10.1007/s00125-012-2481-9.

Rubanyi, G. M. (1988). Vascular effects of oxygen-derived free radicals. *Free Radic Biol Med* 4, 107–120. doi:10.1016/0891-5849(88)90071-8.

Shek, E. W., Brands, M. W., and Hall, J. E. (1998). Chronic leptin infusion increases arterial pressure. *Hypertension* 31, 409–414. doi:10.1161/01.HYP.31.1.409.

Shi, H., and Bartness, T. J. (2001). Neurochemical phenotype of sympathetic nervous system outflow from brain to white fat. *Brain Res Bull* 54, 375–385. doi:10.1016/S0361-9230(00)00455-X.

Sir, A., D'Souza, R. F., Uguz, S., George, T., Vahip, S., Hopwood, M., et al. (2005). Randomized trial of sertraline versus venlafaxine XR in major depression: efficacy and discontinuation symptoms. *J Clin Psychiatry* 66, 1312–20.

Slavin, B. G., and Ballard, K. W. (1978). Morphological studies on the adrenergic innervation of white adipose tissue. *Anat Rec* 191, 377–389. doi:10.1002/ar.1091910310.

Smith, M. M., and Minson, C. T. (2012). Obesity and adipokines: effects on sympathetic overactivity. *J Physiol* 590, 1787–801. doi:10.1113/jphysiol.2011.221036.

Soltis, E. E., and Cassis, L. A. (1991). Influence of perivascular adipose tissue on rat aortic smooth muscle responsiveness. *Clin Exp Hypertens A* 13, 277–296.

Souki, A., Arraíz, N., Prieto, C., Pérez Jiménez, F., Valero, P., Amell, A., et al. (2016). Association between resistin serum levels and dimension and body composition variables in children and adolescents. *Rev médica Chile* 144, 307–16. doi:10.4067/S0034-98872016000300005.

Spengler, R. N., Allen, R. M., Remick, D. G., Strieter, R. M., and Kunkel, S. L. (1990). Stimulation of alpha-adrenergic receptor augments the production of macrophage-derived tumor necrosis factor. *J Immunol* 145, 1430–4.

Spradley, F. T., Ho, D. H., and Pollock, J. S. (2016). Dahl SS rats demonstrate enhanced aortic perivascular adipose tissue-mediated buffering of vasoconstriction through activation of NOS in the endothelium. *Am J Physiol Regul Integr Comp Physiol* 310, R286-96. doi:10.1152/ajpregu.00469.2014.

Steppan, C. M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., et al. (2001). The hormone resistin links obesity to diabetes. *Nature* 409, 307–12. doi:10.1038/35053000.

Stunes, A. K., Reseland, J. E., Hauso, O., Kidd, M., Tommeras, K., Waldum, H. L., et al. (2011). Adipocytes express a functional system for serotonin synthesis, reuptake and receptor activation. *Diabetes Obes Metab* 13, 551–558. doi:10.1111/j.1463-1326.2011.01378.x.

Sturza, A., Leisegang, M. S., Babelova, A., Schröder, K., Benkhoff, S., Loot, A. E., et al. (2013). Monoamine oxidases are mediators of endothelial dysfunction in the mouse aorta. *Hypertension* 62, 140–146. doi:10.1161/HYPERTENSIONAHA.113.01314.

Szasz, T., and Webb, R. C. (2012). Perivascular adipose tissue: more than just structural support. *Clin Sci* 122, 1–12. doi:10.1042/CS20110151.

Tang-Christensen, M., Havel, P. J., Jacobs, R. R., Larsen, P. J., and Cameron, J. L. (1999). Central Administration of Leptin Inhibits Food Intake and Activates the Sympathetic Nervous System in Rhesus Macaques [1]. *J Clin Endocrinol Metab* 84, 711–717. doi:10.1210/jcem.84.2.5458.

Thang, L. V, Demel, S. L., Crawford, R., Kaminski, N. E., Swain, G. M., Van Rooijen, N., et al. (2015). Macrophage depletion lowers blood pressure and restores sympathetic nerve α2-adrenergic receptor function in mesenteric arteries of DOCA-salt hypertensive

rats. *Am J Physiol Heart Circ Physiol* 309, H1186-97. doi:10.1152/ajpheart.00283.2015.

Thase, M. E. (1998). Effects of venlafaxine on blood pressure: A meta-analysis of original data from 3744 depressed patients. *J Clin Psychiatry* 59, 502–508.

Trayhurn, P., and Beattie, J. H. (2001). Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proc Nutr Soc* 60, 329–339. doi:10.1079/PNS200194.

Vargovic, P., Ukropec, J., Laukova, M., Cleary, S., Manz, B., Pacak, K., et al. (2011). Adipocytes as a new source of catecholamine production. *FEBS Lett* 585, 2279–2284. doi:10.1016/j.febslet.2011.06.001.

Vargovic, P., Ukropec, J., Laukova, M., Kurdiova, T., Balaz, M., Manz, B., et al. (2013). Repeated immobilization stress induces catecholamine production in rat mesenteric adipocytes. *Stress* 16, 340–352. doi:10.3109/10253890.2012.736046.

Vasdev, S., Gill, V., and Singal, P. (2007). Role of advanced glycation end products in hypertension and atherosclerosis: therapeutic implications. *Cell Biochem Biophys* 49, 48–63.

Vega, A., Chacón, P., Monteseirín, J., Bekay, R. El, Álvarez, M., Alba, G., et al. (2004). A new role for monoamine oxidases in the modulation of macrophage-inducible nitric oxide synthase gene expression. *J Leukoc Biol* 75, 1093–1101. doi:10.1189/jlb.1003459.

Verhaagh, S., Schweifer, N., Barlow, D. P., and Zwart, R. (1999). Cloning of the mouse and human solute carrier 22a3 (Slc22a3/SLC22A3) identifies a conserved cluster of three organic cation transporters on mouse chromosome 17 and human 6q26-q27. *Genomics* 55, 209–218. doi:10.1006/geno.1998.5639.

Verhagen, S. N., and Visseren, F. L. J. J. (2011). Perivascular adipose tissue as a cause of atherosclerosis. *Atherosclerosis* 214, 3–10. doi:10.1016/j.atherosclerosis.2010.05.034.

Vida, G., Pena, G., Kanashiro, A., del Rocio Thompson-Bonilla, M., Palange, D., Deitch, E. a., et al. (2011). β2-Adrenoreceptors of regulatory lymphocytes are essential for vagal neuromodulation of the innate immune system. *FASEB J* 25, 4476–85. doi:10.1096/fj.11-191007.

Visentin, V., Boucher, J., Bour, S., Prevot, D., Castan, I., Carpene, C., et al. (2005). Influence of high-fat diet on amine oxidase activity in white adipose tissue of mice prone or resistant to diet-induced obesity. *J Physiol Biochem* 61, 343–352.

Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L., and Ferrante, A. W. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112, 1796–1808. doi:10.1172/JCI200319246.

Weston, A. H., Egner, I., Dong, Y., Porter, E. L., Heagerty, A. M., and Edwards, G. (2013). Stimulated release of a hyperpolarizing factor (ADHF) from mesenteric artery perivascular adipose tissue: involvement of myocyte BKCa channels and adiponectin. *Br J Pharmacol* 169, 1500–1509. doi:10.1111/bph.12157.

Weyer, C., Funahashi, T., Tanaka, S., Hotta, K., Matsuzawa, Y., Pratley, R. E., et al. (2001). Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab* 86, 1930–5. doi:10.1210/jcem.86.5.7463.

Wofford, M. (2001). Antihypertensive effect of α- and β-adrenergic blockade in obese and lean hypertensive subjects. *Am J Hypertens* 14, 694–698. doi:10.1016/S0895-7061(01)01293-6.

CHAPTER 2

Perivascular adipose tissue contains functional catecholamines

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Abstract

The sympathetic nervous system and its neurotransmitter effectors are undeniably important to blood pressure control. We made the novel discovery that perivascular adipose tissue (PVAT) contains significant concentrations of catecholamines. We hypothesized that PVAT contains sufficient releasable catecholamines to affect vascular function. HPLC, isometric contractility, immunohistochemistry, whole animal approaches and pharmacology were used to test this hypothesis. In normal rat thoracic aorta and superior mesenteric artery, the indirect sympathomimetic tyramine caused a concentration-dependent contraction that was dependent on the presence of PVAT. Tyramine stimulated release of NE, dopamine (DA) and the tryptamine, serotonin (5-HT), from PVAT isolated from both arteries. In both arteries, tyramine-induced concentration-dependent contraction was rightward-shifted and reduced by the norepinephrine transporter inhibitor nisoxetine (1 μM), the vesicular monoamine transporter tetrabenazine (10 μM) and abolished by the α-adrenoreceptor antagonist prazosin (100 nM). Inhibitors of the DA and 5-HT transporter did not alter tyramine-induced, PVAT-dependent contraction. Removal of the celiac ganglion as a neuronal source of catecholamines for superior mesenteric artery PVAT did not significantly reduce the maximum or shift the concentration dependent contraction to tyramine. Electrical field stimulation of the isolated aorta was not affected by the presence of PVAT. These data suggest that PVAT components that are independent of sympathetic nerves can release NE in a tyramine-sensitive manner to result in arterial contraction. Because PVAT is intimately apposed to the artery, this raises the possibility of local control of arterial function by PVAT catecholamines.

Introduction

Perivascular adipose tissue (PVAT, the fat immediately adjacent to blood vessels) is a fat depot just beginning to be appreciated for the contributions it makes to vascular function, human health and disease (Aghamohammadzadeh et al., 2012; Aghamohammadzadeh and Heagerty, 2012; Brandes, 2007; Chaldakov et al., 2007; Gollasch and Dubrovska, 2004; Szasz and Webb, 2012; Thanassoulis et al., 2012). Since the discovery that this fat could modify agonist-induced contraction (Soltis and Cassis, 1991), a plethora of substances that are vasoactive have been discovered in PVAT. In large part, these substances appear to inhibit arterial contraction (Fesus et al., 2007; Gollasch and Dubrovska, 2004). For example, removal of PVAT enhanced arterial contraction to an exogenous agonist. Similarly, buffer incubated with tissues with intact PVAT caused a relaxation or reduction in contraction in arteries without PVAT. To be fair, studies also support PVAT in promoting arterial contraction (Gálvez-Prieto et al., 2008; Gao et al., 2006; Huang et al., 2010; Lee et al., 2009; Owen et al., 2013; Payne et al., 2012), but the focus has largely been on the anti-contractile factors. While studying the role of kynurenines in PVAT-induced depression of contraction (Watts et al., 2011), we made the novel observation that PVATs in the rat contain substantial concentrations of catecholamines, including dopamine (DA), norepinephrine (NE) and epinephrine (E). As recognized neurotransmitters of the sympathetic nervous system, these substances are critical to basic vascular function and both short and long-term arterial pressure control, making their endogenous function important to understand.

The new finding of significant concentrations of catecholamines in PVAT is the basis for the work presented here. We focused on the effects of the indirect

sympathomimetic tyramine. Tyramine has been used for decades as a measure of the function/effectiveness of the sympathetic nervous system (Broadley, 2010; Burn and Rand, 1958; Nasmyth, 1962), given its ability to be taken up by the norepinephrine transporter (NET), the vesicular monoamine transporter (VMAT) and to displace catecholamines from vesicular stores. We used tyramine as a tool to test whether a functional pool of catecholamines exists in PVAT. An important related issue is whether the catecholamines that are measured in PVAT are independent of sympathetic nerves. We used several approaches to test these ideas. HPLC measures of catecholamine content and release, imaging of NE, isometric contraction and electrical field stimulation as well as whole animal surgery to remove sympathetic nerves provided an integrated study of whether PVAT, in general, provides a pool of catecholamines that are functionally released. We performed this work in two different arteries – the thoracic aorta and superior mesenteric artery – of the normal Sprague-Dawley male rat. These two arteries were used for several reasons. First, the thoracic aorta is a conduit artery while the superior mesenteric artery is a model of a resistance artery. Second, the PVAT of each artery is not the same, with the aorta possessing primarily brown fat while the superior mesenteric artery contains a mixture of white and brown fat (Watts et al., 2011). Third, the aorta possesses minimal sympathetic innervation, while that of the superior mesenteric artery is greater (Spector et al., 1972; Stassen et al., 1998). The outcomes of this work support the existence of an active, functional catecholamine pool in arterial PVAT of the rat.

Methods

Materials

Phenylephrine hydrochloride, acetylcholine hydrochloride, tyramine hydrochloride and inhibitors were purchased from Sigma Chemical Company, St. Louis MO USA [citalopram hydrochloride, desipramine hydrochloride, fluoxetine hydrochloride, nisoxetine hydrochloride, prazosin hydrochloride, tetrabenazine) or Tocris Bioscience (part of R&D Systems, Minneapolis, MN USA; LY53857, GBR12935, sibutramine metabolite 2 BTS54-505).

Animal Model

The male Sprague Dawley rat (225-275 grams or ~ 8-10 weeks of age, Charles River, Indianapolis, IN USA) was used. The total number of rats used was 90. All protocols were approved by the MSU Institutional Animal Care and Use Committee, and follow the "Guide for the Care and Use of Laboratory Animals", 8th edition, 2011. Rats were anesthetized with sodium pentobarbital (60-80 mg/kg, ip) and tested for loss of blink reflex and hind foot retraction when pinched. Tissues were removed for one of the following protocols.

High-Performance Liquid Chromatography

Catecholamine, 5-HT and tyramine measurements were made by homogenizing the tissue in four times their weight of 0.1M percholoric acid, centrifugation and taking samples through a 30 kDa filtration tube, and the filtrate analyzed by HPLC. The HPLC system (ESA Biosciences, Chelmsford MA) consisted of a Coulochem III electrochemical

detector set at -350 mV with separation of the analytes on an HR-80 reverse-phase column (Thermo Scientific, Waltham MA). Cat-A-Phase II (Thermo) was the mobile phase with a flow rate of 1.1 ml/min and the separation column was maintained at 35 ^{o}C. Quantification of the analytes was accomplished by performing a standard curve periodically and the limit of detection was 0.1 ng/ml for the catecholamines and 0.5 ng/ml for 5-HT.

Glyoxylic Acid Staining

Sections (5 mm X 5 mm) of mesentery containing fat cells surrounding an artery-vein pair was removed and pinned to the Sylgard-clad bottom of a 35 mm culture dish and rinsed 3X with phosphate buffered saline (PBS). The mesenteries then were incubated with either PBS or 2% glyoxylic acid in PBS (Sigma Chemical Company, St. Louis MO USA) for 5 minutes. The solutions were emptied from the dishes and the sections blown dry with N_2 gas. The dried sections then were placed on glass slides and heated to 120 °C for 5 miutesn. After allowing the slides to cool to room temperature, a drop (25 µl) of mineral oil was placed on the tissue and a cover slip applied and sealed in place with clear nail polish. The tissues then were imaged on a Nikon upright microscope using a DAPI filter set. Images were captured with a SPOT camera with illumination and exposure optimized for glyoxylic acid-stained sections and applied to all subsequent images.

Immunohistochemistry

Tissues were formalin-fixed or fresh frozen. Sections (8 micron) were taken through immunohistochemistry using a species-specific Vector kit (Burlingame CA, USA). Sections were incubated 24 hours with an NE specific antibody (ab887, Abcam, Cambridge MA USA) or no primary antibody at 4 ^{o}C. Sections were developed using a DAB (3, 3-diaminobenzidine) developing solution (Vector Laboratories, Burlingame CA, USA). Slides were counterstained with Vector Hematoxylin (30 seconds). Sections were photographed on a Nikon TE2000 inverted microscope using MMI® Cellcut Software (MMI, Haslett, MI USA).

Amine Release

The fat immediately surrounding the artery (PVAT) were removed from the artery (whole thoracic aorta, and 1 inch of superior mesenteric artery), and divided in half. All blood clots on the face of PVAT were removed. In a microfuge tube, PVATs were incubated in 500 microliters physiological salt solution [PSS in mM; NaCl 130; KCl 4.7; KH_2PO_4 1.8; $MgSO_4 * 7H_2O$ 1.7; $NaHCO_3$ 14.8; dextrose 5.5; $CaNa_2EDTA$ 0.03, $CaCl_2$ 1.6 (pH 7.2)] containing 1 μM of the monoamine oxidase inhibitor pargyline (Sigma Chemical Company, St. Louis, MO, USA) and vehicle or nisoxetine (1 μM) for 30 minutes at 37 ^{o}C prior to the addition of either vehicle (water) or tyramine (10^{-4} M; Sigma Chemical Co St. Louis, MO, USA). Tissues incubated for one hour at 37 ^{o}C. Buffer was saved for HPLC quantitation of amines and tissues were weighed.

Isometric Contraction

Arteries (endothelium-intact; thoracic aorta, superior mesenteric artery, ~4-5 mm long) cleaned of fat (-PVAT) or with fat intact (+PVAT) were mounted individually in tissue baths for isometric tension recordings using Grass FT03 transducers and PowerLab Data Acquisitions System (ADInstruments, Colorado Springs, CO, USA). Four preparations were made from each thoracic aorta while two were made from the superior mesenteric artery, and PVAT was cleaned of blood clots while minimally handling the PVAT itself. Baths, kept at 37 ^{o}C by a heating circulator, were filled with oxygenated PSS. Rings were placed under optimum resting tension [4 grams for rat thoracic aorta, 1.2 grams for rat superior mesenteric artery] and equilibrated for one hour. An initial concentration of 10 μM phenylephrine (PE) tested arterial viability and validation of an intact endothelial cell layer was tested by acetylcholine (1 μM)-induced relaxation of a half-maximal PE-induced contraction. Tissues were washed until tone returned to baseline. Agonists were added in a cumulative fashion, with significant time (sometimes over 20 minutes) necessary for a response to plateau prior to the next addition. In some experiments, either vehicle (water, 0.1% DMSO) or inhibitor was added for one hour without washing before construction of the concentration response curve. Only one inhibitor was tested in each arterial ring.

For electrical field stimulation, arterial rings +/-PVAT were mounted, in the isolated tissue bath, between two platinum electrodes (positioned within the tissue bath) connected to a Grass Instruments stimulator (S88; Quincy, MA) and maximum electrical stimulus was delivered (30 stimuli, stimulus duration 0.5 ms, frequency 20 Hz, voltage 120 V). If EFS-induced contraction was observed, tetrodotoxin (TTX), a fast sodium channel

inhibitor, was incubated for 30 minutes prior to the stimulus to validate nerve-dependence of contraction. Contractile force was measured as outlined above, with tissues initially contracted to a maximum concentration of PE to validate tissue viability.

Celiac Ganglionectomy

While rats were under general anesthesia (2% isoflurane, oxygen mix), a ventral midline abdominal incision was performed and the small intestines were gently retracted and placed on warm saline soaked gauze. The celiac plexus located between the aorta, celiac artery and mesenteric artery was dissected free and removed (CGx). The small intestines were placed back into the abdominal cavity and lavaged with warm saline. The midline abdominal incision was sutured closed in layers. The sham group (SGx) underwent a sham operation that was performed by accessing and exposing the celiac plexus only. All rats were given an intra-muscular injection of piperacillin. Animals were used after a recovery period of five days. Rats were sacrificed by an intraperitoneal injection of sodium pentobarbital (60-80 mg/kg). The liver, spleen, small intestine, right and left kidney, retroperitoneal fat, superior mesenteric artery and aortic PVAT, and epididymal fat were dissected and stored at -80 ^{o}C prior to isolation of amines. In some experiments, the superior mesenteric artery was used for measurement of isometric contraction.

Statistical Analyses

Data are reported as means±SEM for number of animals indicated in parentheses (N). HPLC are reported as ng/gram tissue. Histochemical and immunohistochemical

images depict sections incubated with and without primary antibody, and are representative of a minimum of four separate animals. Adjustments in brightness and contrast were made to the whole panel of a photograph, not a portion. Contraction (agonist-induced or EFS-induced) is reported as means±SEM as force (milligrams) or as a percentage of the initial contraction to a maximal concentration of phenylephrine (PE, 10 μM). Potency values (-log EC_{50}, M) were calculated as concentrations necessary to cause a half-maximal effect. Where a maximum was not achieved, the values are estimated and true potencies equal or greater than that reported. Either an unpaired Student's t test or repeated measures ANOVA was performed after confirming the normality of data distribution. Equality of data variances was tested using the F test (StatPlus/Mac 2009). Where variances were not equivalent, a Mann-Whitney U test was conducted as a nonparametric measure of two independent groups where appropriate. $p < 0.05$ was considered statistically significant.

Results

PVAT contains catecholamines

Using HPLC, we measured the content of the catecholamines DA, NE and E in fat that surrounds the aorta (aortic PVAT), the brown fat pad (interscapular), fat that surrounds the superior mesenteric artery (RMA PVAT) and the fat directly behind the left kidney (a white adipose tissue, retroperitoneal). Figure 2A demonstrates that PVATs (aortic and mesenteric) contain significant levels of NE relative to the well-known, sympathetically-dependent brown fat pad (scapular). Importantly, both DA and E could be detected in all tissues, but most prominently in the mesenteric PVAT. The NE measured in the mesenteric PVAT was visualized in the cytoplasm of the adipocyte through glyoxylic acid histochemistry (Figure 2B); aortic PVAT was too dense to visualize. NE was also detected immunohistochemically in mesenteric PVAT (Figure 2C), observed primarily in what appears to be adipocyte cytoplasm and consistent with glyoxylic acid staining.

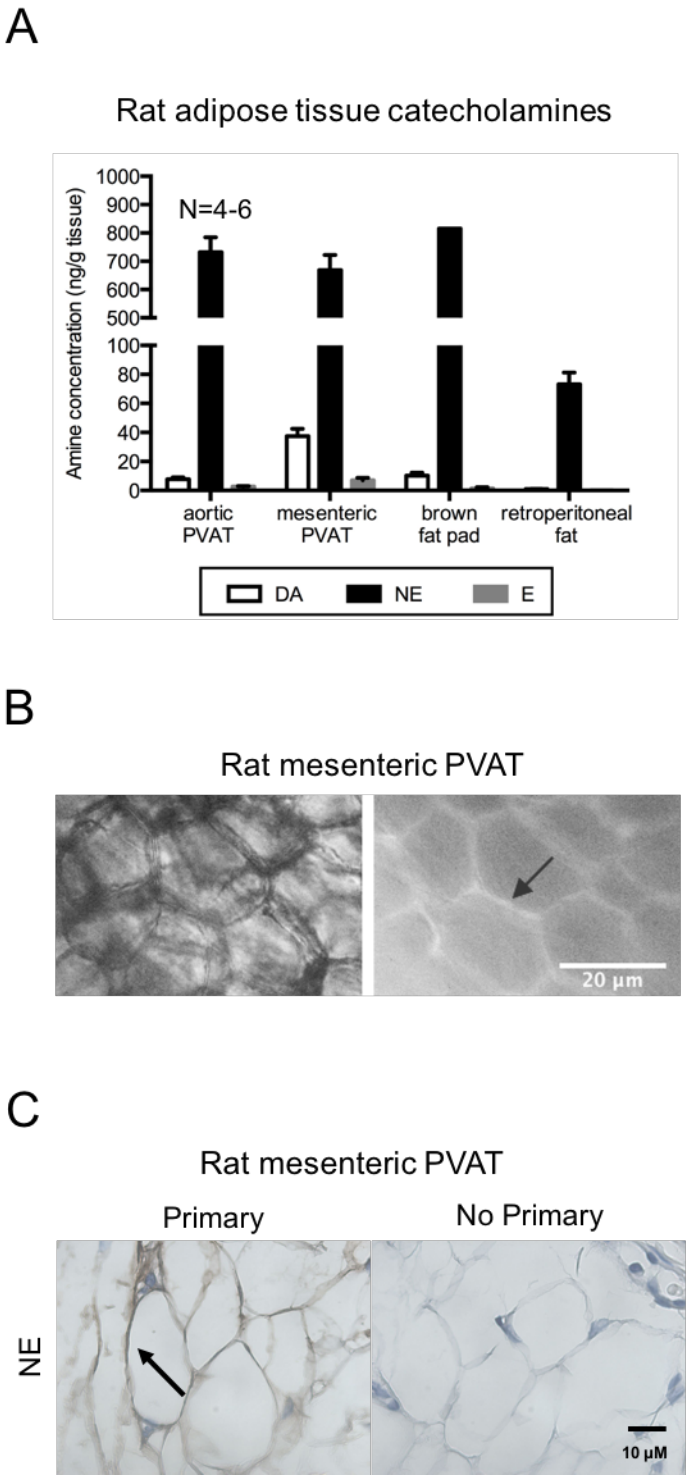


Figure 2. Catecholamines in rat PVAT.

Figure 2. (cont'd)

(A) HPLC measures of catecholamine content (ng/g tissue) in aortic PVAT, brown fat pad (interscapular), mesenteric PVAT and retroperitoneal fat from the same rats. Bars represent means±SEM for number of animals in parentheses. (B) Representative image of glyoxylic acid staining of rat mesenteric PVAT where the left hand side is bright field image, and right hand side is the fluorescent glyoxylic acid image. Representative of four individual animals. (C) Detection of NE in the superior mesenteric PVAT. Representative of four individual animals. Left panel are images from sections exposed with primary antibody (primary), and the right images from sections not exposed to primary antibody (no primary). Arrows point regions of interest.

PVAT has a functional reservoir of catecholamines largely independent of sympathetic nerves

Tyramine (10^{-4} M) stimulated release of NE, DA and 5-HT from mesenteric (Figure 3A) and aortic (Figure 3B) PVATs into the surrounding buffer. Tyramine was detected in HPLC such that absence or addition of tyramine to samples could be appropriately validated. In separate experiments, nisoxetine (1 μM pre-incubation) reduced tyramine-induced NE release in aortic PVAT (tyramine = 623±144 ng/gram tissue, tyramine & nisoxetine = 403±38 ng/gram tissue, $p<0.05$). Tyramine caused a concentration-dependent contraction of the isolated thoracic aorta (RA) and superior mesenteric artery (RMA) in arteries with intact PVAT and minimal contraction in arteries with PVAT removed (Figure 4A and B, respectively). All tissues without PVAT possessed a robust contraction to a maximum concentration of the α_1 adrenoreceptor agonist phenylephrine (PE; values in parentheses in key). A representative tracing of tyramine-induced contraction in the RMA is shown in Figure 5A. Contraction was relatively slow to develop. The α_1 adrenoreceptor antagonist prazosin (1 μM) significantly reduced tyramine-induced contraction when added directly, illustrated in Figure 5A. This was quantified in both arteries when using a ten-fold lower concentration of prazosin (100 nM) (Figure 5B). These data raised the possibility that sympathetic nerve fibers within PVAT could be the source of catecholamines.

Electric field stimulation (EFS; maximum stimulus of 20 Hz) of the RA did not result in robust contraction (< 10% PE contraction) either with or without PVAT, while removal of PVAT from the RMA reduced a 20 Hz-induced contraction (Figure 6A). A 20 Hz stimulus was used as this is a near maximal stimulus in isolated arteries. EFS-induced

20 Hz contraction in the RMA was abolished by the fast sodium channel inhibitor TTX (300 nM) and by prazosin (100 nM), indicating that EFS-induced contraction was mediated primarily by sympathetic nerves and stimulation of adrenoreceptors. TTX (300 nM) did not modify tyramine-induced contraction itself. Importantly, all tissues used in EFS experiments contracted to a maximal concentration of phenylephrine (10 μM, milligrams: RA+PVAT = 1229±264; RA-PVAT: 1911±365; RMA± PVAT = 732±116; RMA -PVAT = 1154±343). The relative NE content of the artery and arterial PVAT is compared in Figure 6B. NE content was significantly higher in aortic PVAT *vs.* the aorta, while NE content was evenly balanced in the artery and PVAT of the RMA. These findings suggest that sympathetic nerves in the rat aortic PVAT are not functional, but are so in the mesenteric PVAT. This lead to the next experiment.

To investigate nerve-dependence of tyramine-induced contraction in the superior mesenteric artery, we removed the celiac ganglion. Celiac ganglionectomy reduced the content of NE in the superior mesenteric artery PVAT by 38%; E and DOPA were similarly reduced when compared to sham ganglionectomized rats (SGx) (Figure 7A). These values are quantitatively lower than those shown in Figure 2. We attribute this to the fact that the tissues from the animals used to generate data in Figure 7 all underwent abdominal surgery, and we have observed this to lower organ catecholamine content. Tyramine-induced contraction was modestly but not significantly reduced in the RMA +PVAT from CGx *vs.* SGx rats (~22% reduction; Figure 7B). Tyramine potency was not different between the two groups. The dramatic reduction of the liver, small intestine and splenic content of NE when comparing ganglionectomized to control (Figure 7C) supports successful ganglionectomy.

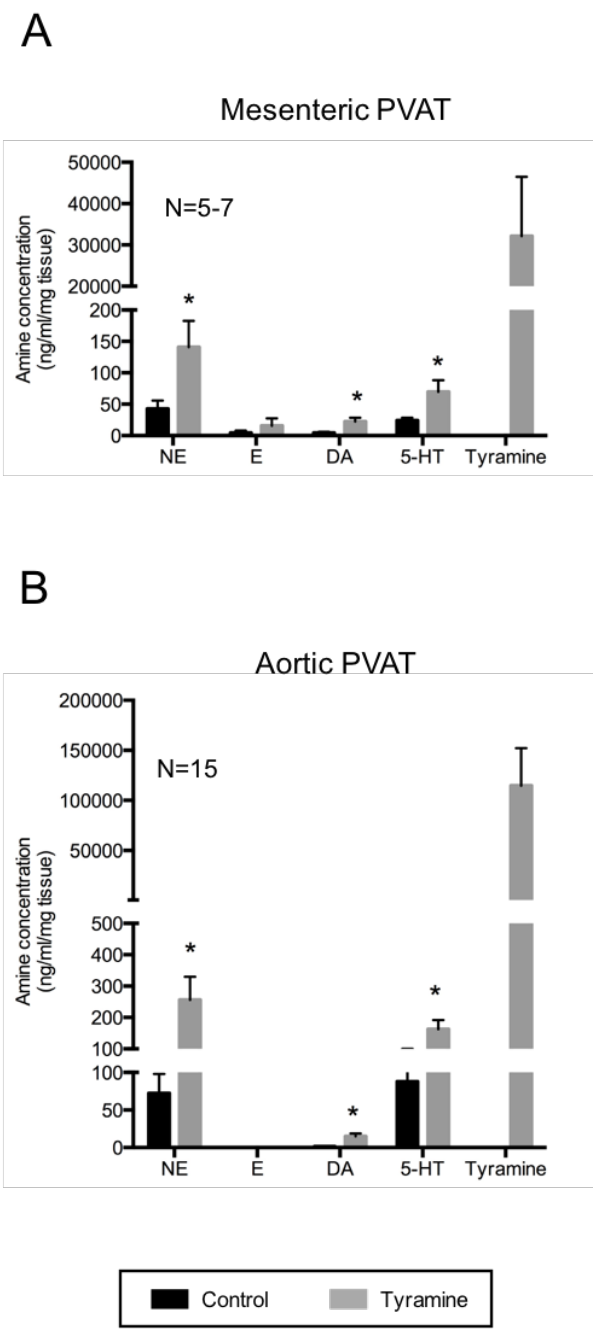


Figure 3. Tyramine-released catecholamines from PVAT.

Mesenteric (A) and aortic (B) PVAT catecholamines as measured through HPLC. Bars represent means±SEM for number of animals in parentheses. Solid bars are values for samples incubated with normal PSS, gray bars for samples incubated with 10^{-4} M tyramine. * indicate statistically significant increase ($p<0.05$) versus appropriate control.

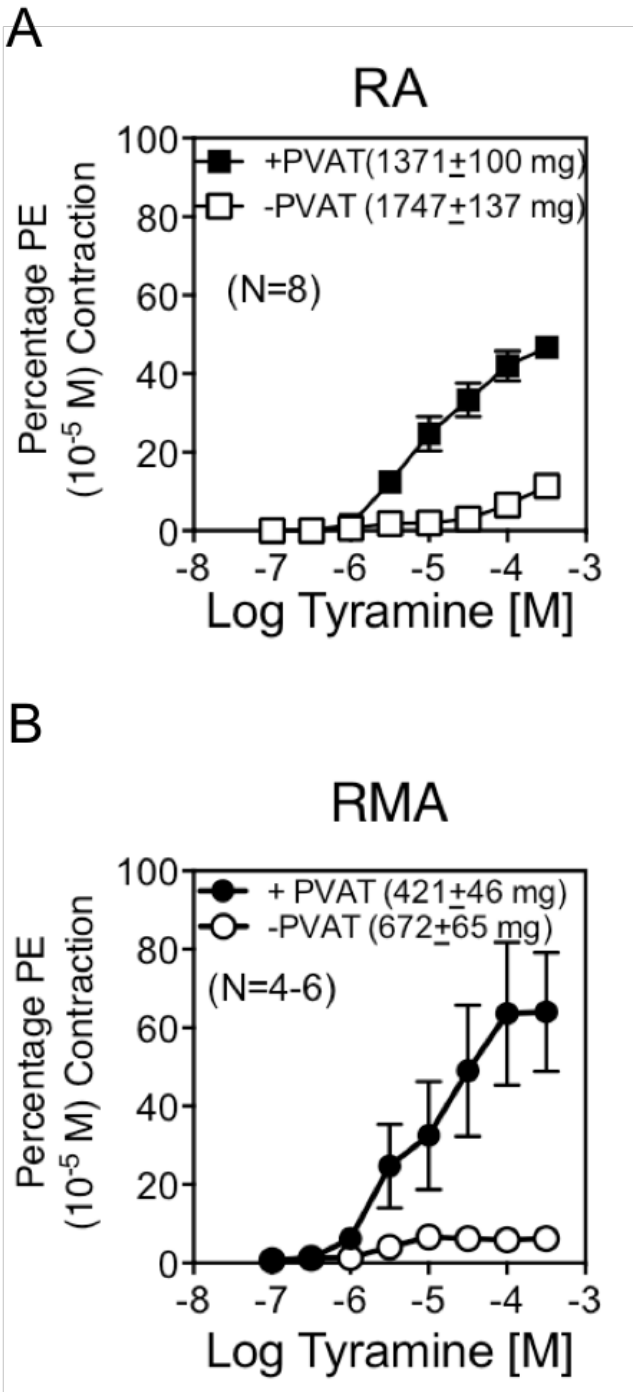
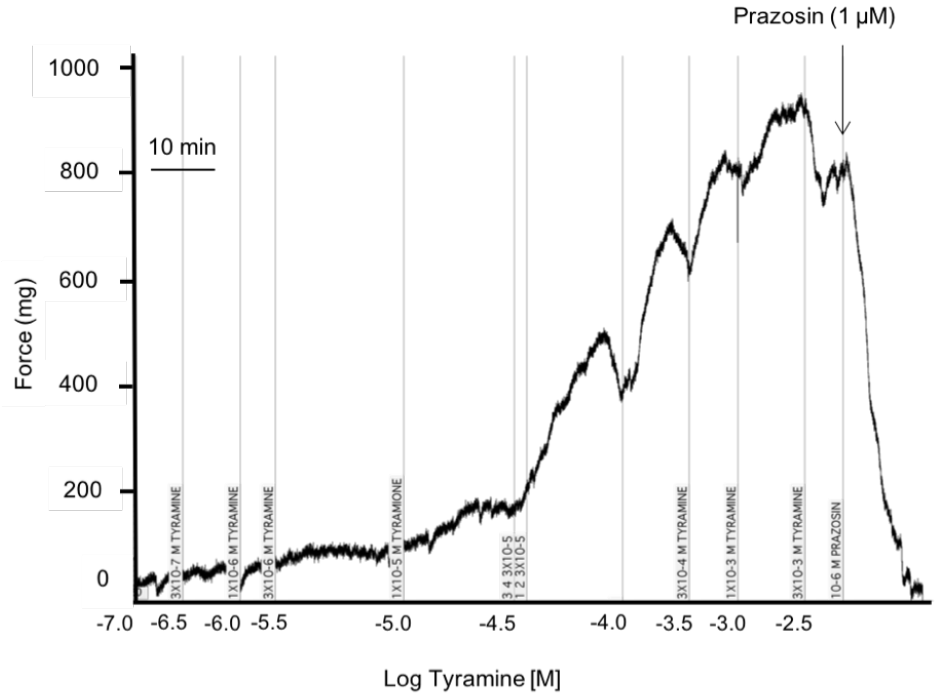


Figure 4. Tyramine-induced contraction in the isolated RA (A), and RMA (B) of the normal Sprague Dawley rat.

Points represent means±SEM for the number of animals in parentheses. Values in key legend are the response in milligrams to 10^{-5} M PE.

A



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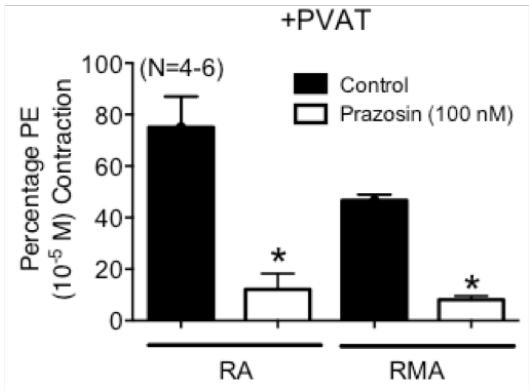

Figure 5. Prazosin's effect on tyramine induced contraction.

(A) Inhibition of tyramine-induced maximum contraction by the α_1 adrenoceptor antagonist prazosin (1 μM). (B) Quantification of inhibition of tyramine-induced contraction by a 10-fold lower concentration of prazosin. Bars represent means±SEM for number of animals in parentheses. * = significant difference *vs* appropriate control values ($p<0.05$).

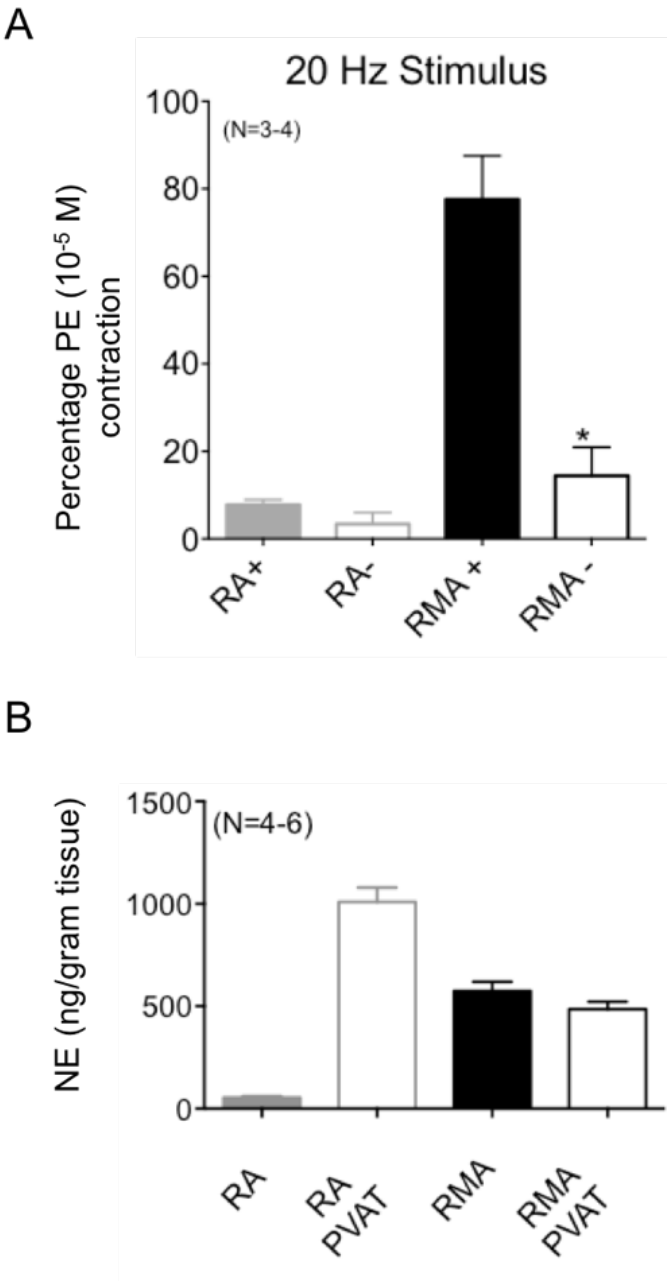

Figure 6. EFS-induced contraction and NE content in the RA and RMA +/-PVAT.

(A) Contraction of isolated RA and RMA +/-PVAT to maximum electrical field stimulation (20 Hz). (B) NE content in artery proper and PVAT around artery for the RA and RMA. Bars represent means±SEM for number of animals in parentheses. * indicate statistically significant increase ($p<0.05$) versus +PVAT values.

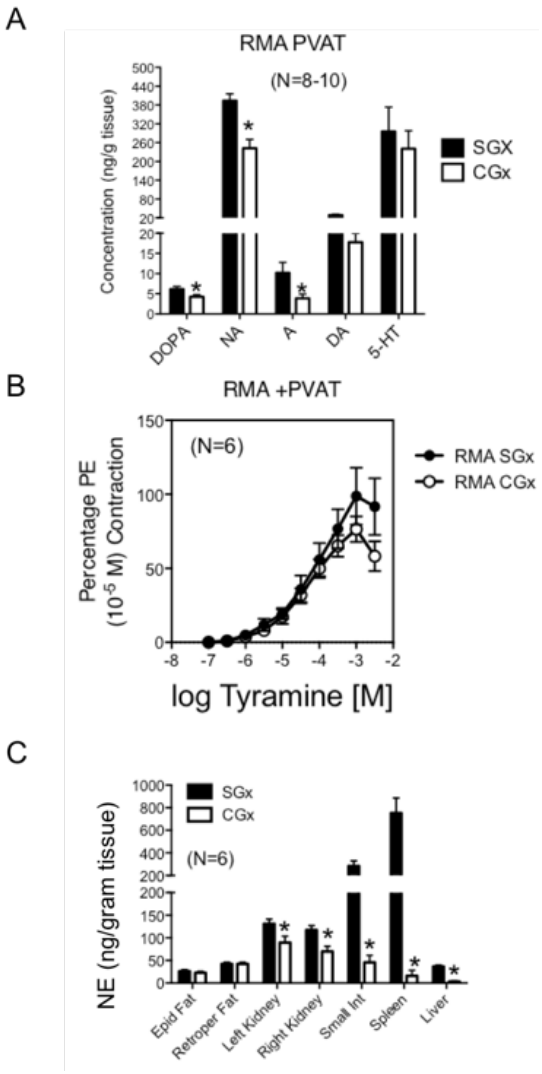

Figure 7. **The effect of CGx on RMA +PVAT amine content and contraction to tyramine.**

(A) Quantitation of RMA PVAT catecholamines in animals with a sham (black; SGx) or (white; CGx) celiac ganglionectomy. (B) Tyramine-induced RMA+PVAT contraction in animals with SGx or CGx surgery. (C) HPLC validation of celiac ganglionectomy through reduced NE content of those tissues known to be innervated by the celiac ganglion (liver, small intestine and spleen). Bars represent means±SEM for number of animals in parentheses. * indicates statistically significant increase ($p<0.05$) versus SGx values.

Tyramine-induced PVAT dependent contraction depends on the NET

Because tyramine-induced contraction was PVAT and not nerve dependent in the RA, we used this artery as the primary model for studying the mechanism of tyramine-induced contraction. Four separate conditions could be tested in rings from the same animal, and the results of experiments investigating the mechanism of tyramine-induced contraction are shown in Figure 8, separated by the vehicle used in each experiment (A: water. B: 0.1% DMSO). Table 1 reports pharmacological parameters (-log EC_{50} M as potency values, maximum contraction) for the data presented in Figure 8 and, for some interventions, the RMA under the conditions described below (shown in Figure 8). Findings using LY53857, GBR 12935 and fluoxetine in the rat aorta are shown only in Table 1 given that they were negative in outcome. Statistical comparisons are not shown in Figure 8 for clarity, but are shown in Table 1.

A group of transporter inhibitors were tested for the ability to shift and reduce tyramine-induced contraction. The serotonin transporter (SERT) inhibitor citalopram did not shift the tyramine contraction curve nor reduced the maximum contraction. All other inhibitors that have significant affinity for NET shifted the concentration-response curve rightward and reduced the maximum contraction to tyramine. This includes desipramine, nisoxetine, and the second metabolite of the NET/SERT inhibitor sibutramine, BTS 54-505. Inhibition of the dopamine transporter inhibitor by GBR12935 and another SERT inhibitor, fluoxetine, did not modify tyramine-induced contraction (Table 1). Importantly, the α_1 adrenoreceptor antagonist prazosin completely prevented tyramine-induced contraction in the thoracic aorta (Figure 8A). 5-HT, though released by tyramine in the RA PVAT, does not participate in tyramine-induced contraction, as the 5-HT_2 receptor

antagonist LY53857 did not modify tyramine-induced contraction (Table 1). Finally, the vesicular monoamine transporter (VMAT) inhibitor tetrabenazine shifted tyramine-induced contraction modestly to the right and reduced maximum contraction compared to vehicle (Figure 8B).

Similar key experiments were performed in the RMA +PVAT and these findings are shown in Table 1. Tetrabenazine inhibition of tyramine-induced contraction in the RMA was qualitatively similar to that observed in the rat aorta. Tyramine-induced contraction was also inhibited by nisoxetine (1 μM), but the maximum contraction recovered to a greater extent in the RMA *vs* RA.

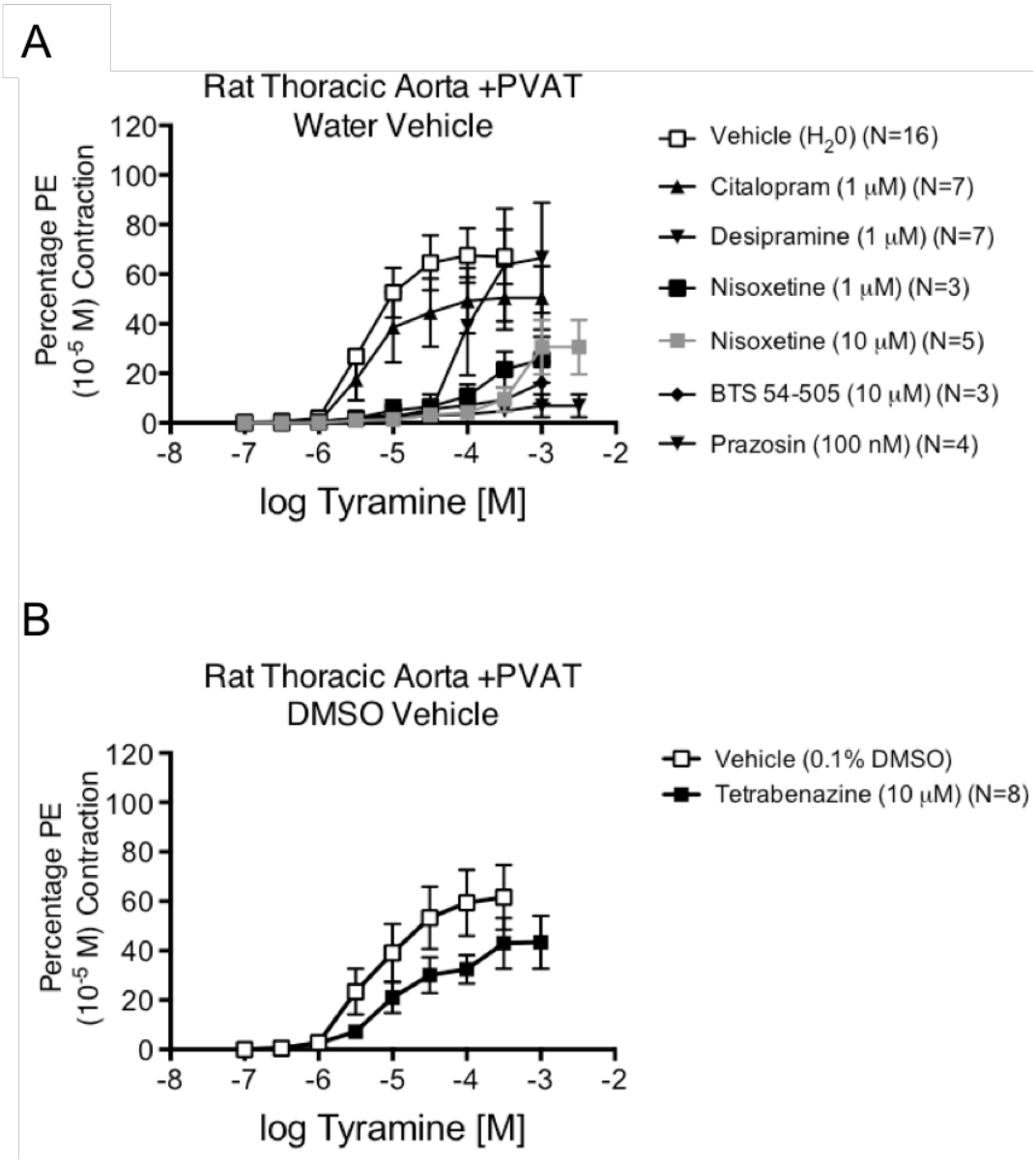

Figure 8. Tyramine-induced contraction in the RA+PVAT in response to inhibitors of NE transport.

(A) Tyramine-induced contraction in the RA +PVAT in the presence of a series of inhibitors compared to water-based vehicle. (B) Tyramine-induced contraction in the RA+PVAT in the presence of the VMAT inhibitor tetrabenazine or DMSO vehicle. Points represent means±SEM for the number of animals in parentheses.

Tissue Intervention	Tyramine -log EC_{50} [M]	Tyramine % PE Contraction
RA + PVAT		
Vehicle (water)	5.34±0.06	67.00±11.1
Citalopram (1 μM)	5.36±0.04	50.40±12.8
Desipramine (1 μM)	4.05±0.01*	66.6±22.0
Nisoxetine (1 μM)	3.65±0.34*	25.50±9.3*
Nisoxetine (10 μM)	3.39±0.07*	30.60±11.0*
BTS 54-505 (10 μM)	NC	16.30±8.4*
Prazosin (100 nM)	NC	6.98±4.6*
LY53857 (100 nM)	5.47±0.03	35.00±8.7
GBR 12935 (100 nM)	5.45±0.05	48.90±16.2
Fluoxetine (100 nM)	5.42±0.07	56.20±15.1
Vehicle (0.1% DMSO)	5.26±0.06	61.62±13.1
Tetrabenazine (10 μM)	4.86±0.13	43.44±10.7*
RMA +PVAT		
Vehicle (0.1 % DMSO)	4.87±0.04	73.80±20.0
Tetrabenazine (10 μM)	4.38±0.03	41.41±10.5*
Nisoxetine (1 μM)	3.54±0.07*	55.70±10.3*
SGx	4.14±0.10	98.80±19.0
CGx	4.46±0.14	76.40±8.6

Table 1. Pharmacological parameters of tyramine-induced contraction in isolated RA and RMA +PVAT. Points represent means±SEM for values calculated from figures presented. NC = not convergent. * indicates $p<0.05$ from vehicle response

Discussion

Since the discovery that substances from PVAT support vascular relaxation, PVAT has primarily been described as a tissue that *reduces* arterial contractility through release of substances like adiponectin or factors that activate arterial K^+ channels (Aghamohammadzadeh et al., 2012; Aghamohammadzadeh and Heagerty, 2012; Brandes, 2007; Chaldakov et al., 2007; Gollasch and Dubrovska, 2004; Szasz and Webb, 2012; Thanassoulis et al., 2012). However, a growing list of papers suggest that PVAT may be the source of contractile substances (or ones that reduce relaxation), especially in a pathological state (Gálvez-Prieto et al., 2008; Gao et al., 2006; Huang et al., 2010; Lee et al., 2009; Owen et al., 2013; Payne et al., 2012). The present study identifies catecholamines as substances that have the potential to participate in the influence of PVAT on arterial function. NE is quantifiable in PVAT, largely independent of sympathetic nerves, and is released by tyramine to cause arterial contraction in a VMAT-, NET- and α_1 adrenoreceptor-dependent manner. To our knowledge, this is the first report supporting the presence of a pool of catecholamines in PVAT, and that this pool can be activated.

Catecholamines are present in PVAT

Adipocytes, mesenchymal adipose derived stem cells, fibroblasts, endothelial cells, small blood vessels, preadipocytes and blood cells make up PVAT (Divoux and Clément, 2011; Kershaw and Flier, 2004; Peinado et al., 2012). Immunohistochemical staining, glyoxylic acid staining and HPLC measurement of NE provide three different measures of the presence of NE in PVAT. The adipocyte cytoplasm appears to be one

site of NE localization in PVAT, evidenced by both glyoxylic acid and immunohistochemical staining. Future experiments will likely need to include electron microscopy to determine whether catecholamines are concentrated vesicularly in adipocytes in the way they are in neurons. Electron microscope images of the epididymal adipocyte show structures consistent with dense core vesicles, but the formal identification of these structures as vesicles was not the focus of that study (Cushman, 1970). Our finding of releasable stores of catecholamines in PVAT is novel, but we acknowledge that one group (Kvetnansky et al., 2012; Vargovic et al., 2011) first published the findings of NE content in white adipose tissue. Thus, PVAT is similar to classical adipose tissue depots in containing catecholamines.

Experiments in both the isolated thoracic aorta and superior mesenteric artery suggest that PVAT contains a substance released by tyramine that results in contraction. These particular PVATs are brown (RA) and a mix of brown and white fat (RMA) (Watts et al., 2011). As tyramine is an indirect sympathomimetic, it was most logical that it is a catecholamine-like substance released, and our results from HPLC studies confirm this idea. Tyramine released NE, DA and 5-HT from PVAT from both RA and RMA PVAT, and tyramine-induced contraction was abolished by blockade of α adrenoreceptors. Where, from within PVAT, do these catecholamines come from? Are they released from the adipocytes and stromal vascular fraction of PVAT, or perhaps nerves that course through PVAT? While we have not investigated the relative distribution of NE in adipocytes and the stromal vascular fraction, we did investigate the potential contribution of sympathetic nerves to NE content in PVAT.

Sympathetic nerves are not necessary for tyramine-induced PVAT-dependent contraction

It is not well known how sympathetic nerves interact with and/or use PVAT for tracking with and innervating blood vessels. We undertook two measures of functional innervation of PVAT, viewing sympathetic nerves as one potential source of the NE released by tyramine. First, we tested whether arteries with and without PVAT would contract to an electrical field stimulus, dissecting whether PVAT contains functional nerves. Second, we removed the celiac ganglion as this is the most logical source for sympathetic innervation of the superior mesenteric artery (Czaja et al., 2002).

Electrical field stimulation allowed us to test the idea that PVAT carries nerve fibers that innervate the artery beneath it. The adventitia of an artery was not removed when PVAT was dissected away, such that reduction of an electrical field stimulated-induced contraction with removal of PVAT would mean the nerve was significantly superficial on the artery. All tissues responded to a maximum electrical field stimulation qualitatively, but there were notable differences in the magnitude of response. In the RMA, removal of PVAT reduced electrical field stimulated-induced contraction by over 80%, indicating that the nerve responsible for contraction was carried in the PVAT, agreeing with previous findings that the RMA is innervated (Stassen et al., 1998) and that EFS-induced contraction is reduced with PVAT removal (Gao et al., 2006). By contrast, the RA+PVAT did not display a robust electrical field stimulated-induced contraction when compared to its contraction to exogenous adrenergic stimulus (PE). There was a trend for RA contraction to be reduced with PVAT removal, but this was not statistically significant. Our findings are in agreement with work published 40 years ago which showed that the RA (without PVAT) has roughly 1/10 the innervation/tyrosine hydroxylase activity of the

mesenteric artery (Spector et al., 1972). The RA has long been recognized as being sparsely innervated (Stassen et al., 1998). We cannot exclude the possibility that sympathetic and sensory nerves were both activated in the aorta and physiologically antagonized one another such that no change in contraction was observed. However, the electrical field stimulated-induced responses in the rat thoracic aorta have primarily been observed as relaxation, not contraction (Park et al., 2000). Moreover, HPLC experiments demonstrated that NE in the RA was distributed differently than in the RMA. Because electrical field stimulation was unable to cause contraction in the RA+PVAT, this suggests that the NE in the PVAT around the RA does not exist in sympathetic nerve terminals and that the PVAT either takes up circulating NE and/or makes NE.

A different approach was necessary for testing the contribution of nerves to tyramine-induced contraction in the RMA. Animals were used 5-7 days after celiac ganglionectomy, a time during which re-innervation does not occur (Kandlikar and Fink, 2011). Ganglionectomy was successful as those organs innervated by the celiac ganglion (small intestine, spleen and liver) showed a profound reduction in NE content. In the PVAT around the superior mesenteric artery from the same rats, NE content fell from 418±30 to 261±38 ng/gram tissue, or a 38% fall. Qualitatively, these values were lower than shared in Figure 2, and we attribute this to the sympathetic discharge that occurs during surgery such that tissues from both the SGx and CGx animals have lower total catecholamine content than those tissues taken from naïve animals. Contraction to tyramine did not fall the same percentage with ganglionectomy. Maximum contraction was 98±19 % *vs.* 76±8 PE contraction, or a 22% reduction, and concentration-dependent contraction was not shifted rightward relative to the curve of RMA from SGx rats. These findings suggest that

a majority of the NE content is not in the nerves in mesenteric PVAT, and that tyramine must stimulate something other than nerves to contract the artery. An alternative possibility is that we did not remove the appropriate innervation source; in other words, the celiac ganglion is not responsible for innervating mesenteric PVAT. This is difficult to understand given the pattern of innervation known for the celiac ganglion (Czaja et al., 2002), but must be offered as an alternative hypothesis. Similarly, it is possible that remnant nerves from which NE could be released still remain in the PVAT of the CGx animals. This would mean that these particular nerves are hardier than those that innervate the spleen and intestine, given that NE content in these tissues dropped dramatically with ganglionectomy; we have no evidence to support or refute this idea.

PVAT adrenergic system contributes to tyramine-induced contraction.

Our studies support the RA as a good model in which to study the mechanism of PVAT catecholamine release because we can largely disregard the involvement of a nerve and focus on contributions made by PVAT. Use of the classic indirect sympathomimetic tyramine (Burn and Rand, 1958; Nasmyth, 1962) revealed that this NE source in PVAT is dynamic. Tyramine is initially taken up by NET (Broadley, 2010; Goldstein, 2008). Though tyramine has been used for decades to assess local sympathetic function, its mechanism to release amines once inside the cell is not completely accepted, with vesicular storage of amines thought to be unnecessary or essential (Broadley, 2010; Goldstein, 2008). Tyramine displacement of NE from storage vesicles is the mostly commonly cited action of tyramine. The released NE builds up cytoplasmically and leaves the cell through reverse transport by proteins such as NET. In this way, tyramine can be

doubly dependent upon NET for its actions. Tyramine-induced contraction is likely caused by amines released from PVAT. Four pieces of evidence support this. First, removal of PVAT significantly reduced tyramine-induced contraction in all vessels tested. Second, tyramine stimulated release of NE from PVAT in a nisoxetine-sensitive manner. Third, compounds that have affinity for NET reduced tyramine-induced contraction. Fourth, prazosin significantly reduced tyramine-induced contraction. Because tyramine-induced contraction was modest in tissues without PVAT and tyramine releases amines, it is unlikely that tyramine is interacting with trace amine associated receptors to stimulate contraction (Maguire et al., 2009; Stalder et al., 2011). Importantly, trace amine associated receptors are not antagonized by prazosin, so our finding that prazosin reduced tyramine-induced contraction in +PVAT arteries suggests tyramine-induced contraction is not a trace amine associated receptor-dependent event, and NE is the most likely effector of tyramine-induced contraction. Tyramine also shows low affinity for α adrenergic receptors in the rat (Anwar et al., 2013). Our findings are consistent with this as tyramine minimally contracted the -PVAT artery which contracted readily to phenylephrine. Importantly, these findings were consistent in two different arteries of the rat.

Because 5-HT and DA were stimulated by tyramine to be released from PVAT, we examined the ability of the SERT inhibitors citalopram, fluoxetine, dopamine transporter (DAT) inhibitor GBR12935 and 5-HT receptor antagonist LY53857 to inhibit tyramine-induced contraction. These interventions were ineffective, and past use of these compounds validate that the concentrations chosen were effective at their target. These

findings suggest that, though released, neither 5-HT nor DA plays a role in tyramine-induced contraction.

Limitations

There are limitations to our study and interpretations. We have used tyramine as the primary indirect sympathomimetic agonist. Many sympathomimetics are based on a similar structure, so we speculate that our findings would be applicable, in general, to these substances. Our findings are presented solely relevant to the rat, though we have observed the same outcomes in vessels from the C57BL/6 mouse (data not shown). Thus, these outcomes are not specific to one species. We were also surprised to observe relatively poor EFS-induced and tyramine-induced contraction in arteries without PVAT. These findings suggest that the innervation within the vessel is not as easily activated as is the one external to the vessel and/or that PVAT removal inherently damages the functioning of the arterial sympathetic network. While this was not so surprising for the RA, it was for the RMA. This calls into question just how deeply a nerve penetrates arteries, with the recognition that arteries differ substantially from one another in this relationship (this study; Stassen et al., 1998). We considered chemically depleting the artery of catecholamines to abolish tyramine-induced contraction, but were concerned that this approach would not reveal nerve *vs.* PVAT-independent function and thus be too non-specific in nature. Our experiments also do not answer whether the adipocytes of PVAT are capable of releasing catecholamines, and this issue is an avenue of future work.

A final unanswered question that deserves discussion is how PVAT catecholamines would be released endogenously. What in the body could tap into, or stimulate release of this functional pool of catecholamines? How does this functional pool contribute to normal vascular tone? There are hints that this system could be important with findings that there is a loss of anti-contractile PVAT capability in disease (Huang et al., 2010). Specifically, production of superoxide in PVAT promotes vasoconstriction in rat vasculature (Gao et al., 2006), and angiotensin II infusion and hypertension changes the function of isolated arteries (Lee et al., 2009). In the pig coronary artery, PVAT potentiation of smooth muscle contraction is augmented in obesity (Owen et al., 2013) and leptin derived from PVAT in a pig model of metabolic syndrome reduces endothelial function (Payne et al., 2010). It is possible that anti-contractile factors, typically produced by PVAT, mask the catecholamine pool which might become functional (revealed) when anti-contractile factors are lost in disease. This remains to be determined.

Conclusion

This study presents the first evidence of a functional pool of catecholamines in PVAT in any species. The discovery of a dynamic pool of catecholamines in PVAT raises the question of whether these local amines regulate vascular tone, as well as what role this pool might play in pathological conditions.

REFERENCES

REFERENCES

Aghamohammadzadeh, R., and Heagerty, A. M. (2012). Obesity-related hypertension: epidemiology, pathophysiology, treatments, and the contribution of perivascular adipose tissue. *Ann Med* 44 Suppl 1, S74-84.

Aghamohammadzadeh, R., Withers, S., Lynch, F., Greenstein, A., Malik, R., and Heagerty, A. (2012). Perivascular adipose tissue from human systemic and coronary vessels: the emergence of a new pharmacotherapeutic target. *Br J Pharmacol* 165, 670–682. doi:10.1111/j.1476-5381.2011.01479.x.

Anwar, M. A., Ford, W. R., Herbert, A. A., and Broadley, K. J. (2013). Signal transduction and modulating pathways in tryptamine-evoked vasopressor responses of the rat isolated perfused mesenteric bed. *Vascul Pharmacol* 58, 140–149. doi:10.1016/j.vph.2012.10.007.

Brandes, R. P. (2007). The fatter the better? Perivascular adipose tissue attenuates vascular contraction through different mechanisms. *Br J Pharmacol* 151, 303–304. doi:10.1038/sj.bjp.0707229.

Broadley, K. J. (2010). The vascular effects of trace amines and amphetamines. *Pharmacol Ther* 125, 363–375. doi:10.1016/j.pharmthera.2009.11.005.

Burn, J. H., and Rand, M. J. (1958). The action of sympathomimetic amines in animals treated with reserpine. *J Physiol* 144, 314–36.

Chaldakov, G. N., Tonchev, A. B., Stankulov, I. S., Ghenev, P. I., Fiore, M., Aloe, L., et al. (2007). Periadventitial adipose tissue (tunica adiposa): enemy or friend around? *Arch Pathol Lab Med* 131, 1766; author reply 1766-7. doi:10.1043/1543-2165(2007)131[1766a:PATTAE]2.0.CO;2.

Czaja, K., Kraeling, R., Klimczuk, M., Franke-Radowiecka, A., Sienkiewicz, W., and Łakomy, M. (2002). Distribution of ganglionic sympathetic neurons supplying the subcutaneous, perirenal and mesentery fat tissue depots in the pig. *Acta Neurobiol Exp (Wars)* 62, 227–234.

Divoux, A., and Clément, K. (2011). Architecture and the extracellular matrix: The still unappreciated components of the adipose tissue. *Obes Rev* 12. doi:10.1111/j.1467-789X.2010.00811.x.

Fesus, G., Dubrovska, G., Gorzelniak, K., Kluge, R., Huang, Y., Luft, F. C., et al. (2007). Adiponectin is a novel humoral vasodilator. *Cardiovasc Res* 75, 719–727. doi:10.1016/j.cardiores.2007.05.025.

Gálvez-Prieto, B., Dubrovska, G., Cano, M. V., Delgado, M., Aranguez, I., González, M. C., et al. (2008). A reduction in the amount and anti-contractile effect of periadventitial

mesenteric adipose tissue precedes hypertension development in spontaneously hypertensive rats. *Hypertens Res* 31, 1415–23. doi:10.1291/hypres.31.1415.

Gao, Y.-J. J., Takemori, K., Su, L.-Y. Y., An, W.-S. S., Lu, C., Sharma, A. M., et al. (2006). Perivascular adipose tissue promotes vasoconstriction: the role of superoxide anion. *Cardiovasc Res* 71, 363–373. doi:10.1016/j.cardiores.2006.03.013.

Goldstein, D. S. (2008). Genotype and vascular phenotype linked by catecholamine systems. *Circulation* 117, 458–61. doi:10.1161/CIRCULATIONAHA.107.745737.

Gollasch, M., and Dubrovska, G. (2004). Paracrine role for periadventitial adipose tissue in the regulation of arterial tone. *Trends Pharmacol Sci* 25, 647–653. doi:10.1016/j.tips.2004.10.005.

Huang, F., Lezama, M. A., Ontiveros, J. A., Bravo, G., Villafana, S., Del-Rio-Navarro, B. E., et al. (2010). Effect of losartan on vascular function in fructose-fed rats: the role of perivascular adipose tissue. *Clin Exp Hypertens* 32, 98–104. doi:10.3109/10641960902993129.

Kandlikar, S. S., and Fink, G. D. (2011). Splanchnic sympathetic nerves in the development of mild DOCA-salt hypertension. *Am J Physiol Heart Circ Physiol* 301, H1965-73. doi:10.1152/ajpheart.00086.2011.

Kershaw, E. E., and Flier, J. S. (2004). Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab* 89, 2548–56. doi:10.1210/jc.2004-0395.

Kvetnansky, R., Ukropec, J., Laukova, M., Manz, B., Pacak, K., and Vargovic, P. (2012). Stress stimulates production of catecholamines in rat adipocytes. *Cell Mol Neurobiol* 32, 801–813. doi:10.1007/s10571-012-9822-6.

Lee, R. M. K. W., Ding, L., Lu, C., Su, L.-Y. Y., and Gao, Y.-J. J. (2009). Alteration of perivascular adipose tissue function in angiotensin II-induced hypertension. *Can J Physiol Pharmacol* 87, 944–53. doi:10.1139/y09-088.

Maguire, J. J., Parker, W. A., Foord, S. M., Bonner, T. I., Neubig, R. R., and Davenport, A. P. (2009). International Union of Pharmacology. LXXII. Recommendations for trace amine receptor nomenclature. *Pharmacol Rev* 61, 1–8. doi:10.1124/pr.109.001107.

Nasmyth, P. A. (1962). An investigation of the action of tyramine and its interrelationship with the effects of other sympathomimetic amines. *Br J Pharmacol Chemother* 18, 65–75.

Owen, M. K., Witzmann, F. A., McKenney, M. L., Lai, X., Berwick, Z. C., Moberly, S. P., et al. (2013). Perivascular adipose tissue potentiates contraction of coronary vascular smooth muscle: influence of obesity. *Circulation* 128, 9–18. doi:10.1161/CIRCULATIONAHA.112.001238.

Park, J. I., Shin, C. Y., Lee, Y. W., Huh, I. H., and Sohn, U. D. (2000). Endothelium-

dependent sensory non-adrenergic non-cholinergic vasodilatation in rat thoracic aorta: involvement of ATP and a role for NO. *J PharmPharmacol* 52, 409–416.

Payne, G. A., Borbouse, L., Kumar, S., Neeb, Z., Alloosh, M., Sturek, M., et al. (2010). Epicardial perivascular adipose-derived leptin exacerbates coronary endothelial dysfunction in metabolic syndrome via a protein kinase C-beta pathway. *Arter Thromb Vasc Biol* 30, 1711–1717. doi:10.1161/ATVBAHA.110.210070.

Payne, G. A., Kohr, M. C., and Tune, J. D. (2012). Epicardial perivascular adipose tissue as a therapeutic target in obesity-related coronary artery disease. *Br J Pharmacol* 165, 659–669. doi:10.1111/j.1476-5381.2011.01370.x.

Peinado, J. R., Pardo, M., de la Rosa, O., Malagón, M. M., and Malagon, M. M. (2012). Proteomic characterization of adipose tissue constituents, a necessary step for understanding adipose tissue complexity. *Proteomics* 12, 607–620. doi:10.1002/pmic.201100355.

Soltis, E. E., and Cassis, L. A. (1991). Influence of perivascular adipose tissue on rat aortic smooth muscle responsiveness. *Clin Exp Hypertens A* 13, 277–296.

Spector, S., Tarver, J., and Berkowitz, B. (1972). Effects of drugs and physiological factors in the disposition of catecholamines in blood vessels. *Pharmacol Rev* 24, 191–202.

Stalder, H., Hoener, M. C., and Norcross, R. D. (2011). Selective antagonists of mouse trace amine-associated receptor 1 (mTAAR1): Discovery of EPPTB (RO5212773). *Bioorganic Med Chem Lett* 21, 1227–1231. doi:10.1016/j.bmcl.2010.12.075.

Stassen, F. R., Maas, R. G., Schiffers, P. M., Janssen, G. M., and De Mey, J. G. (1998). A positive and reversible relationship between adrenergic nerves and alpha-1A adrenoceptors in rat arteries. *J Pharmacol Exp Ther* 284, 399–405.

Szasz, T., and Webb, R. C. (2012). Perivascular adipose tissue: more than just structural support. *Clin Sci* 122, 1–12. doi:10.1042/CS20110151.

Thanassoulis, G., Massaro, J. M., Corsini, E., Rogers, I., Schlett, C. L., Meigs, J. B., et al. (2012). Periaortic adipose tissue and aortic dimensions in the Framingham Heart Study. *J Am Heart Assoc* 1. doi:10.1161/JAHA.112.000885.

Vargovic, P., Ukropec, J., Laukova, M., Cleary, S., Manz, B., Pacak, K., et al. (2011). Adipocytes as a new source of catecholamine production. *FEBS Lett* 585, 2279–2284. doi:10.1016/j.febslet.2011.06.001.

Watts, S. W., Shaw, S., Burnett, R., and Dorrance, A. M. (2011). Indoleamine 2,3-diooxygenase in periaortic fat: mechanisms of inhibition of contraction. *Am J Physiol Heart Circ Physiol* 301, H1236-47. doi:10.1152/ajpheart.00384.2011.

CHAPTER 3

Organic cation transporter 3 contributes to norepinephrine uptake into perivascular adipose tissue

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Abstract

Perivascular adipose tissue (PVAT) reduces vasoconstriction to norepinephrine (NE). A mechanism by which PVAT could function to reduce vascular contraction is by decreasing the amount of NE to which the vessel is exposed. PVATs from male Sprague-Dawley rats were used to test the hypothesis that PVAT has a NE uptake mechanism. NE was detected by HPLC in mesenteric PVAT and isolated adipocytes. Uptake of NE (10 μM) in mesenteric PVAT was reduced by the NE transporter (NET) inhibitor nisoxetine (1 μM; 73.68±7.62%; all values reported as percent of vehicle), the 5-hydroxytryptamine transporter (SERT) inhibitor citalopram (100 nM) with the organic cation transporter 3 (OCT3) inhibitor corticosterone (100 μM; 56.18±5.21%), and the NET inhibitor desipramine (10 μM) with corticosterone (100 μM; 61.18±6.82%). Aortic PVAT NE uptake was reduced by corticosterone (100 μM; 53.01±10.96%). Confocal imaging of mesenteric PVAT stained with 4-[4-(dimethylamino)-styrl]-N-methylpyridium iodide (ASP^+), a fluorescent substrate of cationic transporters, detected ASP^+ uptake into adipocytes. ASP^+ (2 μM) uptake was reduced by citalopram (100 nM; 66.68±6.43%), corticosterone (100 μM; 43.49±10.17%), nisoxetine (100 nM; 84.12±4.24%), citalopram with corticosterone (100 nM and 100 μM, respectively; 35.75±4.21%) and desipramine with corticosterone (10 μM and 100 μM, respectively, 50.47±5.78%). NET protein was not detected in mesenteric PVAT adipocytes. Expression of *Slc22a3* (OCT3 gene) mRNA and protein in PVAT adipocytes was detected by RT-PCR and immunocytochemistry, respectively. These endpoints support the presence of a transporter-mediated NE uptake system within PVAT with a potential mediator being OCT3.

Introduction

Perivascular adipose tissue (PVAT) closely envelops many blood vessels of the body (Gao, 2007). This relationship between PVAT and the blood vessel has earned PVAT its place as the fourth layer of the blood vessel, the “tunica adiposa" (Chaldakov et al., 2007). Beyond providing structural support, PVAT has many roles in modulating blood vessel function (Szasz and Webb, 2012). The release of vasoactive molecules from PVAT influences vascular function by altering the proliferation, migration, inflammation and contraction of vascular smooth muscle (Szasz and Webb, 2012). Interestingly, a releasable pool of catecholamines is present in PVAT (Ayala-Lopez et al., 2014). Although both contractile and anti-contractile substances can be released from PVAT (Ayala-Lopez et al., 2014; Fesus et al., 2007; Gao et al., 2006, 2007; Watts et al., 2013), the presence of PVAT on blood vessels generally reduces vessel contraction in response to various agonists, including norepinephrine (NE) (Soltis and Cassis, 1991). Knowledge on how these mechanisms interact to influence the anti-contractile properties of PVAT in NE-induced contraction is not complete (Kang, 2013).

PVAT’s anti-contractile effect is lost in obesity and hypertension, implicating PVAT as an integral link between both of these diseases (Aghamohammadzadeh and Heagerty, 2012). Over one-third of all adults in the U.S. are hypertensive (Centers for Disease and Prevention, 2011), a condition that significantly increases the risk of death from myocardial infarction or stroke (Lawes et al., 2008). A major risk factor for hypertension is obesity (Henry et al., 2012). Globally, 13% of adults are obese (have a BMI of greater than or equal to 30) [Obesity and overweight (Fact Sheet 311), 2014] and in the U.S. the number is higher with 34.9% of adults classified as obese (Ogden et al., 2014). In obesity,

dysfunction of the anti-contractile effect of PVAT is observed along with overall changes in adipocyte function (Aghamohammadzadeh and Heagerty, 2012). Thus, the relationship between the adipocytes within PVAT and blood vessel function is of interest.

A dynamic adrenergic system that affects blood vessel contraction exists in PVAT (Ayala-Lopez et al., 2014). Catecholamines are released from PVAT upon the addition of tyramine, a sympathomimetic drug, leading to contraction of the rat aorta and the superior mesenteric artery (Ayala-Lopez et al., 2014). Moreover, pharmacological inhibition of NET reduces the PVAT-dependent vascular contraction to tyramine (Ayala-Lopez et al., 2014). Soltis and Cassis discovered that inhibiting NE uptake in the rat aorta abolished the anti-contractile effect of PVAT (Soltis and Cassis, 1991). Collectively, this work led us to investigate the presence of an NE uptake system in PVAT.

This study tests the hypothesis that PVAT takes up NE through molecular transporters and aims to identify the transporters that transport NE in PVAT. Our interest in studying mesenteric PVAT is guided by the knowledge that contraction in the mesenteric resistance arteries increases peripheral resistance, a contributing event towards the elevation of blood pressure. Furthermore, this adipose depot is important for cardiovascular risk. Individuals with large masses of visceral fat have a higher risk of cardiovascular disease than individuals with large masses of subcutaneous fat (Kotchen, 2010).

The PVAT of the mesenteric resistance arteries most closely resembles white adipose tissue in that it contains adipocytes that have large unilocular lipid droplets (Brown et al., 2014). We focus on adipocytes from normal rats as studies of NE transport in adipocytes are sparse and none have been performed on PVAT adipocytes specifically.

To test our hypothesis, we use PVAT from normal male Sprague-Dawley rats for HPLC measures of NE in PVAT and isolated adipocytes. We also measure uptake of NE and use pharmacological inhibitors to transporters to reveal the main transporters that transport NE in PVAT. Confocal microscopy of PVAT is employed using the fluorescent NE transport substrate dye ASP^+ (Schwartz et al., 2003) in addition to immunohistochemistry, immunocytochemistry and gene expression analysis of mesenteric and aortic PVAT to reveal the role of the organic cation transporter 3 (OCT3).

Methods

Materials

Chemicals were purchased from Sigma-Aldrich (Saint Louis, MO USA). The pharmacologic inhibitors nisoxetine (inhibits NET), citalopram (inhibits SERT), corticosterone (inhibits OCT3), and desipramine (inhibits NET and SERT at the concentration used [10 μM]) were purchased from Bio-Techne (Minneapolis, MN USA). Pargyline (an MAO inhibitor), Ro 41-0960 [a catechol-o-methyltransferase (COMT) inhibitor] and NE were purchased from Sigma-Aldrich. 4-[4-(dimethylamino)-styrl]-*N*-methylpyridium iodide (ASP^+) was synthesized and provided by James N. Wilson at the University of Miami, Miami, FL USA (73).

Animal Model

Male Sprague-Dawley rats (225-275 gram or ~8-10 weeks of age, Charles River, Indianapolis, IN USA) were used. All protocols were approved by the MSU Institutional Animal Care and Use Committee and follow the "Guide for the Care and Use of Laboratory Animals", 8th edition, 2011. Rats were anesthetized with sodium pentobarbital (60-80 mg/kg, IP). Anesthesia was verified by lack of paw pinch and eye blink reflexes. Death was assured by pneumothorax and exsanguination after which tissues were removed for one of the following protocols.

NE Uptake

Mesenteric and aortic PVATs were dissected and 20-100 mg of tissue was placed in microcentrifuge tubes containing physiological salt solution (PSS) in mM; 130 NaCl;

4.7 KCl; 1.8 KH_2PO_4; 1.7 $MgSO_4 \cdot 7H_2O$; 14.8 $NaHCO_3$; 5.5 dextrose; 0.03 $CaNa_2$ ethylenediaminetetraacetic acid, 1.6 $CaCl_2$ (pH 7.2) within 30 minutes after the tissue was removed from the rat. Pargyline (10 μM) and Ro 41-0960 (1 μM) were added to the PSS to inhibit NE metabolism. Vehicle or a transporter inhibitor (nisoxetine [1 μM], citalopram [100 nM], corticosterone [100 μM], citalopram [100 nM] with corticosterone [100 μM], desipramine [10 μM], or corticosterone [100 μM] with desipramine [10 μM]) was added for 30 minutes at 37°C. The concentrations were selected based on their specificity for the transporter in question. NE (10 μM) or vehicle (either H_2O or ethanol) was added for another 30 minutes. Tissues were rinsed four times in drug-free PSS and then three times in tissue buffer (0.05 mM sodium phosphate and 0.03 mM citric acid buffer, pH 2.5, in 15% methanol). Samples were saved in tissue buffer and kept at -80°C until assay. The day of the assay the samples were thawed and sonicated for 3 seconds. Samples were centrifuged at 18,000xg for 15 minutes at 4°C, and the supernatant was transferred to new tubes for HPLC analysis. Tissue pellets were dissolved in 1.0 N NaOH and assayed for protein using a Bicinchoninic Acid Protein Assay Kit (cat# BCA1, Sigma-Aldrich).

ASP^+ Uptake

The mesenteric arcade was dissected from Sprague-Dawley rats and stored in PSS without calcium (in mM; 140 NaCl; 5 KCl; 1 $MgCl_2 \cdot 7H_2O$; 10 HEPES; 10 glucose, pH 7.4) at 4°C until use, for up to 5 hours. Immediately prior to experiments, mesenteric resistance arteries with associated PVAT were dissected and pinned onto the Sylgard-coated bottom of an imaging chamber (volume = 1 ml) with the use of a stereomicroscope. Experiments were performed in the dark or under safe lights, at 37°C. The tissue was

superperfused with PSS with calcium (1.8 mM $CaCl_2 \cdot 2H_2O$) and allowed to equilibrate to temperature for 15 min after which a background image was captured. For the ASP^+ concentration-uptake experiment, the tissue was superperfused with PSS containing ASP^+ (1 nM-10 μM) for 10 min and imaged. To test each concentration, a new section of tissue was used from the same animal and the order in which the concentrations were tested was randomized. Each tissue was only used for one condition. For ASP^+-uptake experiments in which inhibitors or NE were used, the tissue was superperfused with an inhibitor of transport, NE (1 mM), or vehicle in PSS for 10 min and an image was captured to assess background fluorescence. ASP^+ (2 μM) was added for 10 min and the tissue was imaged again. For the ASP^+ concentration-uptake experiments and the ASP^+ uptake experiments where nisoxetine, or citalopram were used, tissue imaging was performed with a Leica DMLFSA confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Yokogawa CSU10 spinning disk confocal head (Yokogawa, Tokyo, Japan) coupled to a XR-Mega10 intensified CCD (Stanford Photonics, Palo Alto, CA USA) using a 40x water immersion objective. Illumination was provided by an X-cite Exacte Illuminator (Excelitas Technologies, Waltham, MA USA). Images were recorded with Piper-Control (Stanford Photonics, Palo Alto, CA) and analyzed using ImageJ (ImageJ, NIH). For the corticosterone, citalopram with corticosterone, desipramine, desipramine with corticosterone and NE experiments the protocol was the same as above except that a solid state 488 nm laser was used for illumination and a TurboEX ICCD camera (Stanford Photonics, Palo Alto, CA) controlled by Micro-Manager (Edelstein et al., 2010) was used for image acquisition. Images were captured as stacks of 50 TIFF

(16-bit) images that were then combined with the average z-projection function in Image J. Fluorescence intensity was quantified in relative fluorescent units (RFUs).

Sample Preparation of Mesenteric PVAT, Mesenteric Resistance Vessels, Adipocytes and the SVF

Mesenteric PVAT and mesenteric resistance vessels were dissected in a Sylgard-coated petri dish in PSS with the use of stereomicroscope. Images of the whole mesentery were captured with a Lumix DMC-ZS25 camera (Panasonic, Osaka, Japan) and processed using Adobe Photoshop CC 2014 (Adobe Systems, San Jose, CA USA). The PVAT was either flash frozen for whole PVAT measurements or digested to obtain separate cellular fractions by the following protocol. PVAT was added to 1 ml of PSS with 1 mg/ml collagenase from *Clostridium histolyticum* Type IA (cat #C9891, Sigma-Aldrich) and incubated at 37°C with slow rotation until fully digested (approximately 1 hr). PVAT was centrifuged at 200xg for 5 min and the SVF, which pellets to the bottom, was transferred to a separate tube. Adipocytes and the SVF were washed three times with PSS and centrifuged at 200xg for 10 min. For immunocytochemistry, mesenteric PVAT adipocytes were re-suspended in PSS and centrifuged onto CellTak (cat# 54240, BD Biosciences, Bedford, MA USA) coated slides using a Cytospin 4 cytocentrifuge (700xg for 2 min) and an aliquot was saved for assessing purity using a hemacytometer. For Western blots, the mesenteric PVAT, mesenteric resistance vessels, adipocytes and SVF were added to RIPA buffer solution (cat# R3792, Teknova, Hollister, CA) with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin) into a 2 ml bead tube (Omni International, Kennesaw, GA USA).

Tissues were homogenized using the Omni Bead Ruptor Homogenizer (Omni International) centrifuged for 15 min at 18,000xg and supernatants saved for Western Blot analysis. Supernatants were quantified for protein content by using a Bicinchoninic Acid Protein Assay Kit (cat# BCA1, Sigma-Aldrich). For mRNA isolation and HPLC analysis: the adipocytes and SVF were placed into separate tubes with PSS and centrifuged 200xg for 10 min, after which the supernatant was removed. The tissue was then flash frozen in liquid nitrogen and saved at -80°C until assay. Images of the isolated adipocytes were taken on a Nikon TE2000 inverted microscope with MMI® Cell Tools (Molecular Machines & Industries, Zurich Switzerland).

Western Blot for NET

Fifty micrograms of protein from mesenteric PVAT, mesenteric resistance vessels, adipocytes, stromal vascular fraction (SVF) and vena cava (positive control) were separated on a 10 % SDS gel and transferred to PVDF membrane. The membrane was blocked in 4 % wt/vol chicken egg ovalbumin in tris-buffered saline and tween-20 (TBS-T) for 3 hours at 4 ºC, then incubated with primary antibody [mouse anti-NET (1:500; NET05-2; MAb Technologies, Stone Mountain, GA) and mouse anti-ß-actin (1:2000; A3854; Sigma-Aldrich)] diluted in blocker overnight at 4ºC. The blot was washed with TBS-T (10 minutes each, thrice), and then incubated with IRDye anti-mouse secondary antibody (1:1000; 926-32210; LI-COR Biosciences, Lincoln, NE) diluted in Odyssey Blocking Buffer (927-40000, LI-COR Biosciences) for 1 hour at 4 ºC. The blot was washed with TBS-T (10 minutes each, three times) and developed on the LI-COR Odyssey (LI-COR Biosciences). Densitometric analysis was performed using Image J.

Preparation of Aortic PVAT for PCR and Immunohistochemistry

To obtain samples of aortic PVAT, the thoracic aorta was removed from the rat and placed into PSS. The PVAT was then dissected from the aorta on a Sylgard-coated petri dish with the use of a stereomicroscope. The PVAT was removed and snap frozen in liquid nitrogen for RNA extraction. The Investigative Histopathology Laboratory at Michigan State University prepared the fresh frozen rat aorta slides.

Real-Time PCR

Tissue was homogenized using an Omni Bead Ruptor (Omni International). RNA was extracted with the Quick RNA MiniPrep kit (cat# R1054, Zymo Research Corporation, Irving, CA USA) and purity (260/280 and 260/230 ratios ≥1.8) was verified using a Nanodrop 2000C spectrophotometer (Thermoscientific, Wilmington, DE USA). The mRNA was reverse transcribed with qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD USA). RT-PCR was performed using FAST SYBR Green MasterMix (cat# 4385612, Applied Biosystems, Foster City, CA USA) on the ABI 7500 Fast Real Time PCR system (Life Technologies, Carlsbad, CA USA) and using the following parameters: 95°C for 20 sec, 95°C for 1 sec and 60°C for 20 sec for 40 cycles. Primer sequences for *Slc22a3* amplification are as follows: forward: 5'TAT GCA GCG GAC AGA TAC GG-3', reverse: 5'-AAA ATT CGG TGC AAA CGC CA-3' (Integrated DNA Technologies, Coralville, IA USA). Measures were normalized to beta-2 microglobulin (*B2m*, RT² qPCR Primer Assay, cat# PPR42607A, Qiagen, Valencia, CA USA). A melt curve was performed to verify the presence of one PCR product after the amplification. The data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Immunocyto/histochemistry

Fresh frozen 8-µm aortic tissue sections and adipocyte slides (described above) were fixed in acetone and immunostained using a VECTASTAIN ABC kit (rabbit: cat # PK-4001; Vector Laboratories, Burlingame CA, USA) and an Avidin-Biotin Blocking Kit (SP-2001; Vector Laboratories). The slides were incubated 24 hrs with an anti-OCT3 antibody (1:100, cat# orb107605, Biorbyt, San Francisco, CA USA) or without primary antibody at 4^{o}C. The slides were developed using 3, 3-diaminobenzidine (cat # SK-4100; Vector Laboratories, Burlingame CA USA) and counterstained with Vector Hematoxylin (cat # H-3401, 30 seconds). Imaging was performed on a Nikon TE2000 inverted microscope with MMI® Cell Tools (Molecular Machines & Industries).

High-Performance Liquid Chromatography

PVATs were weighed and homogenized in four times their weight of 0.1 M perchloric acid, centrifuged at 15,000xg for 10 min and the supernatant analyzed by HPLC. Supernatants from uptake experiments were diluted 1:10 in tissue buffer before analysis. The HPLC system consists of a Coulochem III electrochemical detector set at -350 mV with a HR-80 reverse-phase column with Cat-A-Phase II mobile phase (Thermoscientific, Wilmington, DE USA). The separation column was maintained at 35^{o}C with a flow rate of 1.1 ml/min. Quantification was performed by comparing sample area measurements to a calibration curve. Standards were run every 5th sample to verify the identity of our peaks of interest on the chromatogram. The limit of detection was 0.1 ng/ml and NE content was either expressed per weight or by protein content.

Data Analysis

Statistical analyses were performed using GraphPad Prism 6.0 (La Jolla, CA USA). When comparing two groups, either an unpaired Student's t-test was used with similar variances and the Mann-Whitney test was used when the variances were different (as verified by the F-test). When comparing more than two groups, an ANOVA with a Newman Keuls test was used. With non-normally distributed data, the Kruskal-Wallis ANOVA was used followed by the Dunn's test for multiple comparisons. The tests were unpaired. $P<0.05$ was considered statistically significant and the mean ± standard error of the mean (SEM) were reported where appropriate. To calculate percent NE uptake, the concentration of NE in tissues incubated with NE and the pharmacological inhibitor were divided by the concentration of NE in tissue incubated with NE and the vehicle. Image contrast for the ASP^+ experiments was normalized to the brightest image recorded in the data set. Pseudo-colorization of ASP^+ stained images was performed using the "Fire" lookup table and constructed surface plots by applying the "surface plot" function in Image J (version 1.48). All image adjustments in brightness and contrast were made to the whole panel of an image, not a portion. To calculate percent ASP^+ uptake, the F/F_0 (fluorescence intensity ratio) was used, where F is the fluorescence intensity after incubation with the inhibitor and ASP^+ minus the background intensity and F_0, is the fluorescence intensity of ASP^+ incubated with vehicle minus the background intensity.

Results

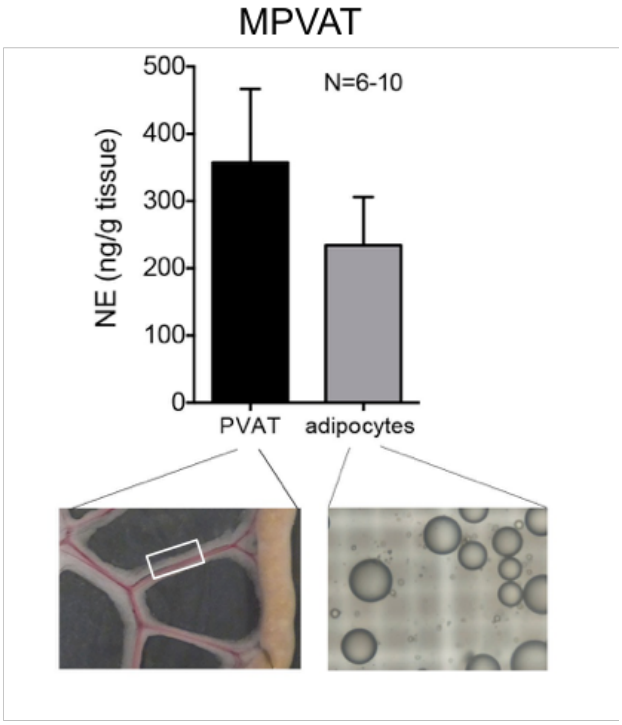
NE is present in rat mesenteric PVAT

A large proportion of the NE found in PVAT is in the adipocyte fraction (Figure 9A). Measures of NE by HPLC in mesenteric PVAT and isolated mesenteric PVAT adipocytes were similar ($p>0.05$; N-9-10). Representative images of each sample are shown in Figure 9A (bottom left and bottom right). The white box highlights a representative area of tissue that was used. One mechanism by which NE could localize to adipocytes in PVAT is through transporter-mediated uptake of extracellular NE, thus we investigated this further.

NE uptake occurs in PVAT

We used a pharmacological approach to test the hypothesis that uptake of NE into PVAT is mediated by transporters. First, we established that NE uptake occurs in PVAT (Figure 9B). PVAT was then incubated with NE (10 μM) or vehicle in PSS for 30 minutes, the tissue was washed to remove excess NE and the NE in the tissue was measured by HPLC. Figure 9B shows that mesenteric PVAT NE content was significantly increased after adding NE (10 μM) compared to the addition of vehicle (18.87±2.42 *vs*. 4.48±1.98 pg/μg protein, respectively, $p<0.05$). This NE uptake could be reduced by the inhibition of NE transport by pre-incubation with inhibitors of NET, SERT and OCT3: nisoxetine (1 μM), citalopram (100 nM) with corticosterone (100 μM) and desipramine (10μM) with corticosterone (100 μM). Desipramine, citalopram or corticosterone alone did not significantly reduce NE uptake compared to vehicle (Figure 10). These data support transporter-mediated uptake of NE in mesenteric PVAT through NET, SERT and OCT3.

A



B

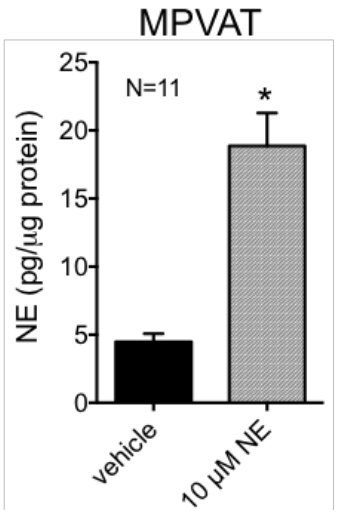


Figure 9. NE is present in PVAT adipocytes and PVAT can take up extracellular NE.

Figure 9 (cont'd)

(A) Mesenteric PVAT adipocytes were isolated and NE content was measured by HPLC. Measures were normalized to tissue weight. Lower left: representative image of mesenteric PVAT used. The white box highlights the portion of PVAT used in the experiments of this study. Lower right: representative image of adipocytes isolated from mesenteric PVAT. (B) Adding of NE (10 μM) to PVAT in PSS for 30 min increased NE accumulation as measured by HPLC and normalized to protein content ($p<0.05$). Bars represent means ±SEM for the N value stated.

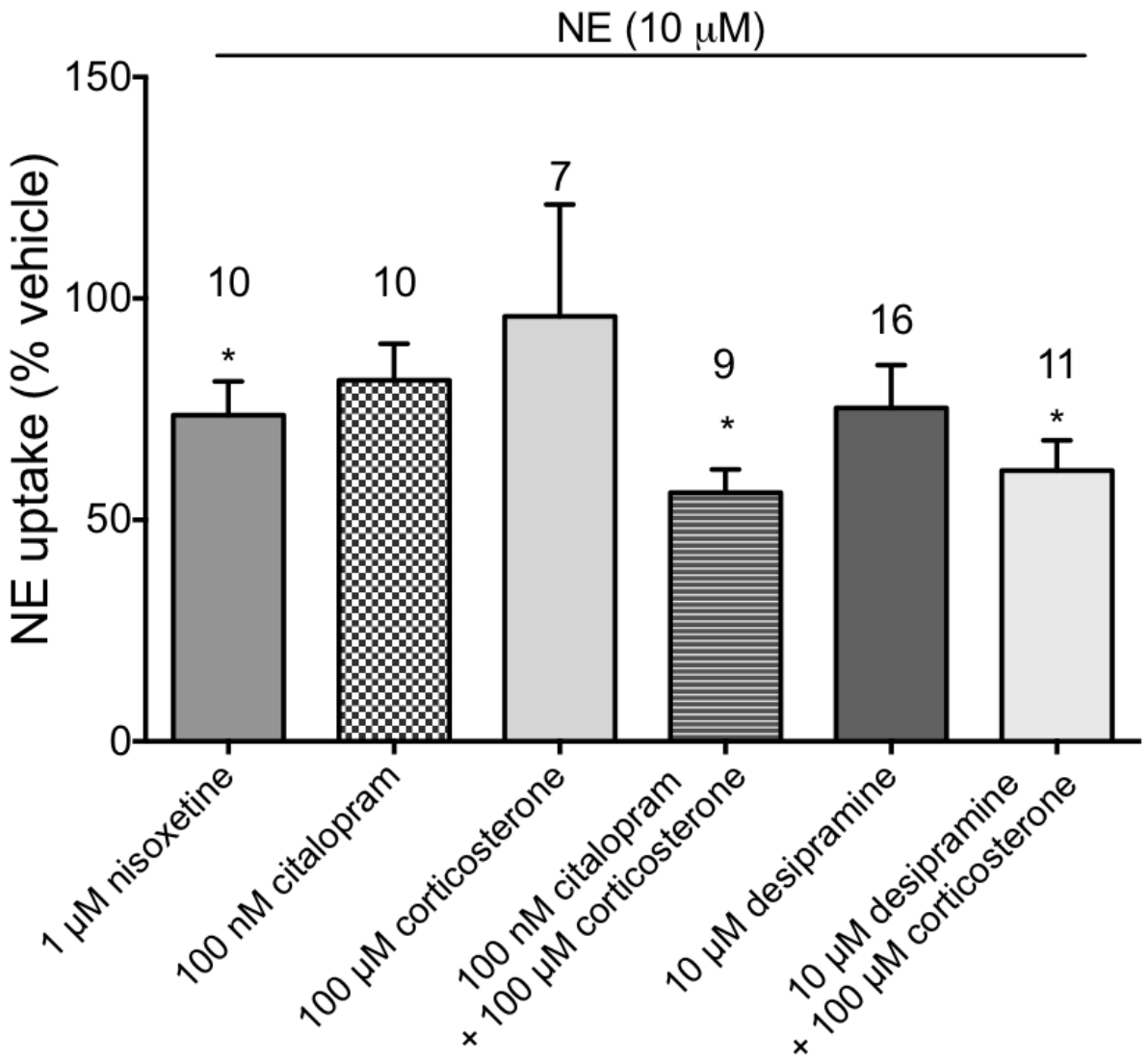


Figure 10. PVAT accumulates NE through transporter-mediated uptake.

Mesenteric PVAT was incubated for 30 min with transporter inhibitors before the addition of 10 μM NE. Data reported as percent uptake from vehicle. Bars represent means ±SEM. Numbers above the bars present the N number for each inhibitor (*$p<0.05$ vs vehicle).

ASP^+, a fluorescent substrate dye for NE transporters, is taken up by PVAT adipocytes

The transporter substrate 4-(-4-(dimethylamino)-styryl)-N-methylpyridinium (ASP^+) was used to identify the presence of NE transporters on mesenteric PVAT (MPVAT) adipocytes. ASP^+ fluoresces upon binding, is transported and accumulates in mitochondria (Schwartz et al., 2003). To determine if ASP^+ would bind to PVAT adipocytes, we added increasing concentrations of ASP^+ to mesenteric PVAT and imaged the tissue by fluorescence microscopy and a graph of the concentration-fluorescence intensity relationship was constructed (Figure 11A). Quantification of the intensity of ASP^+ fluorescence and comparison of fluorescence intensity between vehicle (water) and each concentration revealed a significant increase in fluorescence from vehicle starting at 1 μM ASP^+. Fluorescence intensity saturated the camera at 10 μM ASP^+ (Figure 11B). A concentration of 2 μM ASP^+ was chosen for subsequent experiments to achieve a detectable fluorescence signal while avoiding camera saturation. By imaging the adipocyte at the focal plane that transverses the adipocyte, ASP^+ fluorescence was localized to the periphery of the adipocyte, where the cytoplasm is located (Figure 12A upper panel). A surface plot image of the fluorescence intensity levels with pseudo-colorization shows this more clearly (Figure 12A lower panel). Confocal imaging at the level of the adipocyte nucleus revealed intense staining around the adipocyte nuclei, an area rich in mitochondria in white adipose tissue adipocytes (Figure 12B; upper panel shows the image without color information and the bottom panel shows the image with pseudo-color; arrows are pointing at the perinuclear staining) (DeMartinis et al., 1987). Perinuclear ASP^+ fluorescence in PVAT adipocytes suggests transport of ASP^+ into the adipocyte as opposed to only surface binding.

Pre-incubation of PVAT with an excess of NE (1 mM) reduced ASP^+ uptake (Figure 13). Any residual binding of ASP^+ in the presence of this saturating concentration of NE can be considered non-specific to NE transport mechanisms. To identify which transporters were involved in ASP^+ uptake, mesenteric PVAT was incubated at 37°C in PSS containing an inhibitor of transport or vehicle for 10 min followed by ASP^+ (2 μM) and imaged (Figure 13). Representative images are shown of the fluorescence obtained with vehicle only with ASP^+ (2 μM), background (no ASP^+), and each experiment following pre-incubation with NE or an inhibitor of transport (Figure 13). ASP^+ uptake was significantly reduced by inhibition of NET by nisoxetine (100 nM and 10 μM), SERT by citalopram (100 nM), OCT3 by corticosterone (100 μM), SERT and OCT3 by citalopram with corticosterone (100 nM and 100 μM, respectively), and NET and OCT3 by desipramine with corticosterone (10 μM and 100 μM, respectively). However, uptake was not significantly reduced by desipramine alone (10 μM). Binding and transport of ASP^+ in PVAT adipocytes thus may be mediated by NET, SERT and OCT3, consistent with our studies of NE transport into PVAT (Figure 10).

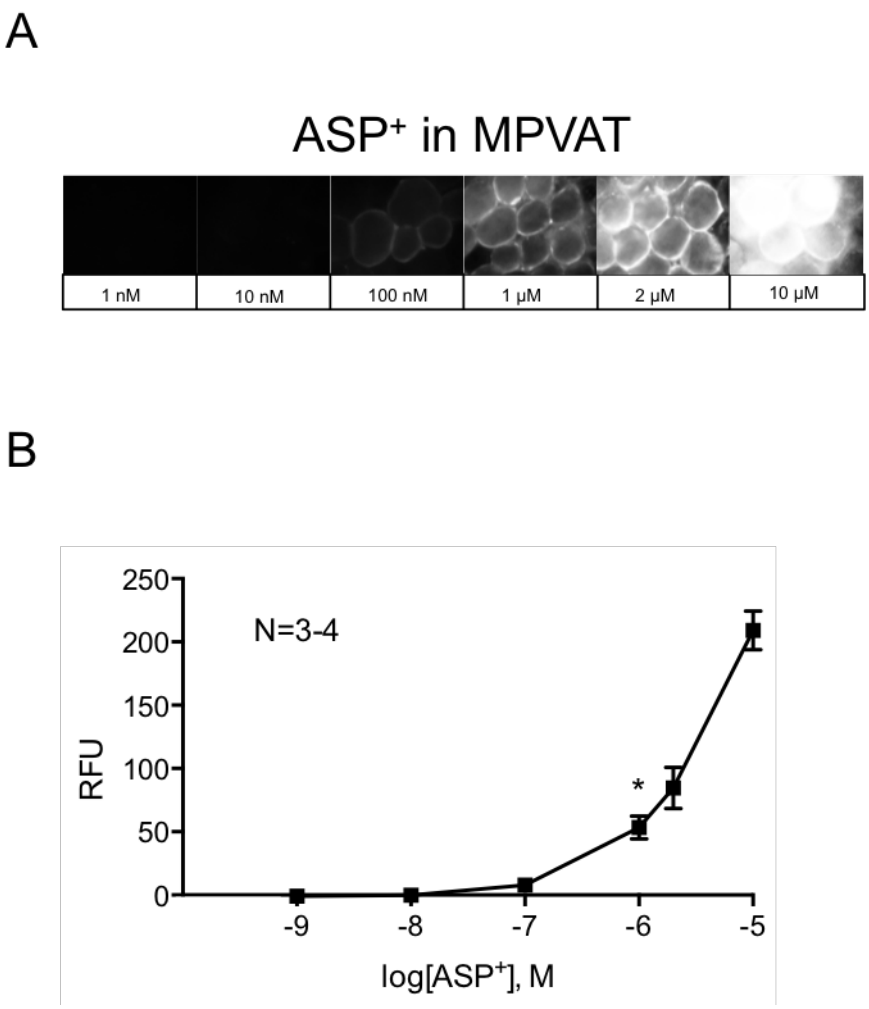


Figure 11. ASP$^+$, a fluorescent substrate of cation transporters, binds to mesenteric PVAT adipocytes.

ASP$^+$ fluorescence in mesenteric PVAT (MPVAT) was tested by adding ASP$^+$ at different concentrations for 10 min and then imaging by fluorescence microscopy. (A) Representative images of ASP$^+$ at each concentration tested. (B) Quantification of ASP$^+$ fluorescence intensity at each concentration is shown (*$p<0.05$, indicates the first concentration with significantly increased fluorescence vs. background). The fluorescence intensity was expressed in RFU=Relative fluorescence units. Points represent means±SEM for number of animals stated (N). Imaged with a 40x objective.

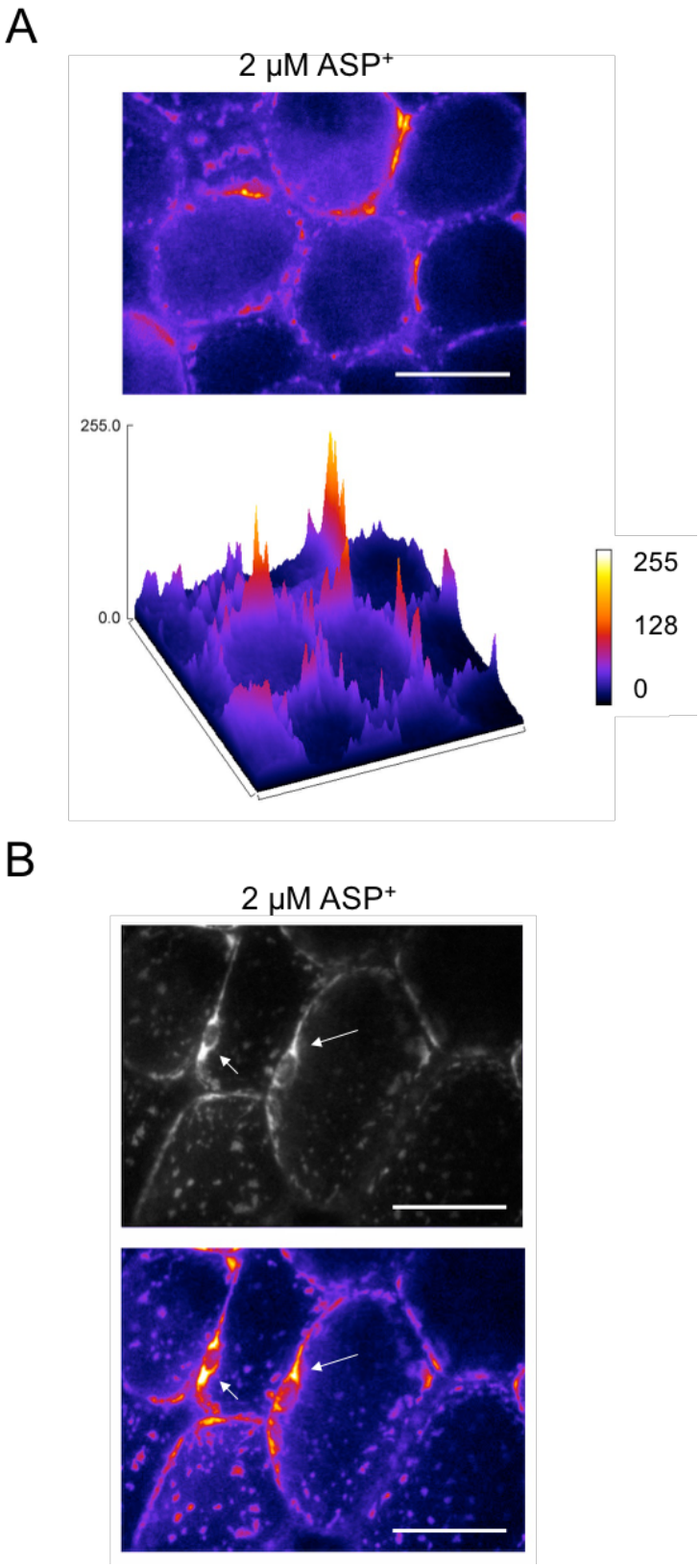


Figure 12. ASP$^+$ is transported into mesenteric PVAT adipocytes.

Figure 12. (cont'd)

ASP^+ (2 μM) was added to mesenteric PVAT for 10 minutes in PSS and imaged by confocal microscopy. (A) A pseudo-colored representation of the fluorescence intensity of ASP^+ (2 μM) binding reveals that ASP^+ binds along on the periphery of the adipocyte in a punctate pattern (top). The surface plot of the same image in the lower panel shows this more clearly (bottom). Refer to the RFU (relative fluorescence units) scale to the right. Lighter (white-yellow) colors indicate higher fluorescence intensities as measured by RFU and darker (violet-black) colors indicate lower fluorescence intensities. (B) Top: Image of PVAT adipocyte imaged at the level of the nucleus. Arrows point to areas of perinuclear staining. Pseudo-colorization of the images (bottom) shows intense staining is present in the perinuclear region. Representative from six animals. Scale bars= 50 microns. Imaged with a 40x objective.

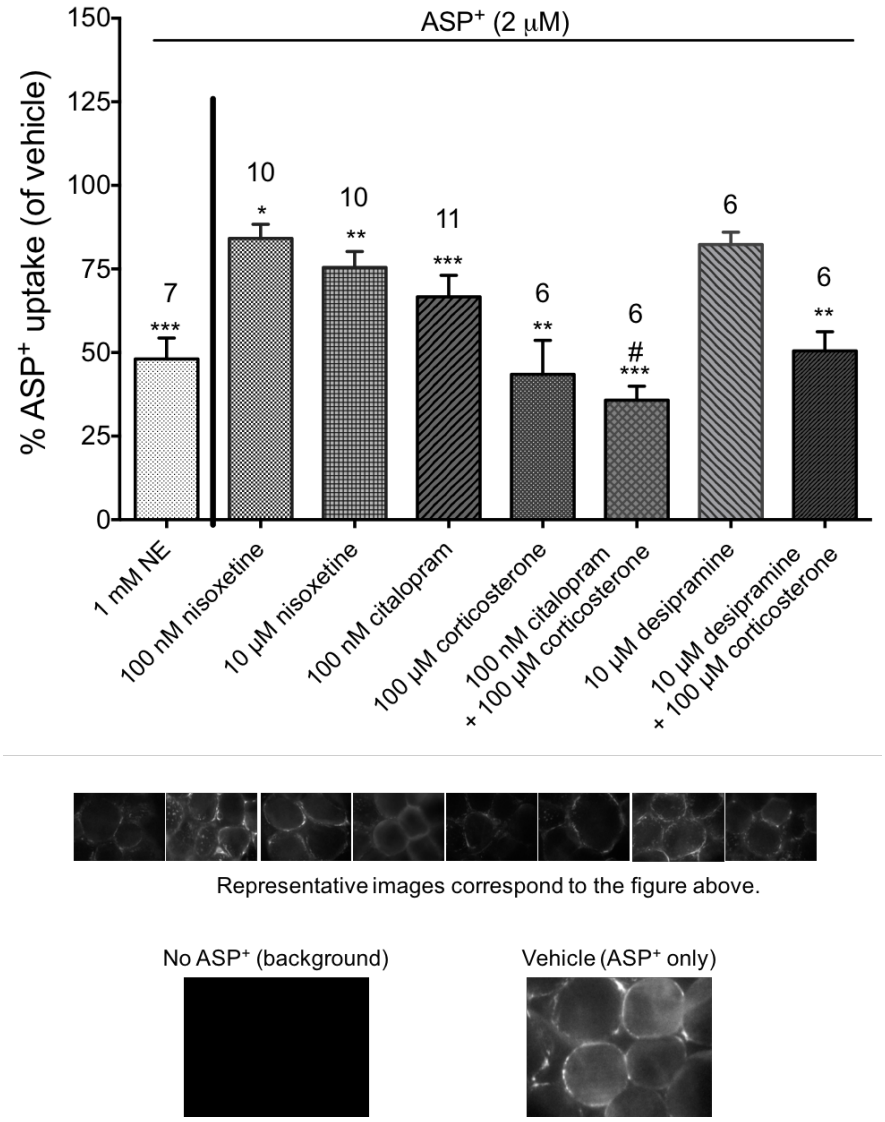


Figure 13. ASP$^+$ uptake is reduced by transporter inhibitors.

Mesenteric PVAT was incubated with inhibitors of transport or vehicle for 10 minutes followed by the addition of ASP$^+$ (2 μM). Data reported as percent uptake from vehicle. The numbers above the bars indicate the N number of animals used in each experiment. Bars represent means ±SEM (*p<0.05, **p<0.005, ***p<0.001 vs vehicle; #p<0.05 vs. corticosterone). Representative images from each experiment are located beneath the corresponding bar.

NET is not present in mesenteric PVAT adipocytes

NET presence in the mesentery was investigated because of the modest effect of nisoxetine on ASP^+ uptake. Protein from mesenteric PVAT, mesenteric resistance vessels, mesenteric PVAT adipocytes, and mesenteric PVAT SVF were assayed for NET by Western blot. We did not observe bands for NET in the adipocytes or the SVF but did observe some faint bands for NET in the mesenteric PVAT, resistance vessels, and the vena cava, our positive control (Figure 14), indicating that NET is most likely not the main transporter that is mediating uptake of NE in PVAT. Corticosterone and corticosterone with citalopram caused the greatest reduction in ASP^+ uptake (Figure 13), therefore we focused on OCT3 for the rest of the studies.

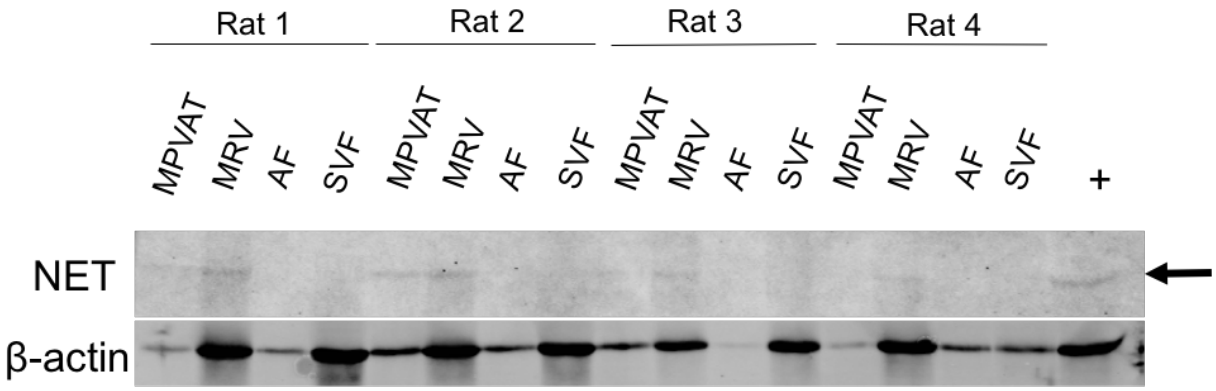


Figure 14. NET is not located in mesenteric PVAT adipocytes.

Whole mesenteric PVAT (MPVAT) was assayed for NET by Western blot along with the mesenteric resistance vessels (MRV), PVAT adipocyte fraction (AF), and the PVAT SVF. The black arrow points to the band of interest. Beta-actin was used as the loading control and the vena cava was used as a positive control. N=4.

OCT3 is present in PVAT adipocytes

PVAT expression of *Slc22a3* (the gene for OCT3) was compared to its relative expression in the heart, a positive control (Chen et al., 2010)(Figure 15A). Mesenteric and aortic PVAT expressed *Slc22a3* at higher relative expression than in the heart (Figures 15A and 15B). Mesenteric PVAT was separated into the adipocyte and SVF to allow for the assay of *Slc22a3* expression in each fraction. Relative expression of *Slc22a3* was higher in adipocytes than in the SVF (Figure 15C). We used the reference gene B2m because it gave us the most similar expression among all of the sample types compared to other housekeepers we assayed (data not shown). The cycles at which each sample reached threshold for B2m were similar between the heart and the mesenteric PVAT (C_T=20.0 and 19.4, respectively), the heart and the aortic PVAT (C_T=17.8 and 17.5, respectively), but were dissimilar for the heart, adipocytes and the SVF (C_T=18.4, 16.7 and 19.9, respectively). This would affect our calculations for relative expression for the last group (C_T for *Slc22a3*=26.9 heart, 23.0 adipocytes, and 30.7 SVF). Immunostaining revealed OCT3 protein in aortic PVAT (Figure 16A) and mesenteric PVAT adipocytes (Figure 16B) using the aorta as a positive control for OCT3 (Verlohren et al., 2004). Aortic staining for OCT3 was located to the tunica media (labeled M) but not the tunica adventitia (labeled V). Immunostaining for OCT3 was present on the periphery of the adipocytes in both aortic and mesenteric PVAT (Figures 16A and B). Immunostaining was not present when the primary antibody was excluded (Figures 16C and D). Inhibiting OCT3 with corticosterone (100 μM) reduced NE uptake in aortic PVAT by 47.0±11.0% (Figure 17). These data support the presence of OCT3 on adipocytes and the potential for OCT3 to transport NE in PVAT.

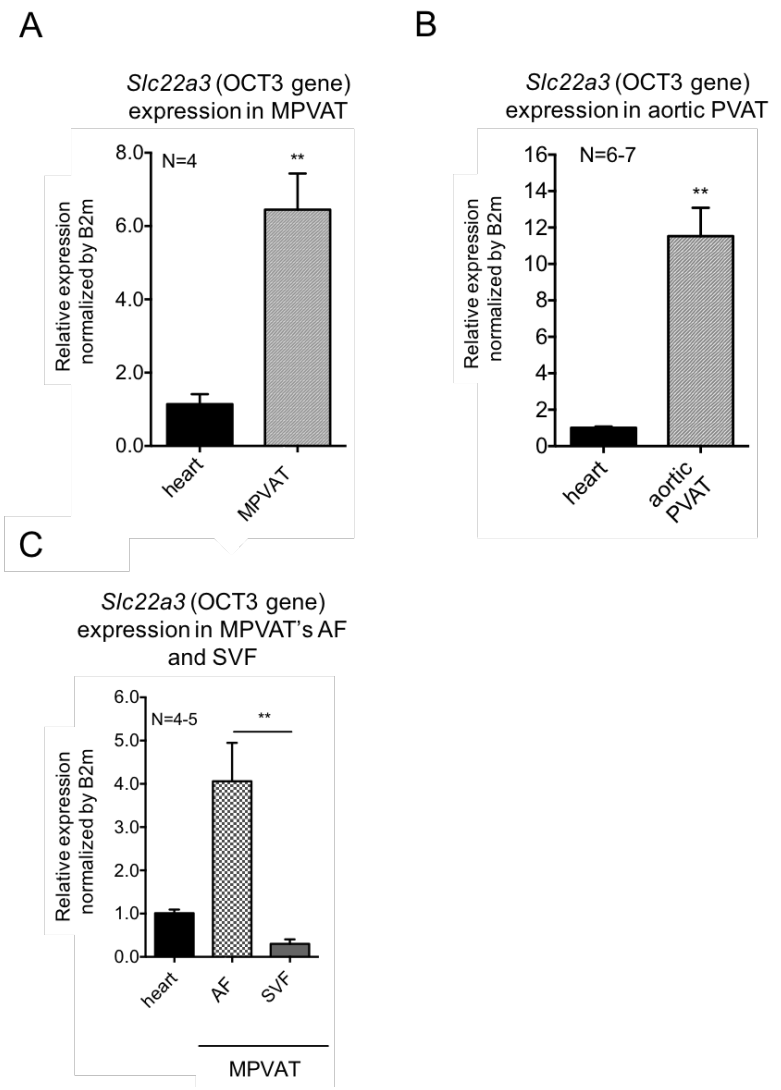


Figure 15. *Slc22a3* mRNA is expressed in mesenteric and aortic PVAT.

(A) Relative mesenteric PVAT (MPVAT) expression of *Slc22a3* mRNA was measured and compared to the heart as a positive control. Measures were normalized to B2M. Bars represent means±SEM, **p<0.005. (B) Whole aortic PVAT relative expression of *Slc22a3* was measured and compared to the heart as a positive control. (C) PVAT adipocyte fraction (AF) relative expression of *Slc22a3* mRNA was higher than the SVF (**p<0.005) when compared to the heart as a positive control. Measures were normalized to B2M. Bars represent means±SEM for the number of animals stated (N), **p<0.005.

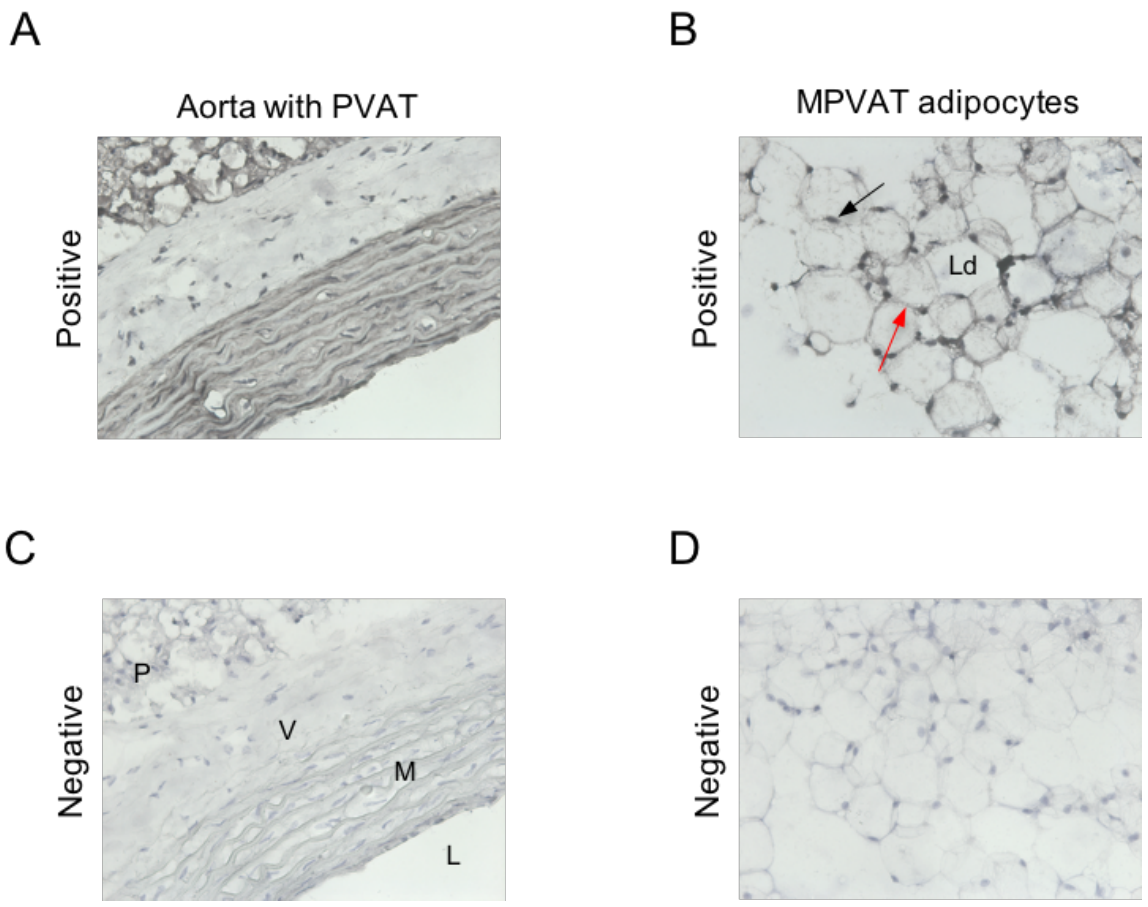


Figure 16. Immunohistochemical and immunocytochemical staining reveals OCT3 presence in aortic PVAT and mesenteric PVAT adipocytes.

(A) The aorta was used as the positive control for OCT3. Aortic staining for OCT3 protein was evident in the media (labeled M) but not the adventitia (labeled V). PVAT (labeled P) around the aorta also stained for OCT3 (L=Lumen). (B) Mesenteric (MPVAT) adipocytes were isolated and stained for OCT3 protein. The black arrow points to the location of adipocyte nuclei and the red arrow points to the location of the cytoplasm of the adipocyte (Ld=lipid droplet). (C) Aorta with PVAT was stained without the inclusion of the primary antibody to serve as a negative control. (D) MPVAT adipocytes were stained without the inclusion of the primary antibody as a negative control. Adipocyte images are representative of four animals. Imaged with a 40x objective. Scale bar= 50 microns. N=4.

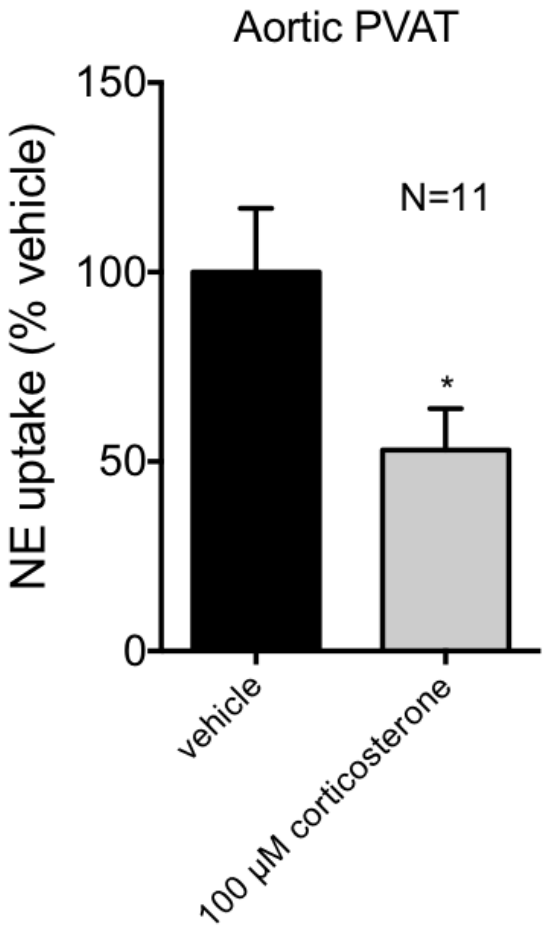


Figure 17. Inhibition of OCT3 with corticosterone reduces NE uptake in the aortic PVAT.

NE uptake of aortic PVAT was assayed after incubation with vehicle or corticosterone (100 μM). Data are reported as percent from vehicle and measures were expressed as NE concentration to protein content. Bars represent means±SEM for the number of animals stated (N), *p<0.05.

Discussion

PVAT modulates blood vessel function (Szasz et al., 2013) and we present evidence that at least part of this can occur through direct NE uptake. The novel discovery of NE uptake by PVAT could present a mechanism by which adipose tissue reduces the local concentration of NE, thereby reducing the ability of NE to interact with the vascular smooth muscle to induce contraction and vasoconstriction. This could be a physiologically relevant mechanism by which PVAT modulates vascular tone. In this study we discuss NE uptake in PVAT with the consideration that most NE that the blood vessel is exposed to is not circulating NE, but rather NE released from sympathetic nerve boutons in and around the blood vessel. Autonomic nervous system interactions with PVAT and its effects on the blood vessel have been recognized (Bulloch and Daly, 2014). Nerves that innervate arteries and their PVAT may release NE that is subsequently taken up by PVAT, thus reducing arterial contraction to NE. In other words, PVAT might serve as a sink or source of NE.

PVAT can take up NE

An adrenergic system exists in adipose tissue, evidenced by the discovery that mesenteric adipose tissue adipocytes synthesize NE and 5-hydroxytryptamine (5-HT) (Stunes et al., 2011; Vargovic et al., 2011). PVATs possess measurable catecholamines (Ayala-Lopez et al., 2014). We found that the adipocyte fraction contains catecholamines (Figure 9A). Other sources of NE could reside in the SVF of PVAT such as macrophages (Brown et al., 2003), lymphocytes (Qiu et al., 2004) and neurons. The catecholamines present in PVAT are releasable by tyramine and support contraction in the rat superior

mesenteric artery independent of sympathetic innervation (Ayala-Lopez et al., 2014). PVAT-dependent contraction to tyramine was reduced by the NET inhibitor nisoxetine (Ayala-Lopez et al., 2014), directing us further into the investigation of NE transport in PVAT. In the present study, we used the transporter inhibitors desipramine [10 μM; k_i (inhibitory constant) for NET: 7.36 nM (Paczkowski et al., 1999) and for SERT:], nisoxetine [1 μM; k_i for NET: 0.46 nM and for SERT: 158 nM (Davids et al., 2002)] and corticosterone [100 μM; OCT3 IC_{50}: 120-290 nM (Koepsell et al., 2007)]. Desipramine, a NET inhibitor, at higher concentrations can also inhibit SERT [K_i: 228 nM (Davids et al., 2002)] and OCT3 [IC_{50} of 700 nM (Zhu et al., 2012)]. Since our experimental samples were intact tissues, not isolated transporters or membranes, each inhibitor was used at a concentration that is ~50-100 times above the K_i values to assure the inhibitors reached their target, considering that they can be metabolized or bound.

Uptake of NE was reduced upon the addition of nisoxetine, citalopram with corticosterone, and desipramine with corticosterone. While the high concentration of nisoxetine reduced uptake on its own, there are two possibilities for why inhibition with two drugs (desipramine/corticosterone and citalopram/corticosterone), as opposed to either of them alone, was necessary to observe a reduction in uptake. First, redundancy of NE transport may exist through different transporters in PVAT. In the brain, the uptake-2 system (another term for OCT3-mediated transport) has been implicated in limiting the reduction of NE, DA and 5-HT uptake by specific inhibitors (Hensler et al., 2013). Therefore, in our system, to significantly reduce NE uptake in PVAT, multiple transporters may have to be targeted. Second, it is possible that transporters on adipocytes heteroligomerize (De Felice and Adams, 2001; Horschitz et al., 2003; Kocabas et al.,

2003). NET and SERT can heteroligomerize but it is debated whether heteroligomerization affects their function. Less is known about oligomerization of OCT3. Homodimerization of rat OCT1 and human OCT2, the other organic cation transporter isoforms, is required for the transporter to be placed on the plasma membrane (Koepsell, 2013). While oligomerization would be interesting to study, is not the focus of this work. It is a possibility that different PVAT depots may contain a different distribution of transporters of NE. Therefore, the applicability of these findings to other PVAT depots is not known outside of rat aortic and mesenteric PVAT.

The cation transporter substrate ASP^+ is taken up by PVAT adipocytes in a NET-, SERT- and OCT3-dependent manner

ASP^+ is a useful experimental tool for probing NE transport, validated previously in NE uptake assays using radiolabeled [^{3}H]NE (Haunso and Buchanan, 2007). ASP^+ fluorescence detection permits its use for live cell imaging to identify potential transporters of NE. Confocal imaging of ASP^+-stained PVAT adipocytes allowed us to visualize the ASP^+ taken into the adipocyte via the observation of bright halos around the adipocyte nuclei. This pattern of perinuclear ASP^+ fluorescence was observed in all six of the tissues imaged. The punctate pattern of ASP^+ fluorescence in the adipocyte is strikingly similar to that observed when adipocyte mitochondria were stained using rhodamine 123 (DeMartinis et al., 1987). This previous study reported nuclear “haloing” when mitochondria were stained using the fluorescent dye similar to what we observed when we applied ASP^+ to PVAT. Furthermore, ASP^+ accumulation in mitochondria after transport into the cell has been shown previously (Blakely et al., 2005), supporting our

observation that ASP^+ was able to bind and be transported into the cell. The finding that ASP^+ was citalopram-sensitive is in line with the finding that adipocytes express functional SERT (Stunes et al., 2011). Moreover, a study by Pizzinat et al. (1999) found that [^{3}H]NE uptake in isolated human adipocytes obtained from abdominal or mammary lipectomies could be reduced by inhibiting the uptake-2 system (OCT3) with disprocynium 24 (Pizzinat et al., 1999). In our study, nisoxetine reduced ASP^+ uptake at both concentrations tested and it inhibited NE uptake in PVAT at 1 μM, a concentration that would be non-specific for NET. Although convincing evidence that NET is present on mesenteric PVAT adipocytes was not found (Figure 14), it is possible that ASP^+ is a more sensitive tool for the detection of NET. We did not investigate the presence of DAT further due to the finding that GBR 12935 [100 nM; a DAT inhibitor; K_i= 3.7 nM (Rothman et al., 2001)] did not reduce ASP^+ fluorescence and mRNA for DAT could not be detected by PCR in mesenteric PVAT in 40 cycles (data not shown). Therefore, it is unlikely that DAT is playing a role in NE uptake in PVAT.

The “anti-contractile” effect of PVAT to NE in the rat thoracic aorta is attenuated by desipramine plus deoxycorticosterone (Soltis and Cassis, 1991) and this observation was the impetus to study PVAT NE transport. Since the anti-contractile effect of PVAT due to NE transport at least in the rat aorta has already been shown (Soltis and Cassis, 1991), we did not pursue these experiments in this manuscript. Instead, we set out to find the mechanism by which PVAT can take up NE. We also used desipramine and corticosterone (similar to deoxycorticosterone in that it inhibits OCT3) in this study to investigate transport. Desipramine alone did not have an effect on ASP^+ or NE uptake, but when added in conjunction with corticosterone we observed a significant reduction in

both assays. We observed similar general patterns of inhibition in NE uptake and ASP^+ studies. Interestingly, in contrast to our NE uptake experiment, which required both desipramine and corticosterone or citalopram and corticosterone to reduce uptake, ASP^+ fluorescence was reduced by pre-incubation with corticosterone or citalopram alone. This could be due to a difference in transporter affinity for ASP^+ *vs.* NE. ASP^+ has been used as a surrogate for [^{3}H]NE, due to the similarity in their pharmacologic profiles but there are differences in their affinity to certain transporters (Haunso and Buchanan, 2007). This was also evidenced by the observation that adding a high concentration of NE to saturate NE transporters failed to abolish ASP^+ staining indicating the presence of ASP^+ fluorescence non-specific to NE transport mechanisms. Schwartz et al (2003) observed non-specific fluorescence of ASP^+ in experiments using HEK cells (Schwartz et al., 2003). Therefore, care needs to be taken when interpreting findings from ASP^+ binding studies to mechanisms of specific NE transport. Both the ASP^+ and NE uptake studies pointed to OCT3 as being important, and thus was the focus in our final studies.

OCT3 is highly expressed in mesenteric and aortic PVAT

OCT3 is a low-affinity, high-capacity uptake transporter, formally known as “EMT” (extraneuronal monoamine transporter) and also termed the “uptake-2 system”, and is broadly expressed in non-neuronal cells. OCT3 expression on PVAT adipocytes has not yet been investigated. We were surprised to find a higher expression of OCT3 in mesenteric and aortic PVAT than in the heart. It is interesting to speculate on the function NE transport molecules on adipocytes when NE activates lipolysis. Adipocytes contain levels of monoamine oxidase activity that are comparable to that of the liver, an organ

high in monoamine metabolizing activity (Pizzinat et al., 1999). This would support the idea that the function of NE transporters deliver NE into the cell to be metabolized.

We observed high expression of OCT3 in aortic PVAT and inhibiting OCT3 reduced NE uptake. The presence of OCT3 in the aorta, a conduit artery, would lead one to question OCT3's role in blood pressure regulation. Ultimately, the main role of OCT3 in PVAT is unknown. Uptake of NE by aortic PVAT was reduced by inhibition of OCT3 with corticosterone. Thus, it could serve to remove excess NE (Pizzinat et al., 1999), but there may be other physiological roles for OCT3 that may not involve blood pressure regulation such as polyamine transport (Sala-Rabanal et al., 2013) and clearance of toxins (Jonker and Schinkel, 2004). Therefore, OCT3 may be more necessary in the aortic PVAT due to its other roles versus the mesenteric resistance PVAT. This would have to be investigated further.

Limitations

Although *in vitro* NE transport influences contractility of blood vessels to NE (Ayala-Lopez et al., 2014; Soltis and Cassis, 1991), there is no confirmation that this occurs *in vivo*. The mesentery and the omentum are considered 'visceral fat' (Ibrahim, 2010; Nguyen et al., 2014) and the adipose tissue around the mesenteric resistance arteries is most specifically referred to as mesenteric PVAT. We show an image of the mesenteric PVAT in Figure 9 to clarify this point. Mesenteric PVAT is a common PVAT depot that studied for its relevance to alteration in vascular response and blood pressure (Galvez et al., 2006; Li et al., 2012; Verlohren et al., 2004), while PVAT around skeletal muscle arteries is more associated with mechanisms of insulin resistance (Bakker et al., 2009;

Hariya et al., 2014; Meijer et al., 2015). Therefore, the mesenteric PVAT is the most relevant PVAT for us to study. Aortic PVAT was included in the IHC, NE uptake and RT-PCR analysis as this is the most discrete and widely studied PVAT depot and therefore useful when comparing findings to other studies. Nonetheless, it should be noted that mesenteric PVAT mechanisms of NE uptake are more physiologically relevant with regards to blood pressure regulation.

The lack of specific antibodies against NET and SERT in which we have confidence for use in adipose tissue, has been an experimental limitation and why a pharmacological approach using well-characterized transporter inhibitors was used to characterize monoamine transporters. The Western blot for NET lacked strong reactivity to the positive control (Figure 14) indicating that a lack of signal in our samples could be attributed to the low affinity of the antibody and not that the transporter is not present. This was the best NE antibody that we had available. Studies employing the use of knock-out animals and/or siRNA towards different molecular transporters could also be helpful to elucidate monoamine transport mechanisms. However, this approach is accompanied with upregulation of transporters to take up NE, facilitated by the promiscuous nature of neurotransmitter transporters (Daws, 2009). This is observed in the brains of NET knock-out mice (Solich et al., 2011), which exhibit increased SERT and DAT expression. Also, mice with reduced SERT expression overexpress OCT3 and exhibit increased 5-HT clearance through OCT3 (Baganz et al., 2008). These points have been discussed in a previous manuscript as it pertains to SERT knock out rats (Linder et al., 2008). The upregulation of transporters in genetic models of deficient transport would thereby make

the interpretations of experimental results arising from these techniques difficult and is why we did not use the OCT3 knock out rodent in these studies.

The anti-contractile effect of PVAT due to NE transport at least in the rat aorta was discovered by Soltis and Cassis (1991) and we present the first biochemical measures to investigate the mechanism by which PVAT can take up NE. This study suggests that NE transport may be involved in the anti-contractile effect of PVAT. We fully recognize that other mechanisms exist that reduce NE-induced contraction such as the release of adiponectin from PVAT adipocytes (Greenstein et al., 2009). The contribution of these mechanisms in a physiological system may dictate the pathology observed in different vascular disorders. A decline in NE transport capacity of PVAT may exacerbate loss of adiponectin followed by adipocyte hypertrophy and dysfunction in disease. Also, in situations of dysfunctional adiponectin release, PVAT NE transport may become a more important mechanism of reducing vascular tone. These are questions we will pursue in the future.

The method in which we sacrifice the animal prior to tissue collection could have a potential effect on adrenergic system activation thus, these factors and how they would affect our end points were considered. Hirota et al. reported inhibition of NE and dopamine release following exposure of rat brain striatal slices to barbiturates, including pentobarbital (Hirota et al., 2000). Other groups have similarly reported an inhibition of NE release (Ida et al., 1990; Mizuno et al., 1994; Shimokawa et al., 1998) or no effect (Kubota et al., 1999; Pashkov and Hemmings, 2002) by pentobarbital in their studies. Our laboratory has previously sacrificed rats using isoflurane or pentobarbital, and we have not observed a difference in tissue catecholamine content with either of them suggesting

similar functions of uptake and release. In the present study, most of the experiments allow comparisons within each animal by reporting percent NE uptake (opposed to absolute NE values) and every animal is sacrificed the same way (with pentobarbital). Thus, we hope that small changes in baseline NE would not affect our results.

Great care was taken to clean our adipocyte fraction from any contaminating cells. A possibility is that there is some contamination of nerve fibers and SVF cells in the adipocyte isolates, and this is why it was especially revealing to observe fluorescent labeling of PVAT adipocytes with ASP^+. With confocal microscopy, it was possible to confirm that ASP^+ was labeling adipocytes and could readily distinguish if blood vessels or other structures that were in the visual field were exhibiting fluorescence with ASP^+.

Conclusion

This study identified NE transport in PVAT and found OCT3 to be the prime candidate transporter of NE within the PVAT adipocyte. This is the first report of NE transport in PVAT adipocytes. This is also the first time that ASP^+ has been applied to the study of adipose tissue. Experiments using ASP^+ may be extended to compare NE transport in disease models of obesity and hypertension to investigate the regulation and dysfunction of NE transport.

The present manuscript identified NE uptake into PVAT, as part of a larger project defining the physiology and pathophysiology of an endogenous adrenergic system in PVAT. Adipose tissue maintains high expression of the amine metabolizing enzymes monoamine oxidase and semicarbazide sensitive amine oxidase (Abella et al., 2004). Therefore, it is likely that one way PVAT interacts with NE is by breaking it down with amine oxidases. Relative to NE storage, tyramine (a sympathomimetic drug)-induced PVAT-dependent contraction of the rat thoracic aorta and the rat superior mesenteric artery was reduced by tetrabenazine (a VMAT inhibitor). This finding, published by our group, supports that local stores of NE in PVAT could contribute to vascular contraction (Ayala-Lopez et al., 2014). Collectively, these findings will define an adrenergic system in PVAT that we can then investigate in obesity.

This study sheds light on the interaction between PVAT and the blood vessel within a local adrenergic system. PVAT's role in reducing vascular contraction in health could be, in part, due to NE uptake into PVAT, and this mechanism of NE removal may be dysfunctional in diseases of altered vascular tone. The scarcity of information on transporters of NE and NE uptake in PVAT led us to study normal (non-disease model)

rats to test whether uptake is an important physiological mechanism in PVAT. Understanding the normal functional characteristics of NE uptake in the non-diseased rodent allows us to know what to look for when investigating a disease model. In this study, we developed an assay (confocal microscopy of ASP^+ staining of adipose tissue) that we and/or other investigators could use to investigate PVAT mechanisms in an obese and/or hypertensive model organism. The present study is a first step in this direction.

REFERENCES

REFERENCES

Abella, A., Garcia-Vicente, S., Viguerie, N., Ros-Baro, A., Camps, M., Palacin, M., et al. (2004). Adipocytes release a soluble form of VAP-1/SSAO by a metalloprotease-dependent process and in a regulated manner. *Diabetologia* 47, 429–438.

Aghamohammadzadeh, R., and Heagerty, A. M. (2012). Obesity-related hypertension: epidemiology, pathophysiology, treatments, and the contribution of perivascular adipose tissue. *Ann Med* 44 Suppl 1, S74-84.

Ayala-Lopez, N., Martini, M., Jackson, W. F., Darios, E., Burnett, R., Seitz, B., et al. (2014). Perivascular adipose tissue contains functional catecholamines. *Pharmacol Res Perspect* 2, e00041. doi:10.1002/prp2.41.

Baganz, N. L., Horton, R. E., Calderon, A. S., Owens, W. A., Munn, J. L., Watts, L. T., et al. (2008). Organic cation transporter 3: Keeping the brake on extracellular serotonin in serotonin-transporter-deficient mice. *Proc Natl Acad Sci U S A* 105, 18976–18981. doi:10.1073/pnas.0800466105.

Bakker, W., Eringa, E. C., Sipkema, P., and van Hinsbergh, V. W. (2009). Endothelial dysfunction and diabetes: roles of hyperglycemia, impaired insulin signaling and obesity. *Cell Tissue Res* 335, 165–189. doi:10.1007/s00441-008-0685-6.

Blakely, R. D., Defelice, L. J., and Galli, A. (2005). Biogenic amine neurotransmitter transporters: just when you thought you knew them. *Physiology* 20, 225–231. doi:10.1152/physiol.00013.2005.

Brown, N. K., Zhou, Z., Zhang, J., Zeng, R., Wu, J., Eitzman, D. T., et al. (2014). Perivascular adipose tissue in vascular function and disease: a review of current research and animal models. *Arterioscler Thromb Vasc Biol* 34, 1621–1630. doi:10.1161/ATVBAHA.114.303029.

Brown, S. W., Meyers, R. T., Brennan, K. M., Rumble, J. M., Narasimhachari, N., Perozzi, E. F., et al. (2003). Catecholamines in a macrophage cell line. *J Neuroimmunol* 135, 47–55. doi:10.1016/S0165-5728(02)00435-6.

Bulloch, J. M., and Daly, C. J. (2014). Autonomic nerves and perivascular fat: interactive mechanisms. *Pharmacol Ther* 143, 61–73. doi:10.1016/j.pharmthera.2014.02.005.

Centers for Disease, C., and Prevention (2011). Vital signs: prevalence, treatment, and control of hypertension--United States, 1999-2002 and 2005-2008. *MMWR Morb Mortal Wkly Rep* 60, 103–108.

Chaldakov, G. N., Tonchev, A. B., Stankulov, I. S., Ghenev, P. I., Fiore, M., Aloe, L., et al. (2007). Periadventitial adipose tissue (tunica adiposa): enemy or friend around? *Arch Pathol Lab Med* 131, 1766; author reply 1766-7. doi:10.1043/1543-

2165(2007)131[1766a:PATTAE]2.0.CO;2.

Chen, L., Pawlikowski, B., Schlessinger, A., More, S. S., Stryke, D., Johns, S. J., et al. (2010). Role of organic cation transporter 3 (SLC22A3) and its missense variants in the pharmacologic action of metformin. *Pharmacogenet Genomics* 20, 687–699. doi:10.1097/FPC.0b013e32833fe789.

Davids, E., Zhang, K., Kula, N. S., Tarazi, F. I., and Baldessarini, R. J. (2002). Effects of norepinephrine and serotonin transporter inhibitors on hyperactivity induced by neonatal 6-hydroxydopamine lesioning in rats. *J Pharmacol Exp Ther* 301, 1097–1102.

Daws, L. C. (2009). Unfaithful neurotransmitter transporters: focus on serotonin uptake and implications for antidepressant efficacy. *Pharmacol Ther* 121, 89–99. doi:10.1016/j.pharmthera.2008.10.004.

DeMartinis, F. D., Ashkin, K. T., and Lampe, K. T. (1987). Fluorescence detection of mitochondrial clusters in mammalian white fat cells in vivo. *Am J Physiol* 253, C783-91.

Edelstein, A., Amodaj, N., Hoover, K., Vale, R., Stuurman, N., and Arthur Edelstein Karl Hoover, Ron Vale, and Nico Stuurman, N. A. (2010). Computer Control of Microscopes Using μManager. *Curr Protoc Mol Biol* 14.20.1-14, Unit14.20. doi:10.1002/0471142727.mb1420s92.

De Felice, L. J., and Adams, S. V (2001). Serotonin and norepinephrine transporters: possible relationship between oligomeric structure and channel modes of conduction. *Mol Membr Biol* 18, 45–51.

Fesus, G., Dubrovska, G., Gorzelniak, K., Kluge, R., Huang, Y., Luft, F. C., et al. (2007). Adiponectin is a novel humoral vasodilator. *Cardiovasc Res* 75, 719–727. doi:10.1016/j.cardiores.2007.05.025.

Galvez, B., De Castro, J., Herold, D., Dubrovska, G., Arribas, S., Gonzonlez, M. C., et al. (2006). Perivascular adipose tissue and mesenteric vascular function in spontaneously hypertensive rats. *Arter Thromb Vasc Biol* 26, 1297–1302. doi:10.1161/01.ATV.0000220381.40739.dd.

Gao, Y.-J. J. (2007). Dual modulation of vascular function by perivascular adipose tissue and its potential correlation with adiposity/lipoatrophy-related vascular dysfunction. *Curr Pharm Des* 13, 2185–2192. doi:10.2174/138161207781039634.

Gao, Y.-J. J., Lu, C., Su, L.-Y. Y., Sharma, A. M., and Lee, R. M. K. W. (2007). Modulation of vascular function by perivascular adipose tissue: the role of endothelium and hydrogen peroxide. *Br J Pharmacol* 151, 323–331. doi:10.1038/sj.bjp.0707228.

Gao, Y.-J. J., Takemori, K., Su, L.-Y. Y., An, W.-S. S., Lu, C., Sharma, A. M., et al. (2006). Perivascular adipose tissue promotes vasoconstriction: the role of superoxide anion. *Cardiovasc Res* 71, 363–373. doi:10.1016/j.cardiores.2006.03.013.

Greenstein, A. S., Khavandi, K., Withers, S. B., Sonoyama, K., Clancy, O., Jeziorska, M., et al. (2009). Local inflammation and hypoxia abolish the protective anticontractile properties of perivascular fat in obese patients. *Circulation* 119, 1661–1670. doi:10.1161/CIRCULATIONAHA.108.821181.

Hariya, N., Mochizuki, K., Inoue, S., Morioka, K., Shimada, M., Okuda, T., et al. (2014). Insulin resistance in SHR/NDmc-cp rats correlates with enlarged perivascular adipocytes and endothelial cell dysfunction in skeletal muscle. *J Nutr Sci Vitaminol* 60, 52–59.

Haunso, A., and Buchanan, D. (2007). Pharmacological characterization of a fluorescent uptake assay for the noradrenaline transporter. *J Biomol Screen* 12, 378–384. doi:10.1177/1087057107299524.

Henry, S. L., Barzel, B., Wood-Bradley, R. J., Burke, S. L., Head, G. A., and Armitage, J. A. (2012). Developmental origins of obesity-related hypertension. *Clin Exp Pharmacol Physiol* 39, 799–806. doi:10.1111/j.1440-1681.2011.05579.x.

Hensler, J. G., Artigas, F., Bortolozzi, A., Daws, L. C., De Deurwaerdere, P., Milan, L., et al. (2013). Catecholamine/Serotonin interactions: systems thinking for brain function and disease. *Adv Pharmacol* 68, 167–197. doi:10.1016/B978-0-12-411512-5.00009-9.

Hirota, K., Kudo, M., Kudo, T., Kitayama, M., Kushikata, T., Lambert, D. G., et al. (2000). Barbiturates inhibit K(+)-evoked noradrenaline and dopamine release from rat striatal slices--involvement of voltage sensitive Ca(2+) channels. *Neurosci Lett* 291, 175–8.

Horschitz, S., Hummerich, R., and Schloss, P. (2003). Functional coupling of serotonin and noradrenaline transporters. *J Neurochem* 86, 958–965.

Ibrahim, M. M. (2010). Subcutaneous and visceral adipose tissue: structural and functional differences. *Obes Rev* 11, 11–8. doi:10.1111/j.1467-789X.2009.00623.x.

Ida, Y., Tsuda, A., Tsujimaru, S., Satoh, M., and Tanaka, M. (1990). Pentobarbital attenuates stress-induced increases in noradrenaline release in specific brain regions of rats. *Pharmacol Biochem Behav* 36, 953–956.

Jonker, J. W., and Schinkel, A. H. (2004). Pharmacological and physiological functions of the polyspecific organic cation transporters: OCT1, 2, and 3 (SLC22A1-3). *J Pharmacol Exp Ther* 308, 2–9. doi:10.1124/jpet.103.053298.

Kang, Y. S. (2013). Obesity associated hypertension: new insights into mechanism. *Electrolyte Blood Press* 11, 46–52. doi:10.5049/EBP.2013.11.2.46.

Kocabas, A. M., Rudnick, G., and Kilic, F. (2003). Functional consequences of homo- but not hetero-oligomerization between transporters for the biogenic amine neurotransmitters. *J Neurochem* 85, 1513–1520.

Koepsell, H. (2013). Polyspecific organic cation transporters and their biomedical relevance in kidney. *Curr Opin Nephrol Hypertens* 22, 533–538. doi:10.1097/MNH.0b013e328363ffaf.

Koepsell, H., Lips, K., and Volk, C. (2007). Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. *Pharm Res* 24, 1227–1251. doi:10.1007/s11095-007-9254-z.

Kotchen, T. A. (2010). Obesity-Related Hypertension: Epidemiology, Pathophysiology, and Clinical Management. *Am J Hypertens* 23, 1170–1178. doi:10.1038/ajh.2010.172.

Kubota, T., Anzawa, N., Hirota, K., Yoshida, H., Kushikata, T., and Matsuki, A. (1999). Effects of ketamine and pentobarbital on noradrenaline release from the medial prefrontal cortex in rats. *Can J Anaesth* 46, 388–392. doi:10.1007/BF03013235.

Lawes, C. M., Hoorn, S. Vander, and Rodgers, A. (2008). Global burden of blood-pressure-related disease, 2001. *Lancet* 371, 1513–1518. doi:10.1016/S0140-6736(08)60655-8.

Li, R., Andersen, I., Aleke, J., Golubinskaya, V., Gustafsson, H., and Nilsson, H. (2012). Reduced anti-contractile effect of perivascular adipose tissue on mesenteric small arteries from spontaneously hypertensive rats : Role of Kv7 channels. *Eur J Pharmacol*, 1–6. doi:10.1016/j.ejphar.2012.09.026.

Linder, A. E., Diaz, J., Ni, W., Szasz, T., Burnett, R., and Watts, S. W. (2008). Vascular reactivity, 5-HT uptake, and blood pressure in the serotonin transporter knockout rat. *Am J Physiol Hear Circ Physiol* 294, H1745-52. doi:10.1152/ajpheart.91415.2007.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402–408. doi:10.1006/meth.2001.1262.

Meijer, R. I., Serné, E. H., Korkmaz, H. I., van der Peet, D. L., de Boer, M. P., Niessen, H. W. M., et al. (2015). Insulin-induced changes in skeletal muscle microvascular perfusion are dependent upon perivascular adipose tissue in women. *Diabetologia* 58, 1907–15. doi:10.1007/s00125-015-3606-8.

Mizuno, T., Ito, E., and Kimura, F. (1994). Pentobarbital sodium inhibits the release of noradrenaline in the medial preoptic area in the rat. *Neurosci Lett* 170, 111–113.

Nguyen, N. L. T., Randall, J., Banfield, B. W., and Bartness, T. J. (2014). Central sympathetic innervations to visceral and subcutaneous white adipose tissue. *Am J Physiol Regul Integr Comp Physiol* 306, R375-86. doi:10.1152/ajpregu.00552.2013.

Obesity and overweight (Fact Sheet 311) (2014). 2015. Available at: http://www.who.int/mediacentre/factsheets/fs311/en/.

Ogden, C. L., Carroll, M. D., Kit, B. K., and Flegal, K. M. (2014). Prevalence of

childhood and adult obesity in the United States, 2011-2012. *Jama* 311, 806–814. doi:10.1001/jama.2014.732.

Paczkowski, F. A., Bryan-lluka, L. J., Pörzgen, P., Brüss, M., and Bönisch, H. (1999). Comparison of the Pharmacological Properties of Cloned Rat , Human , and Bovine Norepinephrine Transporters 1. *J Pharmacol Exp Ther* 290, 761–767.

Pashkov, V. N., and Hemmings, H. C. (2002). The effects of general anesthetics on norepinephrine release from isolated rat cortical nerve terminals. *Anesth Analg* 95, 1274–81, table of contents.

Pizzinat, N., Marti, L., Remaury, A., Leger, F., Langin, D., Lafontan, M., et al. (1999). High expression of monoamine oxidases in human white adipose tissue: evidence for their involvement in noradrenaline clearance. *Biochem Pharmacol* 58, 1735–1742.

Qiu, Y. H., Peng, Y. P., Jiang, J. M., and Wang, J. J. (2004). Expression of Tyrosine Hydroxylase in Lymphocytes and Effect of Endogenous Catecholamines on Lymphocyte Function. *Neuroimmunomodulation* 11, 75–83. doi:10.1159/000075316.

Rothman, R. B., Baumann, M. H., Dersch, C. M., Romero, D. V, Rice, K. C., Carroll, F. I., et al. (2001). Amphetamine-type central nervous system stimulants release norepinephrine more potently than they release dopamine and serotonin. *Synapse* 39, 32–41. doi:10.1002/1098-2396(20010101)39:1<32::AID-SYN5>3.0.CO;2-3.

Sala-Rabanal, M., Li, D. C., Dake, G. R., Kurata, H. T., Inyushin, M., Skatchkov, S. N., et al. (2013). Polyamine transport by the polyspecific organic cation transporters OCT1, OCT2, and OCT3. *Mol Pharm* 10, 1450–8. doi:10.1021/mp400024d.

Schwartz, J. W., Blakely, R. D., and DeFelice, L. J. (2003). Binding and transport in norepinephrine transporters. Real-time, spatially resolved analysis in single cells using a fluorescent substrate. *J Biol Chem* 278, 9768–9777. doi:10.1074/jbc.M209824200.

Shimokawa, A., Jin, Q. H., Ishizuka, Y., Kunitake, T., Takasaki, M., and Kannan, H. (1998). Effects of anesthetics on norepinephrine release in the hypothalamic paraventricular nucleus region of awake rats. *Neurosci Lett* 244, 21–4.

Solich, J., Faron-Gorecka, A., Kusmider, M., Palach, P., Gaska, M., and Dziedzicka-Wasylewska, M. (2011). Norepinephrine transporter (NET) knock-out upregulates dopamine and serotonin transporters in the mouse brain. *Neurochem Int* 59, 185–191. doi:10.1016/j.neuint.2011.04.012.

Soltis, E. E., and Cassis, L. A. (1991). Influence of perivascular adipose tissue on rat aortic smooth muscle responsiveness. *Clin Exp Hypertens A* 13, 277–296.

Stunes, A. K., Reseland, J. E., Hauso, O., Kidd, M., Tommeras, K., Waldum, H. L., et al. (2011). Adipocytes express a functional system for serotonin synthesis, reuptake and receptor activation. *Diabetes Obes Metab* 13, 551–558. doi:10.1111/j.1463-1326.2011.01378.x.

Szasz, T., Bomfim, G. F., and Webb, R. C. (2013). The influence of perivascular adipose tissue on vascular homeostasis. *Vasc Heal Risk Manag* 9, 105–116. doi:10.2147/VHRM.S33760.

Szasz, T., and Webb, R. C. (2012). Perivascular adipose tissue: more than just structural support. *Clin Sci* 122, 1–12. doi:10.1042/CS20110151.

Vargovic, P., Ukropec, J., Laukova, M., Cleary, S., Manz, B., Pacak, K., et al. (2011). Adipocytes as a new source of catecholamine production. *FEBS Lett* 585, 2279–2284. doi:10.1016/j.febslet.2011.06.001.

Verlohren, S., Dubrovska, G., Tsang, S. Y., Essin, K., Luft, F. C., Huang, Y., et al. (2004). Visceral periadventitial adipose tissue regulates arterial tone of mesenteric arteries. *Hypertension* 44, 271–276. doi:10.1161/01.HYP.0000140058.28994.ec.

Watts, S. W., Dorrance, A. M., Penfold, M. E., Rourke, J. L., Sinal, C. J., Seitz, B., et al. (2013). Chemerin connects fat to arterial contraction. *Arter Thromb Vasc Biol* 33, 1320–1328. doi:10.1161/ATVBAHA.113.301476.

Zhu, H., Appel, D. I., Gründemann, D., Richelson, E., and Markowitz, J. S. (2012). Evaluation of organic cation transporter 3 (SLC22A3) inhibition as a potential mechanism of antidepressant action. *Pharmacol Res* 65, 491–496. doi:10.1016/j.phrs.2012.01.008.

CHAPTER 4

Perivascular adipose tissue's amine oxidases: Impact on NE-induced contraction of mesenteric resistance arteries

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Abstract

Perivascular adipose tissue (PVAT) can decrease vascular contraction to NE. A mechanism for this could be the degradation of vasoactive amines by PVAT, yet this has not been demonstrated. We tested the hypothesis that metabolism of vasoactive amines by mesenteric PVAT (MPVAT) could affect NE-induced contraction of the mesenteric resistance arteries. Mesenteric resistance vessels (MRV) and MPVAT from male Sprague-Dawley rats were used. RT-PCR and Western blots were performed to detect which amine metabolizing enzymes are present in the MRV and MPVAT. The Amplex® Red Monoamine Oxidase Assay was used to quantify oxidase activity in the MRV, MPVAT, and the adipocyte fraction and stromal vascular fraction (SVF) isolated from MPVAT. The functional contribution of NE metabolism in third-order mesenteric arteries with or without PVAT was determined by measuring isometric contraction to NE. RT-PCR and Western blots revealed that enzymes capable of metabolizing NE, semicarbazide sensitive amine oxidase (SSAO), monoamine oxidase A (MAO-A), and monoamine oxidase B (MAO-B) are expressed in MRV and MPVAT. The substrates tyramine or benzylamine (both at 1 mM) increased amine oxidase activity in the MRV, MPVAT and its adipocyte fraction (AF). Tyramine, but not benzylamine, drove oxidase activity in the SVF. In both the MPVAT and isolated adipocytes, tyramine- and benzylamine-driven oxidase activity was decreased by inhibiting SSAO with semicarbazide (1 mM). Benzylamine-driven, but not tyramine-driven, oxidase activity in the MRV was also reduced by semicarbazide. By contrast, no reduction in oxidase activity in all sample types was observed with use of the monoamine oxidase (MAO) inhibitors, clorgyline (1 μM- MAO-A) or pargyline (1 μM- MAO-B). Inhibition of MAO-A/B (10 μM pargyline) or

SSAO (1 mM semicarbazide) individually did not alter contraction to NE. However, inhibition of both MAO and SSAO reduced contraction of mesenteric arteries with PVAT to NE but not in arteries cleaned of PVAT. These findings support that most of the amine oxidase activity in MPVAT can be attributed to SSAO and adipocytes within MPVAT are a source of SSAO activity. PVAT's metabolism of NE contributes to the anti-contractile effect of PVAT on arteries exposed to NE.

Introduction

Perivascular adipose tissue (PVAT) makes up the fourth layer of most blood vessels and is part of an “adipose organ” (Cinti, 2012). Its role in creating the vascular environment and regulating vascular function is particularly interesting. PVAT is appreciated for its effects on vascular tone, from mediating relaxation of arteries (Brandes, 2007; Fesus et al., 2007) to stimulating contraction by the release of catecholamines (Ayala-Lopez et al., 2014; Gao et al., 2006). In normal (non-disease) models, PVAT reduces contraction of the underlying arteries to several agonists, such as norepinephrine (NE), phenylephrine and angiotensin II (Löhn et al., 2002; Soltis and Cassis, 1991). However, in diseases such as hypertension and obesity, PVAT physiology changes and loses its anti-contractile effect (Aghamohammadzadeh and Heagerty, 2012).

PVAT causes a right-shift in the concentration-response curve of the thoracic aorta to NE, a sympathetic neurotransmitter and endogenous vasoconstrictor (Soltis and Cassis 1991). Our group demonstrated that PVAT affects arterial contraction through the release and uptake of NE (Ayala-Lopez et al., 2014, 2015). This collective evidence supports the existence of an adrenergic system within PVAT. The question remains if metabolism of NE occurs within PVAT. Amine metabolism in PVAT presents a mechanism, by which PVAT can inactivate vasoactive amines and reduce the contractile response.

We hypothesized that PVAT has amine metabolizing activity. The NE metabolizing enzymes monoamine oxidases A (MAO-A) and monoamine oxidase B (MAO-B) are present in human white adipose tissue adipocytes isolated from mammary and

abdominal lipectomies (Pizzinat et al., 1999), yet their presence and activity in PVAT are currently unknown. Catechol-o-methyl transferase (COMT), also metabolizes NE and is highly expressed in the liver, kidney and brain (Myöhänen et al., 2010). At least one study provides evidence for COMT's presence in adipose tissue (canine subcutaneous) (Belfrage et al., 1977). However, studies investigating COMT in human or rat PVAT have not been done. Lastly, the dual function enzyme, semicarbazide sensitive amine oxidase (SSAO), also known as vascular adhesion protein-1 (VAP-1), catalyzes the deamination of amines, in addition to its other function as a leukocyte adhesion molecule. SSAO is shed from the plasma membrane of adipocytes and is elevated in plasma during cardiovascular disease (Abella et al., 2004) and atherosclerosis (Karadi et al., 2002). NE is a substrate for SSAO in rat brown adipose tissue (Barrand and Callingham, 1982). However SSAO's role in PVAT-mediated alterations of vascular contraction to NE has not yet been investigated.

To study NE metabolism in PVAT, we used mesenteric resistance arteries with and without PVAT. Additionally, we separated PVAT into its fractional components, the adipocyte fraction (AF) and the stromal vascular fraction (SVF) for some of the experiments. Our focus was on the mesenteric fat because the mesenteric resistance arteries are important to the regulation of systemic blood pressure. Further, increased visceral fat, which includes mesenteric fat, is associated with increased cardiovascular risk (Kotchen, 2010). Sympathetic nervous system activity is increased in obesity-related hypertension (Hall et al., 2010). Thus, investigating PVAT's mechanisms of handling NE, the sympathetic neurotransmitter, is relevant in understand the pathology in this disease. Gene expression analysis and Western blots were used to identify which NE metabolizing

enzymes were present in mesenteric resistance vessels (MRV- pooled mesenteric resistance arteries and veins) and in their surrounding MPVAT. We carried out functional assays using tyramine and benzylamine, commonly accepted substrates of MAO and SSAO (Visentin et al., 2005), to drive oxidase activity. Isolation of adipocytes and the SVF allowed us to understand which constituents of PVAT contained amine oxidase activity. Furthermore, to determine the enzyme(s) responsible for the observed amine oxidase activity, we used pharmacological inhibitors of amine oxidases. The functional relevance of our findings to arterial contraction was tested in isolated third-order mesenteric arteries with and without PVAT exposed to inhibitors of MAO-A/B, COMT and SSAO before cumulative addition of NE. Our work presents the first insights into the ability of PVAT to metabolize amines and its impact on arterial contraction to NE.

Methods

Chemicals

Norepinephrine hydrochloride, nisoxetine hydrochloride, semicarbazide hydrochloride, and Ro 41-0960 were purchased from Sigma-Aldrich (St. Louis, MO). Pargyline hydrochloride for the contractility experiments was purchased from Cayman Chemical (Ann Arbor, MI). Pargyline and clorgyline used in the oxidase assay experiments were supplied within the Amplex® Red Monoamine Oxidase Assay Kit (cat# A12214, ThermoFisher Scientific, Grand Island, NY USA).

Animals

Male Sprague-Dawley rats (225-275 gram or ~8-10 weeks of age, Charles River, Indianapolis, IN USA) were used. All protocols were approved by the MSU Institutional Animal Care and Use Committee and follow the "Guide for the Care and Use of Laboratory Animals", Eighth edition, 2011. Rats were anesthetized with sodium pentobarbital (60-80 mg/kg, IP). Anesthesia was verified by lack of paw pinch and eye blink reflexes. Death was assured by pneumothorax and exsanguination after which tissues were removed for one of the following protocols.

Tissue Dissection

The liver was collected, snap frozen in liquid N_2 and saved to serve as a positive control for the RT-PCR and Western blot assays. Mesentery, brain and aorta were collected in physiological salt solution (PSS); in mM; 130 NaCl; 4.7 KCl; 1.8 KH_2PO_4; 1.7 $MgSO_4 \cdot 7H_2O$; 14.8 $NaHCO_3$; 5.5 dextrose; 0.03 $CaNa_2$ ethylenediaminetetraacetic acid,

1.6 $CaCl_2$ (pH 7.2). A portion the rat brain corresponding to the midbrain and pons was dissected and the aorta was cleaned of PVAT. Both samples were then saved in 50 mM potassium phosphate buffer for the oxidase activity assay. The same tissues (brain and aorta without PVAT) from a separate set of animals were dissected, frozen in liquid N_2 and saved for RT-PCR. MPVAT and the associated mesenteric resistance arteries and veins (MRV) were separated out in a Sylgard®-coated petri dish in PSS with the aid of a stereomicroscope. The MPVAT was either used for fractionation, frozen in liquid N_2 for protein isolation, or saved in 50 mM potassium phosphate buffer for the oxidase assay. The MRV were either frozen in liquid N_2 for protein or RNA isolation, or saved in 50 mM potassium phosphate buffer. Samples for the oxidase activity assays were saved at -20 °C and used within one week. Mesenteric resistance arteries (2 mm in length) with or without PVAT for the use in isometric contraction experiments were dissected out in a Sylgard®-coated petri dish in PSS with the aid of a stereomicroscope.

Adipocyte and SVF Isolation

MPVAT was added to 1 mL of PSS with 1 mg/mL collagenase from Clostridium histolyticum type IA (cat# C9891, Sigma) and incubated at 37°C with gentle agitation until fully digested. The sample was centrifuged at 200xg for 5 minutes after which the SVF was transferred into a separate tube. The fractions were then washed six times by adding 1 mL of PSS and centrifuging at 200xg for 10 minutes. Purity of the isolation (>95% adipocytes) was verified by counting the adipocytes *vs.* non-adipocytes present with a hemocytometer. Phase contrast images of the fractions were taken with a 20X objective (Hi PLAN I 20X/ 0.30 PH1) on an inverted microscope (DMi1 [Leica, Buffalo Grove, IL,

USA)] using the Leica Application Suite (LAS). The PSS was then removed and the samples were placed in 50 mM potassium phosphate buffer to be used in the oxidase assay before freezing or snap frozen in liquid N_2 for protein isolation.

Real-Time PCR

All tissues (brain, liver, MRV, MPVAT and aorta) were homogenized using the Bead Ruptor 24 (Omni International, NW Kennesaw, GA). RNA was extracted with the Quick RNA MiniPrep kit (cat# R1054, Zymo Research Corporation, Irving, CA USA) and purity (260/280 and 260/230 ratios ≥1.8) was verified using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Wilmington, DE USA). The mRNA (1 μg) was reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (cat# 4368814, ThermoFisher Scientific). RT-PCR was performed using PerfeCTa FastMix II, ROX (cat# 95119, Quanta Biosciences, Gaithersburg, MD USA) on the ABI 7500 Fast Real Time PCR system (Life Technologies, Carlsbad, CA USA) with the following parameters: 95°C for 20 seconds, 95°C for 1 seconds and 60°C for 20 seconds for 40 cycles. Taqman Primers were purchased from ThermoFisher Scientific. The sequences are proprietary. Thus, we have listed the catalog numbers which are as follows: *Aoc3* (cat# 4448892, assay ID: Rn01452826_m1), *Comt* (cat#4448892, assay ID: Rn01404927_g1: *Actb* (cat#4448892, assay ID: Rn00667869_m1), *Maoa* (cat#4448892, assay ID: Rn01430950_m1), *Maob* (cat#4448892, assay ID: Rn00566203_m1). Measures were normalized to β-actin (*Actb*) and expressed as fold change relative to the positive control tissue as described by Livak and Schmittgen (2001).

Western Blots

MPVAT and MRV protein was isolated in phosphate buffered saline (PBS) with protease inhibitors (1 mM sodium orthovanadate, 100 µg/ml aprotinin/leupeptin and 1 mM phenylmethylsulfonyl fluoride) and homogenized using the Bead Ruptor 24 (Omni International). The protein from the positive controls was isolated as follows. Stomach fundus and gut mucosa were dissected from the rat and were placed into RIPA buffer (cat# R3792, Teknova, Hollister, CA) with the above protease inhibitors before homogenizing with the Bead Ruptor 24 (Omni International) and centrifuging at 10,000 rpm for 10 minutes. Protein from the aorta was isolated in 1X lysis buffer (62.5 mM Tris HCl pH 7.8, 2% SDS, 10 % glycerol) with the above protease inhibitors, frozen in liquid N_2, homogenized with mortar and pestle followed by a centrifugation at 11,000 rpm for 10 minutes. Supernatants were then separated. The Jurkat whole cell lysate was purchased from Santa Cruz Biotechnology (cat# SC-2204, Dallas, TX). The protein concentrations was determined using the Bicinchoninic Acid Protein Assay Kit (cat# BCA1, Sigma-Aldrich). The protein samples (50 µg) were separated on 10% SDS polyacrylamide gels using the Bio-Rad Mini Protean 3 system. Protein was transferred to PVDF-FL (MAO-A) or nitrocellulose (MAO-B, COMT, VAP1) and blocked for 3 hours at 4°C in 4% chicken egg ovalbumin (MAO-A, β-actin), LI-COR Odyssey Blocking Buffer (MAO-B, COMT) or 5% bovine serum albumin (VAP-1). Primary antibody [MAO-A, 1:200 (epitope corresponds to amino acids 458–527 of MAO-A of human origin; Santa Cruz Biotechnology, SC-20156); MAO-B, 1:200 (epitope near the C-terminus of MAO-B of human origin; Santa Cruz Biotechnology, SC-18401); COMT, 1:200 (epitope corresponding to amino acids 1-271 of COMT of human origin; Santa Cruz Biotechnology,

SC-25844); VAP1, 1:200 (epitope near the C-terminus of VAP-1 of human origin; Santa Cruz Biotechnology, SC-13741); or β-actin, 1:2000 (Sigma, A3854)] was incubated overnight in blocking buffer at 4°C. Antibody was recovered and blots were washed with TBS + 0.1% Tween 20 (TBS-T) for 10 minutes at 4°C (three times). Blots were then incubated with species-specific LI-COR IRDye 800 secondary antibody (1:1000, MAO-A, MAO-B, COMT, VAP1) or LI-COR IRDye 700 secondary antibody (1:1000, β-actin) in LI-COR Odyssey Blocking Buffer for 1 hour at 4°C, followed by washes with TBS-T for 10 minutes at 4°C (three times). Bands were visualized using the LI-COR Odyssey or LI-COR FC system. Densitometry was completed with Image J.

Oxidase Activity Assay

Samples were homogenized with the Bead Ruptor 24 (Omni International). The samples were centrifuged at 600xg for 10 minutes and the supernatant transferred to new tubes. The protein concentration was measured using a Bicinchoninic Acid Protein Assay Kit (cat# BCA1, Sigma-Aldrich). MPVAT and the adipocyte fraction (AF) samples were loaded into a black clear-bottom 96-well plate at 20 mg of protein per well. Twice this protein, 40 mg, was loaded for the MRV, the SVF, brain (positive control for MAO) and aorta (positive control for SSAO). More protein was used for non-adipocyte containing tissues because the baseline H_2O_2 production in these extracts was less. Thus, more was loaded to ensure H_2O_2 would be detected. Samples in 1X reaction buffer were added into each well of a 96-well plate with an inhibitor of metabolism/or vehicle. The plate was incubated at room temperature for 30 minutes. Then, the substrate was added (either tyramine or benzylamine; 1 mM), with 100 µL of the Amplex® Red Reagent to start the

reaction. The plate was incubated for another 30 minutes at room temperature after which the fluorescence was read at 530-560 nm excitation and 590 nm emission on the Infinite M1000 PRO (Tecan Group Ltd, Männedorf, Switzerland). Measures were compared to a resorufin standard curve and expressed as pmol/min/mg of protein.

Isometric Contraction

Rat third-order mesenteric resistance arteries cleaned of fat (-PVAT) or with fat intact (+PVAT) were mounted into a Multi Wire Myograph System 620M (Danish Myo Technology, Denmark). Data were acquired using a PowerLab Data Acquisitions unit (ADInstruments, Colorado Springs, CO, USA). Baths contained warmed, oxygenated PSS. Rings were pulled to optimum resting tension (13.3 kpa) with the aid of the normalization module (Danish Myo Technology) and equilibrated for one hour with washes every 20 minutes. The arteries were exposed to an initial concentration of 60 mM KCl to test viability. Tissues were washed and tone returned to baseline and exposed to another concentration of 60 mM KCl to elicit the maximum contraction. The 60 mM KCl maximum contraction was what we normalized the force of contraction to. Tissues were then washed and returned to baseline. Either vehicle or inhibitor was added for one hour without washing. NE was then added in a cumulative fashion, with significant time necessary for a response to plateau prior to the next addition. Tissues were washed and a final 60 mM KCl addition was performed to test for tissue viability at the end of the experiment. Data was expressed as percent maximum contraction.

Statistical Analysis

Data are reported as means±SEM for number of animals indicated by N or near each bar within the graphs. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). RT-PCR relative fold change means were compared with a one-way ANOVA followed by the Holm-Sidak's multiple comparisons test for parametric data sets (*Maob* expression) and the Kruskal-Wallis followed by the Dunn's test for multiple comparisons for the non-parametric data sets (*Maoa, Comt* and *Aoc3* expression). Western blot densitometry was statistically analyzed with a paired Student's t-test. Oxidase activity results reported in Figures 22 and 24 were analyzed by a two-way ANOVA followed by Sidak's multiple comparisons test. Outliers were identified and removed following a Grubb's test. Oxidase activity inhibition results reported in Figures 23 and 25 were analyzed by a Kruskal-Wallis test followed by the Dunn's test for multiple comparisons. Contraction was reported as means±SEM as a percentage of the initial contraction to 60 mM KCl. Potency means ($-\log EC_{50}$, M) were calculated using GraphPad Prism 6.0 as concentrations necessary to cause a half-maximal effect. Where a maximum was not achieved, the values are estimated and true potencies equal or greater than that reported. Unpaired Student's t-tests were performed to compare $\log EC_{50}$ values between tissues with PVAT (+PVAT) and vehicle *vs.* +PVAT and inhibitor. Maximum contraction means were compared with a one-way ANOVA followed by a Kruskal-Wallis test followed by the Dunn's test for multiple comparisons. $P<0.05$ was considered statistically significant.

Results

Aoc3 and Maoa mRNA are the highest amine metabolizers expressed in MPVAT

MPVAT and the underlying artery-vein pair (Figure 18) were dissected from male Sprague-Dawley rats and analyzed for the expression of amine metabolizing enzyme genes. Relative *Maoa* expression in the MPVAT was similar to that in the brain (0.71±0.15 fold change; Figure 19A), the positive control for *Maoa* and *Maob* (Jahng et al., 1997). However, MRV expression of *Maoa* was significantly lower than that in the brain but not significantly lower than the MPVAT. *Maob* expression was significantly lower in both the MRV and in the MPVAT compared to the brain positive control (0.04±0.01 [MRV] and 0.07±0.01 [MPVAT] fold change; Figure 19B). When compared to the liver, a positive control tissue for *Comt* expression (Karhunen et al., 1994), MRV and MPVAT had low levels of *Comt* expression (less than a 0.01-fold change from liver for both; Figure 19C). By contrast, *Aoc3* was expressed at higher levels in the MPVAT (2.45±0.45 fold change; Figure 19D) *vs.* the MRV (0.48±0.01 fold change) when compared relative to the aorta, a positive control for *Aoc3* expression (Wanecq et al., 2006). The number of animals used for each sample type was five with the exception of the MRV (four).

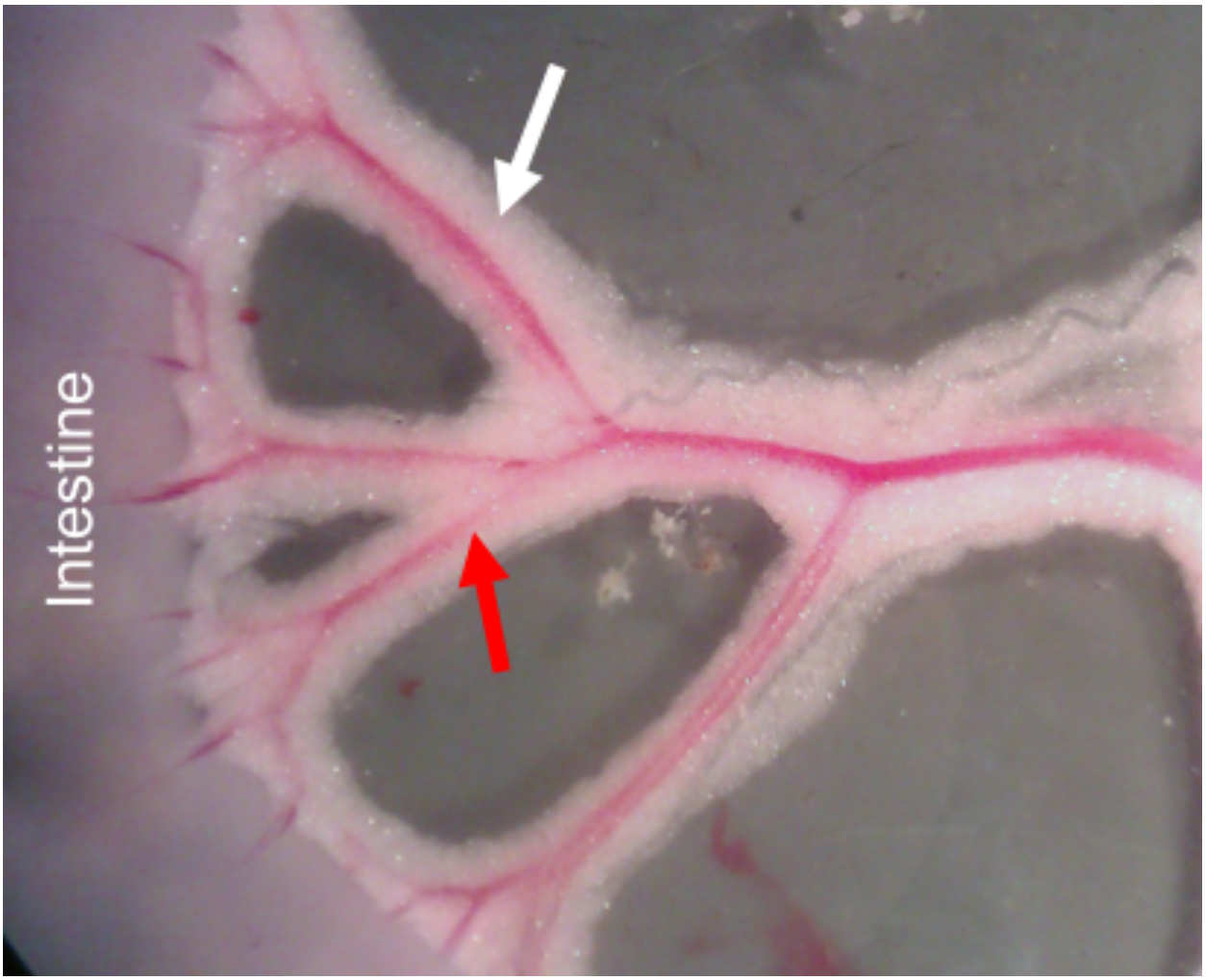


Figure 18. Image representing the rat mesenteric vessels and PVAT used in this study.

The location of the mesenteric resistance artery-vein pair used is indicated by the red arrow and their associated MPVAT is indicated by the white arrow.

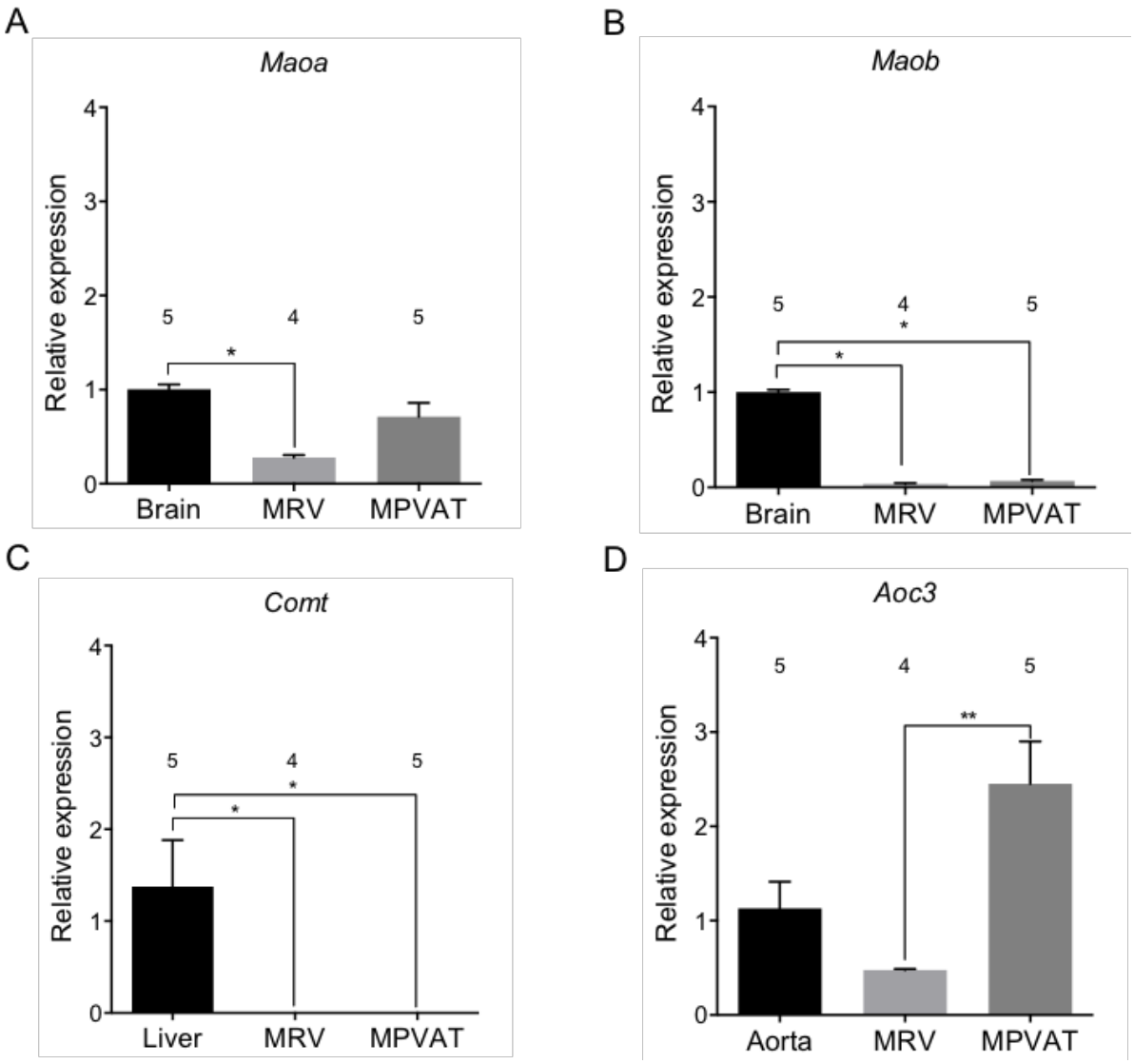


Figure 19. *Maoa* and *Aoc3* are highly expressed in rat mesenteric PVAT.

Amine oxidase gene expression of (A) *Maoa*, (B) *Maob*, (C) *Comt* and (D) *Aoc3* (gene for SSAO) in mesenteric resistance vessels (MRV) and in mesenteric PVAT (MPVAT) normalized to β-actin as the reference gene. Bars represent means±SEM. *p<0.05, **p<0.01 *vs.* positive control tissue. The number above each bar indicates the number of animals used.

MAO-A, MAO-B, and SSAO but not COMT protein is present in MRV and MPVAT

Western blot analysis of protein isolated from the MRV and associated MPVAT for N=4 rats, revealed presence of MAO-A, MAO-B and SSAO (Figure 20A). Consistent with there being no difference between the mRNA expression for *Maoa* in the MRV and MPVAT, there was no statistical difference in relative protein signal between the MRV and MPVAT ($p=0.07$; Figure 20A and B). MAO-B protein signal was present in both sample types but not consistently observed across all of our samples (Figure 20A). Only two out of the four protein samples had bands for MAO-B in the MRV and three out of the four protein samples had bands for MAO-B in the MPVAT. Densitometry reflected this in the size of the error for each sample type. There was no statistical difference between MAO-B presence in MRV and MPVAT ($p=0.25$, Figure 20C). MAO-B signal in the bands that were observed was low compared to the positive control, the stomach fundus. Bands for COMT were not detected in either the MRV or the MPVAT (Figure 20A and D). However, the positive control, Jurkat cell lysate, did have a band for COMT (Figure 20A). Signal for SSAO was consistently present in both the MRV and the MPVAT (Figure 20A). While *Aoc3* mRNA was significantly higher in the MPVAT compared to the MRV (Figure 20D), relative SSAO protein levels calculated by densitometry were not significantly different between the two sample types (Figure 20E, $p=0.08$). From this we conclude that COMT has little to no presence in MRV and in MPVAT while, MAO-B has variable presence in both the MRV and in MPVAT. On the other hand, MAO-A and SSAO are present in both the MRV and the MPVAT.

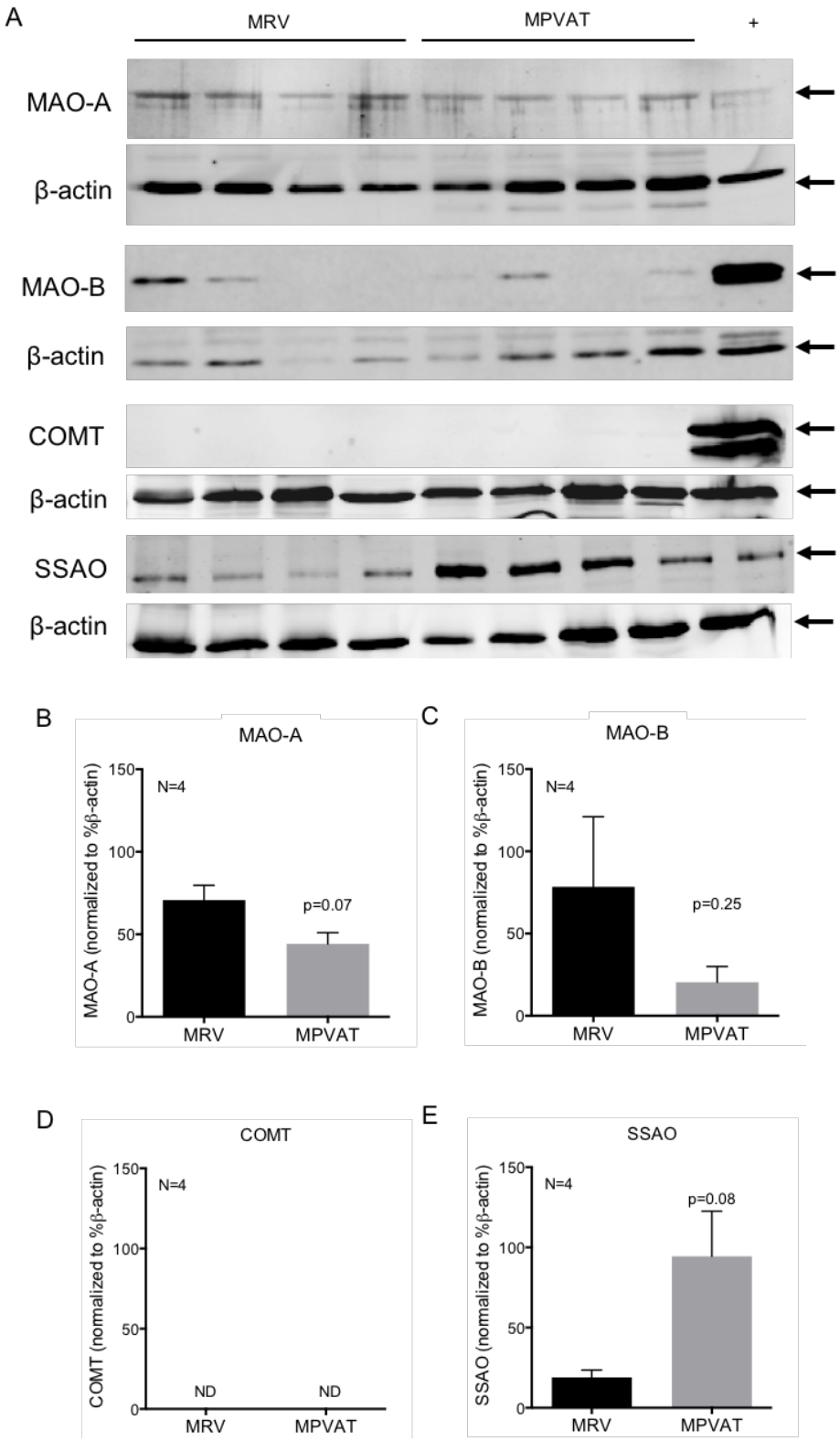


Figure 20. MAO-A, MAO-B and SSAO but not COMT are present in rat MRV and MPVAT.

Figure 20. (cont'd)

(A) Western blot analysis of metabolism enzymes MAO-A, MAO-B, COMT and SSAO in mesenteric resistance vessels (MRV) and in mesenteric PVAT (MPVAT) from four animals. Positive controls were gut mucosa for MAO-A, stomach fundus for MAO-B, Jurkat cells for COMT and aorta for SSAO (N=4 rats). Densitometry analysis of Western blot bands for (B) MAO-A, (C) MAO-B, (D) COMT, (E) SSAO. ND= bands not detected. Bars represent the means±SEM. Differences were not statistically significant. Thus, exact p-values were given.

SSAO mediates tyramine and benzylamine-induced amine oxidase activity in MPVAT

Oxidation of amines in the MRV and in the MPVAT could alter vascular tone through the removal of vasoactive amines and through the release of the reaction product H_2O_2. To quantify the amount of oxidase activity in these tissues, the MRV and MPVAT were carefully separated. The MRV samples contained both the artery and vein. MPVAT was split into its constituent fractions, the AF and the SVF. A microscopic image of the separated fractions is shown in Figure 21, which is represents all AF and SVF used in this study. Tyramine, a substrate for MAO-A, MAO-B and SSAO, was added to the sample extracts to drive oxidase activity. Addition of tyramine increased the oxidase activity (H_2O_2 produced) in the MRV by 3.10±0.81 fold, MPVAT by 2.56±0.18 fold, the AF by 2.24±0.22 fold, and the SVF by 3.58±0.91 fold compared to vehicle (H_2O) (Figure 22). The brain is known to contain both MAO-A and B activity but little SSAO activity (Castillo et al., 1999; Kalaria et al., 1988). Thus, this tissue was tested to serve as a positive control for the MAOs. The brain had 17.61±3.43 fold the oxidase activity in response to tyramine compared to vehicle. The aorta, the positive control for SSAO, had 2.68±0.39 fold the activity to tyramine compared to vehicle.

To determine which amine oxidase(s) was/were contributing to the tyramine-driven H_2O_2 production in the mesentery, the MRV, whole MPVAT, AF and the SVF were incubated with inhibitors to each of the amine oxidases (MAO-A, MAO-B and SSAO) before adding tyramine. Clorgyline, an irreversible inhibitor of MAO-A, was used due to its specificity to MAO-A [IC_{50} of 0.03 μM and 8 μM at MAO-B (Ozaita et al., 1997)] and lack of inhibition of SSAO (Clarke et al., 1982). Pargyline at a 1 μM concentration was used to specifically inhibit MAO-B (K_i 0.5 μM) and a higher concentration (10 μM) was

used to inhibit both MAO-A and B [K_i for pargyline at MAO-A= 15 μM (Fowler, Mantle, and Tipton 1982)]. Semicarbazide was used to specifically inhibit SSAO, at a concentration (1 mM) routinely used in monoamine assays to selectively inhibit SSAO activity (K_i 15 μM; Lizcano *et al.*, 1996; Repessé *et al.*, 2015). Oxidase activity was quantified as the amount of H_2O_2 produced per minute normalized to the amount of protein.

Tyramine-driven oxidase activity in the MRV was not reduced by the addition of clorgyline or pargyline (Figure 23A). There was some reduction with 1 mM semicarbazide (Figure 23A). However, the reduction was not statistically significant. On the other hand, semicarbazide significantly reduced (almost by 100%) the tyramine-driven oxidase activity in the MPVAT and the AF (Figures 23B and C).

Oxidase activity in the SVF was not significantly reduced by inhibition of the MAOs or SSAO (Figure 23D). However, with each inhibitor (clorgyline, pargyline, and semicarbazide) there were non-significant reductions in oxidase activity. There was not one enzyme that predominated in activity over the other. Thus, each amine oxidase (MAO-A, MAO-B, and SSAO) may contribute to oxidase activity in the SVF. The positive control for MAO, the brain, had reduced oxidase activity to clorgyline (1 μM- specific for MAO-A), pargyline (1 μM- specific for MAO and 10 μM- inhibits both MAO-A and MAO-B), but no reduction with the SSAO inhibitor semicarbazide (Figure 23E). These are the results that we were expecting for this type of tissue. Oxidase activity in the aorta, our positive control for SSAO, was abolished by 1 mM semicarbazide (Figure 23F). By contrast, there was no reduction in oxidase activity by clorgyline (1 μM) or pargyline (1

μM). These results for our controls validate that our assay can detect and distinguish between SSAO and MAO activity.

Benzylamine is another substrate that we used in the oxidase activity assays. Benzylamine differs from tyramine in that it is a substrate for MAO-B and SSAO only (and not MAO-A). Addition of benzylamine to MRV increased oxidase activity 2.44±0.13 fold compared to addition of vehicle (Figure 24). The MPVAT and the adipocyte fraction also had an increase in oxidase activity compared to vehicle (2.08±0.13 fold and 1.98±0.20 fold, respectively; Figure 24). However, benzylamine did not significantly increase the oxidase activity in the SVF compared to vehicle. Oxidase activity in both control tissues, the brain and aorta, was also increased with the addition of benzylamine by 9.33±2.16 and 2.04±0.15 fold, respectively (Figure 24). Amine oxidase activity in the MRV was abolished by the SSAO inhibitor, semicarbazide, and was reduced in the MPVAT and the AF (Figures 25A, B, and C). This is different from what was observed with tyramine, where we observed a reduction that was not significant in tyramine-driven oxidase activity in the MRV with semicarbazide. Tyramine is a poor substrate for SSAO compared to benzylamine with a K_m of 17.6 mM. Whereas, the K_m of benzylamine for SSAO is 161 μM (Precious and Lyles, 1988). Thus, a small amount of the tyramine-driven oxidase activity could have been due to other amine oxidases other than SSAO. The SVF amine oxidase activity was not significantly reduced by any of the inhibitors. However, there was some reduction in oxidase activity with 1 mM semicarbazide ($p=0.09$). The brain, the positive control for MAO-B, contained oxidase activity that was completely abolished by inhibition of MAO-B, but was not affected by inhibition of SSAO (Figure 25E). Furthermore, the positive control for SSAO, the aorta (Figure 25F) was not affected by MAO-B inhibitor,

pargyline. These results indicate that the oxidase activity with benzylamine as a substrate can detect and distinguish between MAO-B and SSAO activity.

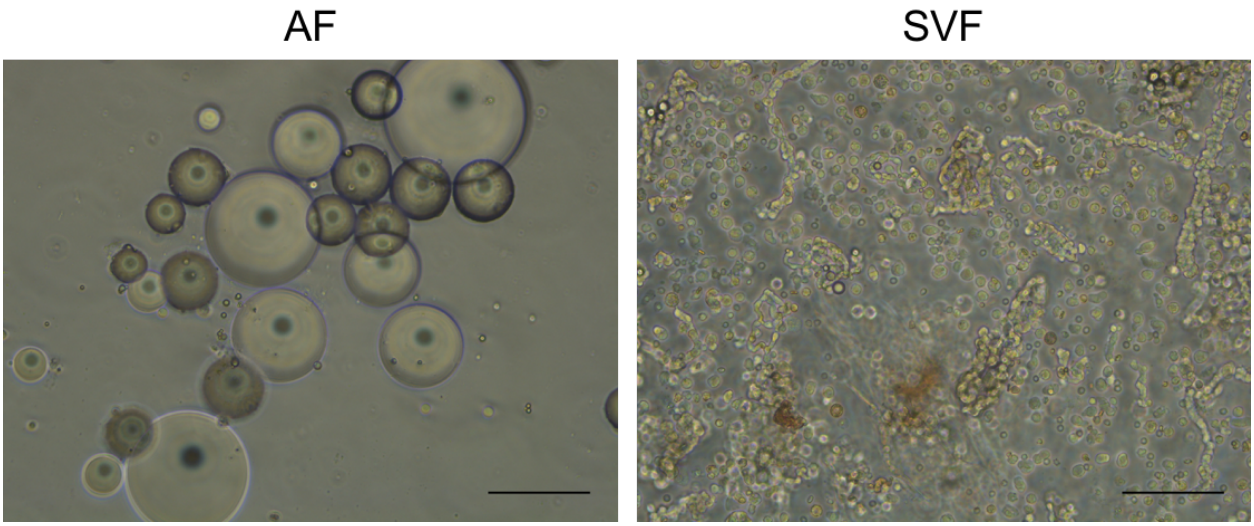


Figure 21. Image representing the AF and the SVF isolated from whole MPVAT. Representative of the following experiments where the AF (left) and SVF (right) were used. Phase contrast images were taken with a 20X objective. The black scale bar= 100 μM.

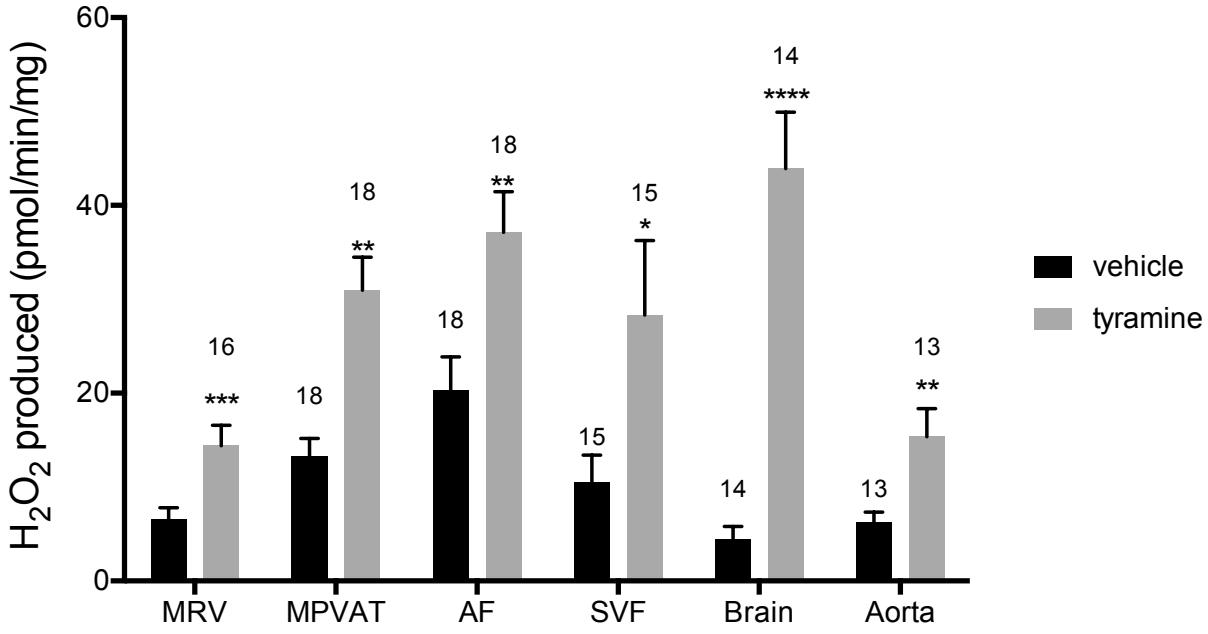


Figure 22. Tyramine drives amine oxidase activity in MPVAT and the MRV.

Tyramine, a substrate for amine oxidases, increased the oxidase activity in the mesenteric resistance vessels (MRV), mesenteric PVAT (MPVAT), adipocyte fraction (AF), and the stromal-vascular fraction (SVF). The brain and aorta were also tested as positive controls. Bars represent the means±SEM for the number of animals indicated above each bar. *p<0.05, **p<0.01, ****p<0.001 *vs.* control of that same sample type.

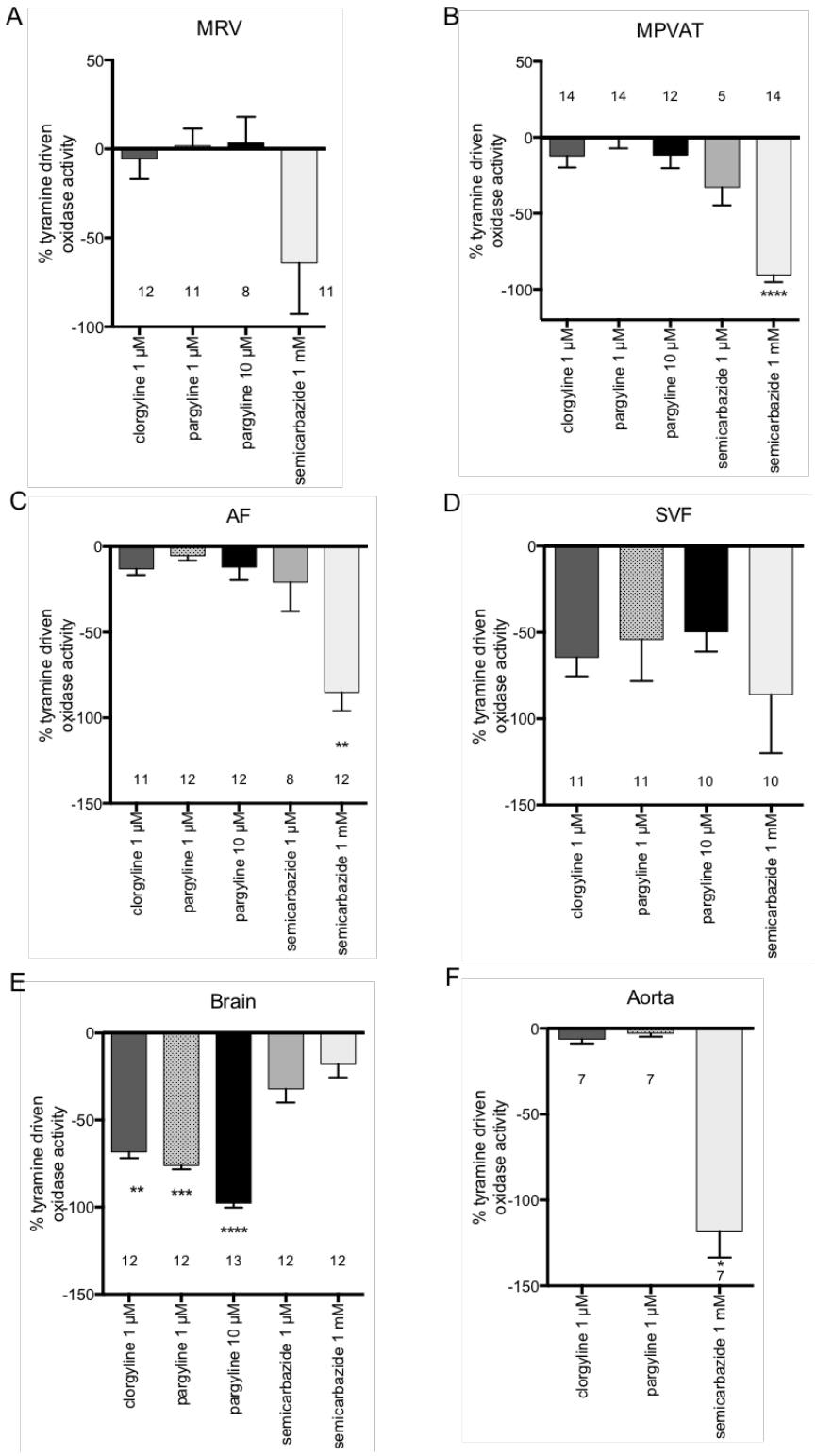


Figure 23. SSAO mediates tyramine-driven amine oxidase activity in the MPVAT, AF but not in the SVF or the MRV.

Figure 23. (cont'd)

Percent inhibition of tyramine driven oxidase activity by pharmacological inhibitors of MAO-A (1 μM clorgyline), MAO-B (1 μM pargyline), both MAO-A and MAO-B (10 μM pargyline) or SSAO (1 mM semicarbazide) in the (A) mesenteric resistance vessels (MRV), (B) mesenteric PVAT (MPVAT), (C) adipocyte fraction (AF), (D) stromal vascular fraction (SVF), and the positive controls: (E) brain (positive control for MAO-A/B), and (F) aorta (positive control for SSAO). Bars represent the means±SEM for the number of animals indicated near each bar. *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs.* vehicle control (no inhibition).

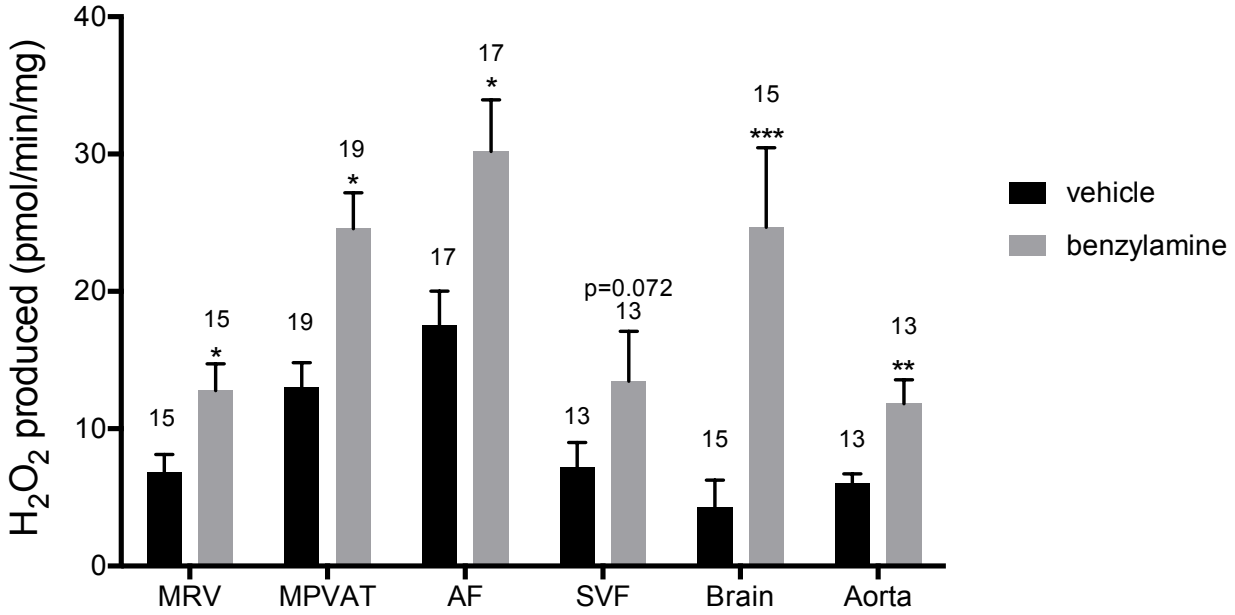


Figure 24. Benzylamine drives amine oxidase activity in MPVAT, AF, MRV but not the SVF.

MPVAT= mesenteric PVAT, AF= adipocyte fraction, MRV= mesenteric resistance vessels, SVF= stromal vascular fraction. The brain and aorta were also tested as positive controls. Bars represent the means±SEM for the number of animals indicated above each bar. *p<0.05, **p<0.01, ****p<0.001 *vs.* control of that same sample type.

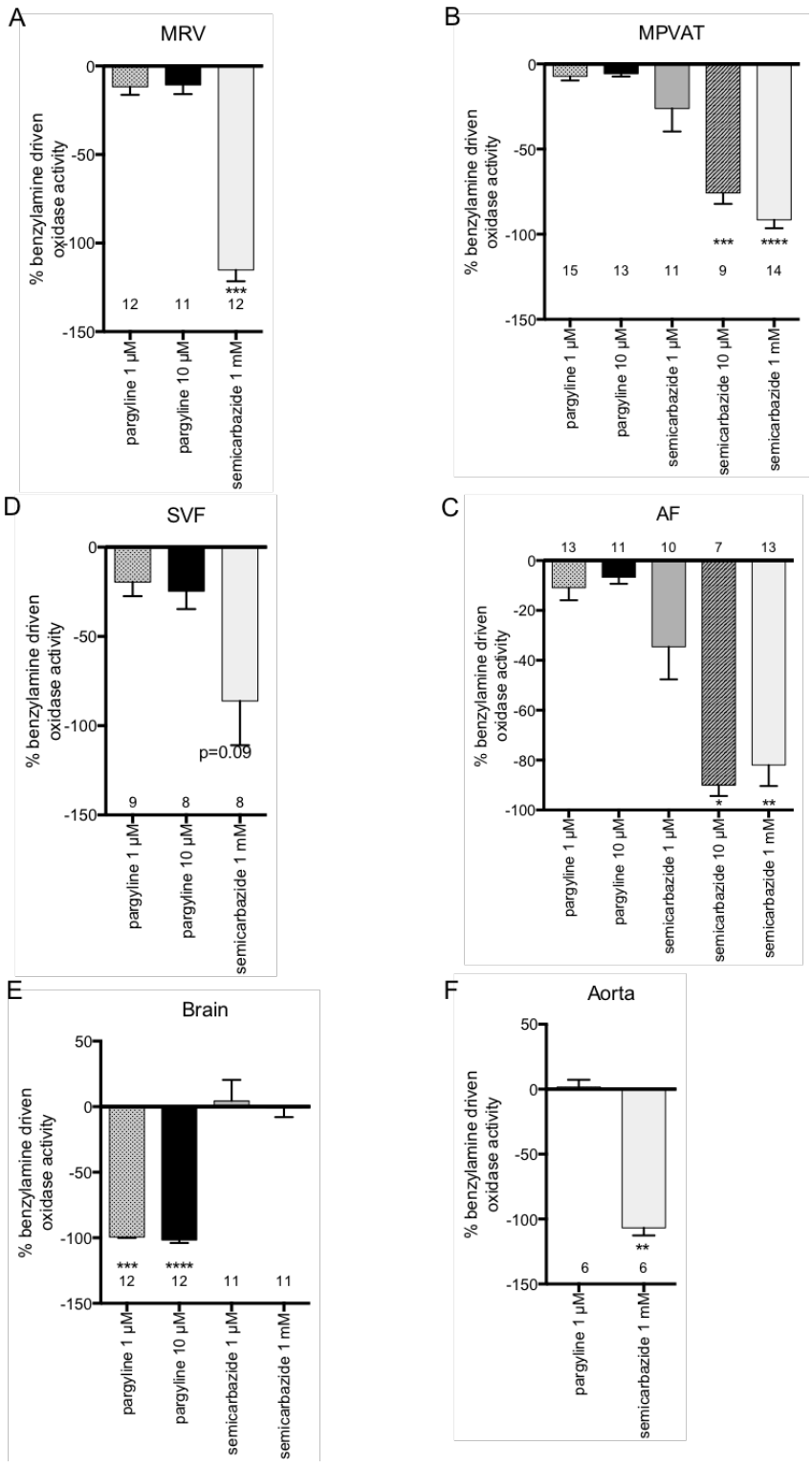


Figure 25. SSAO mediates benzylamine-driven amine oxidase activity in the MRV, MPVAT, and the AF but not in the SVF.

Figure 25. (cont'd)

Percent inhibition of benzylamine driven oxidase activity by pharmacological inhibitors of MAO-B (1 μM pargyline), both MAO-A and MAO-B (10 μM pargyline) or SSAO (1 mM semicarbazide) in the (A) mesenteric resistance vessels (MRV), (B) mesenteric PVAT (MPVAT), (C) adipocyte fraction (AF), (D) stromal vascular fraction (SVF), and the positive controls: (E) brain (positive control for MAO-A/B), and (F) aorta (positive control for SSAO). Bars represent the means±SEM for the number of animals indicated above each bar. *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs.* vehicle control (no inhibition).

NE metabolism contributes to the anti-contractile effect of PVAT

Third-order mesenteric resistance arteries with (+) or without (-) PVAT were mounted on a four-channel wire myograph and incubated with either vehicle or an inhibitor prior to the generation of a NE concentration-response curve. The presence of PVAT caused a right-shift of the NE-response curve (N=24) (Figure 26A). NE had a -logEC_{50} [M] of 5.64±0.05 in mesenteric resistance arteries cleaned of PVAT (-PVAT with vehicle) and a -logEC_{50} [M] of 5.08±0.06 in arteries with PVAT (+PVAT with vehicle). Individual inhibition of MAO-A and B by 10 μM pargyline (Figure 26B), COMT by 1 μM Ro 41-0960 (Figure 26C), or SSAO by 1 mM semicarbazide (Figure 26D) did not cause a shift in the concentration-response curve of arteries with or without PVAT compared to vehicle. However, a left-shift of the +PVAT curve was observed with incubation with semicarbazide + 10 μM pargyline (SP) *vs.* vehicle (Figure 26E), or with all three inhibitors (SPR= 1 mM semicarbazide + 10 μM pargyline + 1 μM Ro 41-0960; Figure 26F) suggesting that multiple NE metabolizers contribute to PVAT's anti-contractile effect on mesenteric resistance arteries to NE. The pharmacological parameters for Figures 26B-F are listed in Table 2.

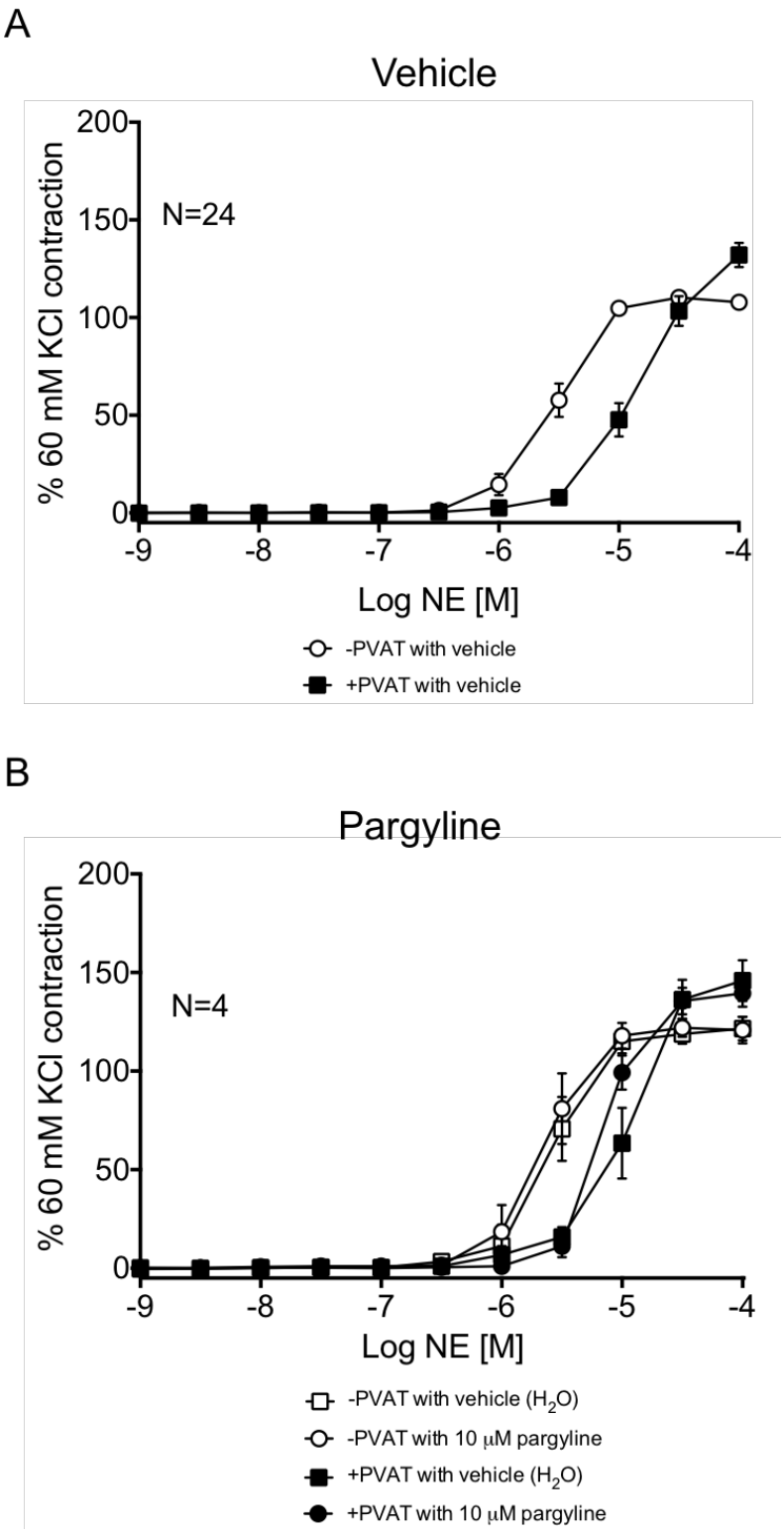
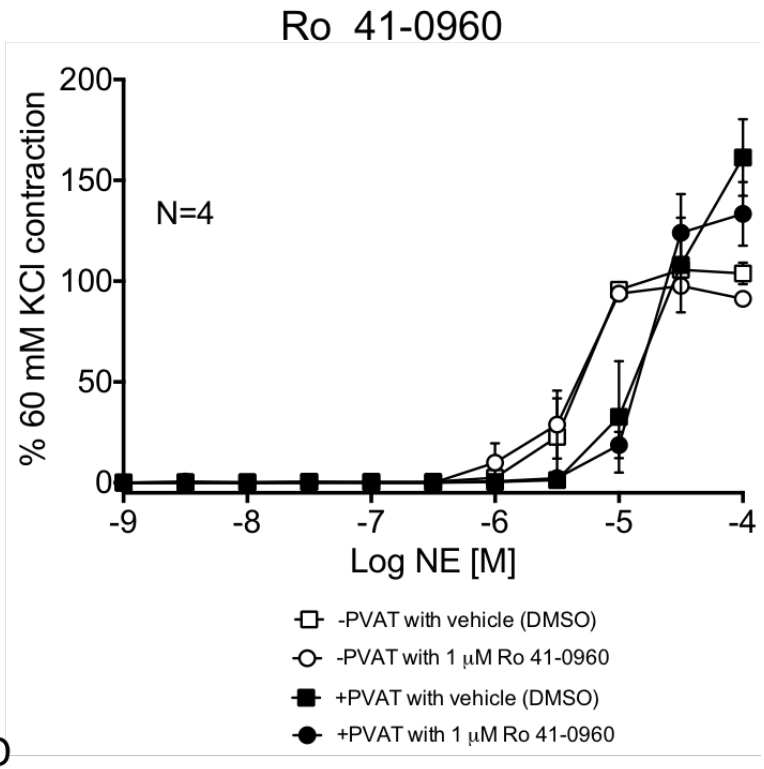


Figure 26. Metabolism of NE by PVAT reduces contraction of rat mesenteric resistance arteries.

Figure 26. (cont'd)

C



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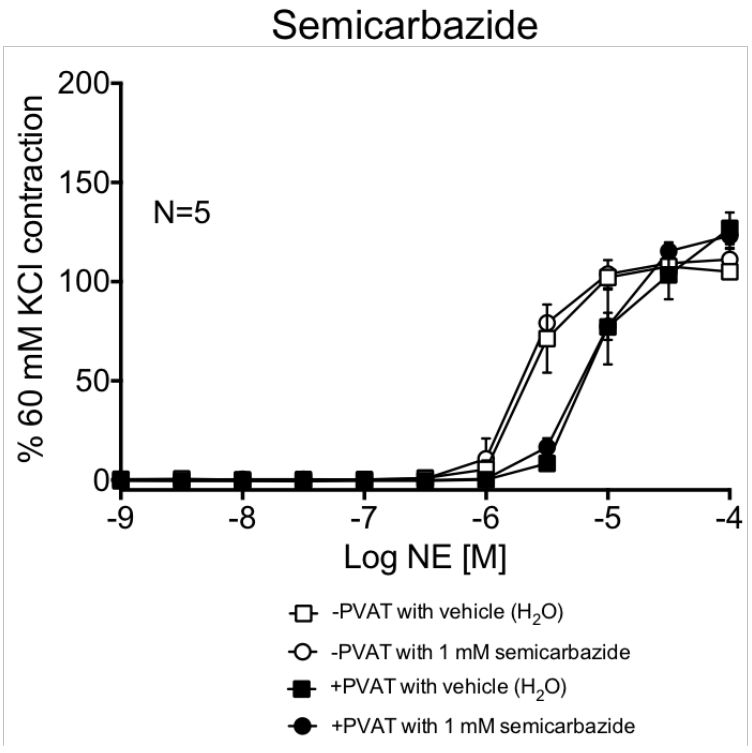
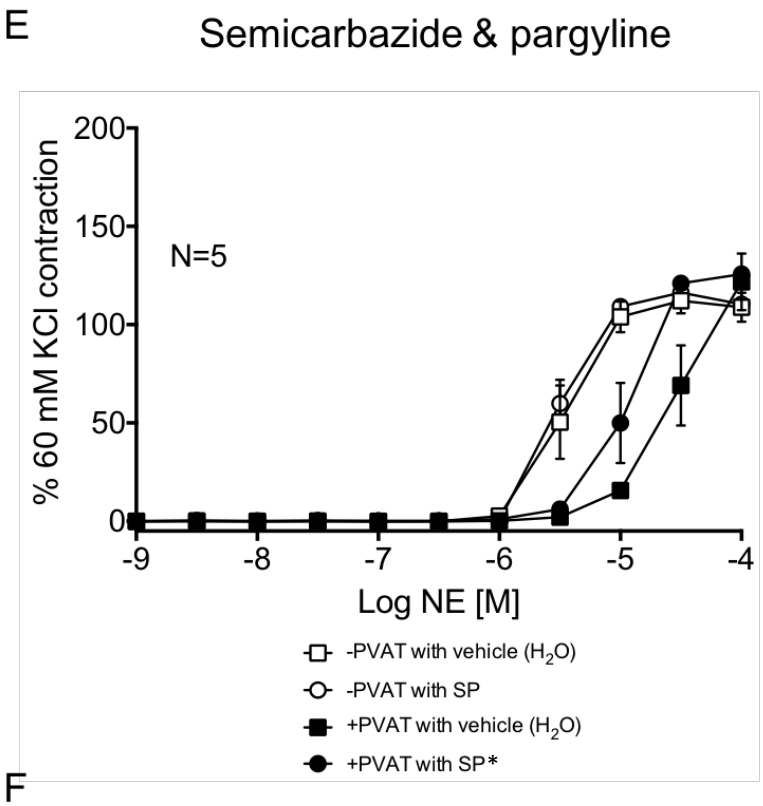


Figure 26. (cont'd)

E



F

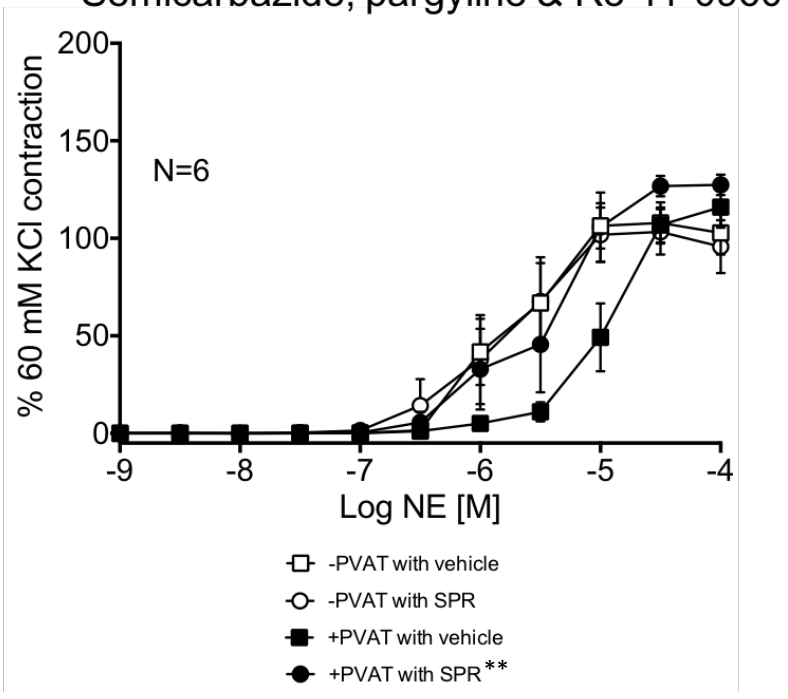


Figure 26. (cont'd)

NE-induced contraction of the rat mesenteric resistance arteries with or without PVAT and with or without (vehicle) the following inhibitors of NE metabolism. (A) Contraction of MRV with (+) and without (-) PVAT to NE. NE curves after an hour incubation with (B) 10 μM pargyline (inhibits MAO-A and B), (C) 1 μM Ro 41-0960 (inhibits COMT), (D) 1 mM semicarbazide (inhibits SSAO), (E) SP= 1 mM semicarbazide and 10 μM pargyline (used to inhibit both MAO-A/B and SSAO), (F) SPR= 1 mM semicarbazide, 10 μM pargyline and 1 μM Ro 41-0960 (used in combination to inhibit all possible metabolizers of NE). Force of contraction was normalized to the percent of 60 mM KCl contraction. Bars represent means±SEM. N= the number of animals used in each group. *$p<0.05$, **$p<0.01$ *vs.* +PVAT with vehicle control (no inhibition).

PVAT	Inhibitor	-LogEC$_{50}$ (M)	Max Contraction %60 mM KCl
-	vehicle	5.74±0.11	121.51±5.98
-	10 μM pargyline	5.83±0.12	120.82±6.73
+	vehicle	5.31±0.15	145.90±10.40
+	10 μM pargyline	5.40±0.15	139.47±6.84
-	vehicle	5.36±0.10	103.88±5.33
-	1 μM Ro 41-0960	5.38±0.08	91.23±2.57**
+	vehicle	5.04±0.19	161.29±19.02
+	1 μM Ro 41-0960	5.00±0.15	133.38±15.85
-	vehicle	5.66±0.09	105.03±3.57
-	1 mM semicarbazide	5.73±0.08	111.17±5.42
+	vehicle	5.22±0.11	126.92±8.00
+	1 mM semicarbazide	5.29±0.09	123.13±6.05
-	vehicle	5.55±0.10	108.83±7.34
-	SP	5.61±0.09	110.35±3.20
+	vehicle	4.73±0.11	121.78±14.43
+	SP	5.13±0.11 *	125.70±2.56
-	vehicle	5.83±0.11	102.80±11.16
-	SPR	5.84±0.13	95.77±13.53
+	vehicle	5.10±0.09	115.96±10.62
+	SPR	5.71±0.14 **	127.47±5.28

Table 2. Pharmacological parameters of isolated mesenteric resistance arteries with (+) or without (-) PVAT.

Points represent mean±SEM for values calculated from figures presented in Figure 8. *p<0.05, **p<0.01 *vs.* +PVAT with vehicle response.

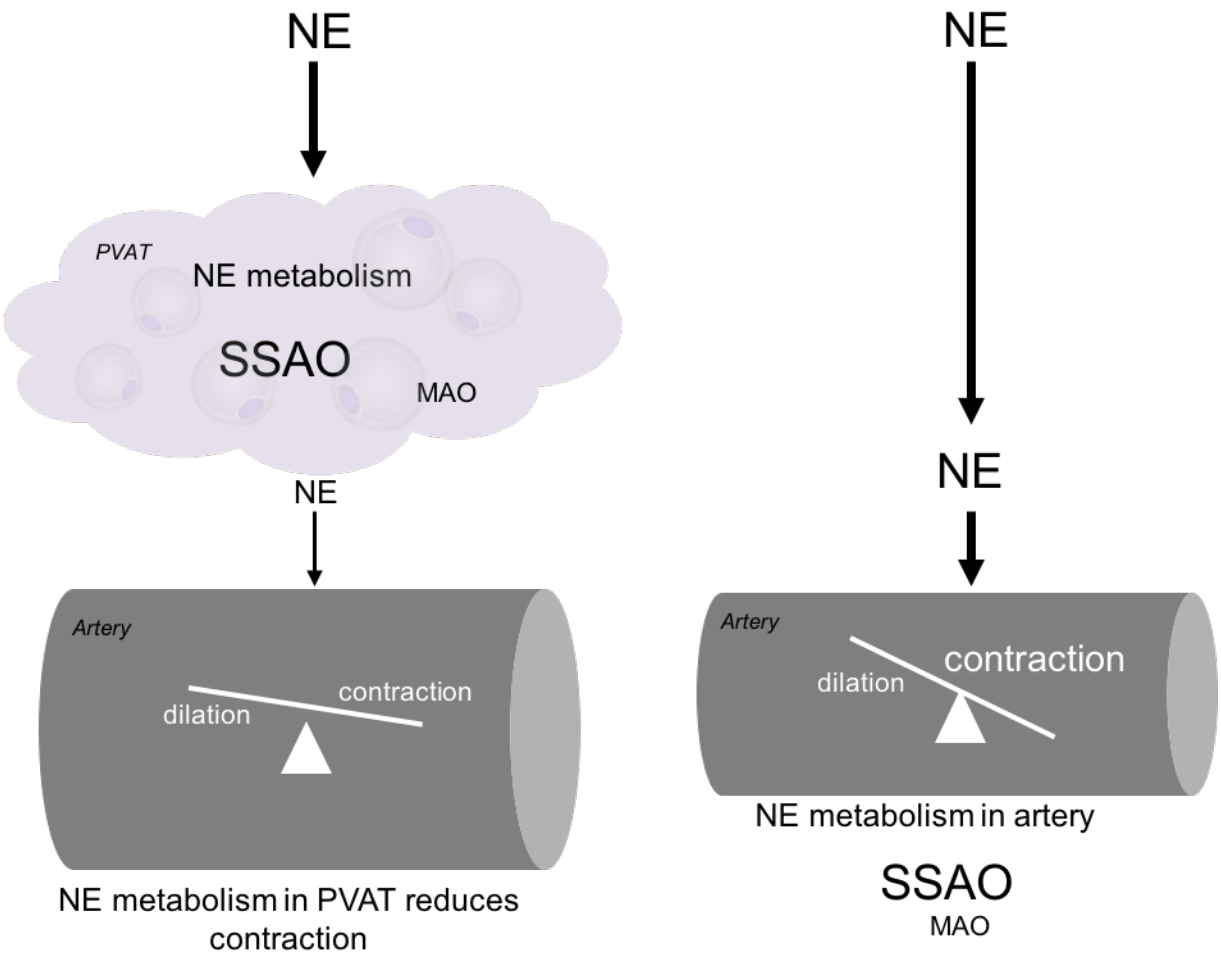


Figure 27. Diagram of NE handling in PVAT.

The hypothesized mechanism for handling of NE by MPVAT and the MRV is shown above. PVAT contributes to metabolism of NE from the adventitial side of the artery. Without PVAT, more NE reaches the artery to cause contraction.

Discussion

PVAT contains releasable catecholamines as well as a system for uptake of NE (Ayala-Lopez et al., 2014, 2015). However, a mechanism for metabolism for amines in PVAT has not been investigated. This study demonstrates that the amine oxidase SSAO is present and highly active in rat MPVAT and MPVAT adipocytes. We also demonstrated that amine metabolism contributes to the protective effect of PVAT.

Adipocytes in human abdominal and mammary white adipose tissue (WAT) contain both MAO-A and MAO-B (Pizzinat et al., 1999). In our study, we also observed expression of mRNA and presence of protein for MAO-A in MPVAT. However, the expression of *Maob* was low and the presence of MAO-B protein in whole MPVAT was not consistently present. This could be that the MAO-B is found in nerves or other cell types and the amount of these cell types was different within each portion of the tissue studied from each animal. In addition to *Maoa*, *Aoc3* was highly expressed in MPVAT. This is consistent with our observation that SSAO protein is present in MPVAT. However, more expression of *Aoc3* was present in MPVAT compared to the MRV but the amount of protein for SSAO was not different between the two tissues. Presence of SSAO in MPVAT is consistent with previous findings that SSAO is highly expressed in adipocytes and has several roles such as adipogenesis (Mercier et al., 2001). We also located SSAO in the MRV. In vascular smooth muscle cells, SSAO has roles in differentiation and the activation of glucose transport (El Hadri et al., 2002).

We used antibodies already validated or took steps to validate the antibodies. The MAO-A antibody had been previously validated by our lab (Linder et al., 2008). MAO-A was present in both the MRV and the MPVAT. The bands located on the Western blots

for MAO-B were verified by including a competing peptide in a validation experiment (data not shown). We were not able to use this approach to validate the band for COMT because a competing peptide for the COMT antibody used was not commercially available. However, the Western data was consistent with low expression levels of mRNA detected in the PCR reaction. We located the appropriate band of interest for SSAO on the Western blot after including a competing peptide in a validation experiment (data not shown).

We previously localized the organic cation transporter 3 (OCT3) to the PVAT adipocyte (Ayala-Lopez et al., 2015) and identified OCT3 as a functional mechanism for adipocytes to take up NE. MAOs are present intracellularly. Thus, if MAO-A is involved in the metabolism of amines in MPVAT, access to MAO would require an internalization of the amines from the extracellular environment because the OCT3 is one way that NE can enter adipocytes to be metabolized. Therefore, the substrates necessary for amine oxidases have the potential to be present intracellularly where they can interact with and be readily metabolized by MAO. Pizzinat et al. (1999) suggested that the uptake-2 system (OCT3) in adipocytes would take up NE to be inactivated by MAO (Pizzinat et al., 1999). However, MAO activity in MPVAT was low in our study. This may be due to substrate specificity differences across species and/or difference adipose depots. SSAO, on the other hand, is present on the plasma membrane of adipocytes where it is shed in a process that is regulated by TNF-alpha and insulin (Abella et al., 2004). Entrance of catecholamines into adipocytes would not be required for SSAO action. Thus, metabolism of NE in PVAT may occur through transport-dependent and independent processes.

Although, MAO-A is present in MPVAT, and has been previously reported to be a potential amine metabolizer in adipose tissue, functionally SSAO was more active to the substrates used in our assay. Previously, Bour et al (2007) reported high MAO-A and SSAO activity in the AF from human subcutaneous adipose tissue (Bour et al., 2007) and Stock and Westermann (1963) reported monoamine oxidase activity in rat epididymal adipose tissue. However, studies in PVAT specifically had not been performed. In the present study, we compared the amine oxidase activity from the MRV and the MPVAT to attain a whole picture of oxidase activity in the vascular environment. SSAO was the predominant oxidase enzyme active in MPVAT as evidenced by a drastic reduction in oxidase activity upon SSAO inhibition. Surprisingly, we did not detect MAO-A activity in MPVAT although MAO-A protein and its mRNA was detected. One explanation for this is that the MAO-A activity in MPVAT was low compared to the high SSAO activity and fell below our level of detection since we loaded a small amount of protein as to not go over the limit of detection based on our standard curve. Thus, the high SSAO activity could have masked lower monoamine oxidase activity. The inclusion of the brain and aorta as controls provided us with assurance that our assay could in fact detect MAO and SSAO activity. SSAO activity in the brain is low and restricted to the microvessels (Castillo et al., 1999), allowing its use as a negative control for SSAO. Our assay detected MAO-A and MAO-B activity in the brain but did not detect SSAO activity. The oxidase assay detected SSAO activity in the positive control for SSAO, the aorta. MAO-A and MAO-B activity in the aorta was not detected. Low or no MAO activity in the aorta was expected as Sturza et al. (2013) were able to measure MAO-A activity in the aorta only after it was induced

by LPS or angiotensin II pre-treatment. It may be that induction of MAO-A activity must occur to observe appreciable MAO-A-dependent H_2O_2 production.

Due to the close proximity of the artery and vein in the mesenteric third order branches, both the artery and vein were used in the oxidase assays. Thus, our results of these assays inform us of the contribution of both vessels to oxidase activity. However, in the isometric contraction experiments, we used only arteries and not veins. A limitation to our findings are that the two substrates used in the oxidase activity assay, tyramine and benzylamine, are not the likely endogenous substrates for amine oxidases in PVAT. However, these substrates were used as they are well characterized substrates for MAO and SSAO allowing us to come to more accurate conclusions as to which enzymes are active. Aminoacetone and methylamine are the proposed endogenous substrates for the human SSAO (Lyles 1995). Other candidates for endogenous substrates include 5-HT and NE. Both NE and 5-HT have been found to be substrates of SSAO in rat brown adipose tissue (Barrand and Callingham, 1982). However, this has not yet been directly tested using PVAT. Unfortunately, the possible substrate that we are interested in for its vasoactive effects, NE, could not be used in the oxidase assay because of its quick oxidization by the reagent in the Amplex® Red assay (Elliott et al., 1989b; Zhao et al., 2012). Thus, one of the limitations in our study is that observed amine oxidase activities to the substrates tested may be different than the activity to endogenous substrates, especially when compared across species and adipose depots.

Another possibility is that degradation of amines by oxidation occurred by other enzymes not tested, especially in the MRV and the SVF where we were less successful at significantly inhibiting the oxidase activity with the specific inhibitors used. For example,

ceruloplasmin, an adipokine that is increased in the adipose tissue of obese humans (Arner et al., 2014), has broad specificity for degrading various amines including NE, epinephrine, dihydroxyphenylalanine (DOPA) and 5-HT (Gutteridge and Stocks, 1981).

Amine metabolism in isolated rat mesenteric arteries has been found to mostly be through SSAO, with less involvement from MAO-A (Elliott et al., 1989a). Elliott, Callingham, and Sharman (1989c) studied the effect of amine metabolism on tyramine-induced contraction of rat mesenteric arteries. They observed that inhibiting MAO or SSAO individually (with clorgyline and MDL 72145, respectively) did not cause a shift in the tyramine response curve. However, a shift was achieved upon blocking both MAO and SSAO. In our studies, with NE as the agonist opposed to tyramine, we did not observe a shift in the NE-response curves of mesenteric arteries cleaned of PVAT whether we individually inhibited MAO or SSAO or inhibited them both. However, arteries with PVAT still attached demonstrated a left-shift to NE when both oxidases (MAO and SSAO) were inhibited, supporting the presence of a redundant system for inactivating vasoactive amines. A more pronounced shift was achieved when MAO, SSAO and COMT were inhibited suggesting a possible role for COMT in NE metabolism in PVAT. This is in contrast to our Western and PCR data indicating low COMT presence and gene expression when both the arteries and veins were assayed together. One possibility for this is that COMT is present in the arteries and is less so in the veins. Combining the two like we did (MRV) could have lowered the overall content of COMT compared to total MRV RNA and protein content. The arteries +PVAT exposed to vehicle in the experiment with both semicarbazide and pargyline (Figure 26E) had a -logEC_{50} lower than the arteries +PVAT exposed to vehicle in the experiment with all three inhibitors (semicarbazide,

pargyline and Ro 41-0960; 4.73±0.11 *vs.* 5.10±0.09). Thus, this far shift to the right in the arteries +PVAT with this particular set of animals may have been more difficult to overcome with the inhibitors. We conclude that SSAO and to a lesser extent, MAO, are the main contributors to amine metabolism in MPVAT and the MRV.

Our proposed hypothesis is illustrated as a diagram in Figure 27. On the left, an artery with PVAT is exposed to NE. The NE subsequently is metabolized in the PVAT by the high SSAO activity and possibly by MAO. A small amount of NE reaches the artery relative to the amount of NE originally released and less contraction of the artery occurs. On the right, an artery with the PVAT removed is exposed to NE. The full amount of NE reaches the artery to cause contraction. A small amount of NE may be metabolized within the artery by SSAO and possibly by MAO but this is not enough to reduce contraction.

An alternative explanation for how inhibition of both MAO-A and SSAO could increase contraction to NE is through the production of H_2O_2, which limits endothelial NO formation and reduces endothelium-dependent relaxation (Sturza et al., 2013). On the other hand, metabolism of 5-HT by MAO in the rat basilar artery was shown to lead to H_2O_2 generation, which by opening BK_{Ca2+} channels, potentiated contraction (Poon et al., 2010). The effect that H_2O_2 generation resulting from NE oxidation has on PVAT's anti-contractile effect remains to be investigated.

SSAO is being investigated due to its importance in inflammation and its utility as a biomarker for atherosclerosis (Karadi et al., 2002; Mészáros et al., 1999). Besides its function in degrading vasoactive amines, SSAO activity in MPVAT may also influence vascular inflammation and damage. Both SSAO and MAO activity are high in mice and dogs fed high fat diets compared to normal fat diet (Visentin et al., 2005; Wanecq et al.,

2006). In obesity, high SSAO and MAO activity in the vicinity of the vessel could have effects on vascular contraction as well as more chronic effects of vascular remodeling. SSAO and MAO expression is increased during adipogenesis (Bour et al., 2007). By contrast, adipose tissue MAO activity was decreased in abdominal subcutaneous biopsies from obese men compared to non-obese men. However, SSAO activity was unchanged (Visentin et al., 2004). Investigation into SSAO and MAO activity in PVAT during obesity in necessary.

Conclusion

The MRV and MPVAT with its corresponding AF and SVF contain amine oxidase activity. Tyramine and benzylamine addition increases in H_2O_2 production in MPVAT and the AF through SSAO. NE metabolism in PVAT decreases the contraction response to NE in rat mesenteric resistance arteries. Thus, amine metabolism in PVAT protects arteries from contraction to NE. Further studies are necessary to understand how this system functions in human PVAT and how it may be altered in diseases such as obesity where adipocyte dysfunction occurs and adipose inflammation is increased.

REFERENCES

REFERENCES

Abella, A., Garcia-Vicente, S., Viguerie, N., Ros-Baro, A., Camps, M., Palacin, M., et al. (2004). Adipocytes release a soluble form of VAP-1/SSAO by a metalloprotease-dependent process and in a regulated manner. *Diabetologia* 47, 429–438.

Aghamohammadzadeh, R., and Heagerty, A. M. (2012). Obesity-related hypertension: epidemiology, pathophysiology, treatments, and the contribution of perivascular adipose tissue. *Ann Med* 44 Suppl 1, S74-84.

Arner, E., Forrest, A. R. R., Ehrlund, A., Mejhert, N., Itoh, M., Kawaji, H., et al. (2014). Ceruloplasmin is a novel adipokine which is overexpressed in adipose tissue of obese subjects and in obesity-associated cancer cells. *PLoS One* 9, e80274. doi:10.1371/journal.pone.0080274.

Ayala-Lopez, N., Jackson, W. F., Burnett, R., Wilson, J. N., Thompson, J. M., and Watts, S. W. (2015). Organic cation transporter 3 contributes to norepinephrine uptake into perivascular adipose tissue. *Am J Physiol - Hear Circ Physiol* 309, H1904–H1914. doi:10.1152/ajpheart.00308.2015.

Ayala-Lopez, N., Martini, M., Jackson, W. F., Darios, E., Burnett, R., Seitz, B., et al. (2014). Perivascular adipose tissue contains functional catecholamines. *Pharmacol Res Perspect* 2, e00041. doi:10.1002/prp2.41.

Barrand, M. A., and Callingham, B. A. (1982). Monoamine oxidase activities in brown adipose tissue of the rat: some properties and subcellular distribution. *Biochem Pharmacol* 31, 2177–84.

Belfrage, E., Fredholm, B. B., and Rosell, S. (1977). Effect of catechol-O-methyl-transferase (COMT) inhibition on the vascular and metabolic responses to noradrenaline, isoprenaline and sympathetic nerve stimulation in canine subcutaneous adipose tissue. *Naunyn Schmiedebergs Arch Pharmacol* 300, 11–7.

Bour, S., Daviaud, D., Gres, S., Lefort, C., Prévot, D., Zorzano, A., et al. (2007). Adipogenesis-related increase of semicarbazide-sensitive amine oxidase and monoamine oxidase in human adipocytes. *Biochimie* 89, 916–925. doi:10.1016/j.biochi.2007.02.013.

Brandes, R. P. (2007). The fatter the better? Perivascular adipose tissue attenuates vascular contraction through different mechanisms. *Br J Pharmacol* 151, 303–304. doi:10.1038/sj.bjp.0707229.

Castillo, V., Lizcano, J. M., and Unzeta, M. (1999). Presence of SSAO in human and bovine meninges and microvessels. *Neurobiol* 7, 263–272.

Cinti, S. (2012). The adipose organ at a glance. 594, 588–594.

doi:10.1242/dmm.009662.

Clarke, D. E., Lyles, G. A., and Callingham, B. A. (1982). A comparison of cardiac and vascular clorgyline-resistant amine oxidase and monoamine oxidase: Inhibition by amphetamine, mexiletine and other drugs. *Biochem Pharmacol* 31, 27–35. doi:http://dx.doi.org/10.1016/0006-2952(82)90231-3.

Elliott, J., Callingham, B. A., and Sharman, D. F. (1989a). Metabolism of amines in the isolated perfused mesenteric arterial bed of the rat. *Br J Pharmacol* 98, 507–14.

Elliott, J., Callingham, B. A., and Sharman, D. F. (1989b). Semicarbazide-sensitive amine oxidase (SSAO) of the rat aorta. Interactions with some naturally occurring amines and their structural analogues. *Biochem Pharmacol* 38, 1507–1515.

Elliott, J., Callingham, B. A., and Sharman, D. F. (1989c). The influence of amine metabolizing enzymes on the pharmacology of tyramine in the isolated perfused mesenteric arterial bed of the rat. *Br J Pharmacol* 98, 515–22.

Fesus, G., Dubrovska, G., Gorzelniak, K., Kluge, R., Huang, Y., Luft, F. C., et al. (2007). Adiponectin is a novel humoral vasodilator. *Cardiovasc Res* 75, 719–727. doi:10.1016/j.cardiores.2007.05.025.

Fowler, C. J., Mantle, T. J., and Tipton, K. F. (1982). The nature of the inhibition of rat liver monoamine oxidase types A and B by the acetylenic inhibitors clorgyline, l-deprenyl and pargyline. *Biochem Pharmacol* 31, 3555–61.

Gao, Y.-J. J., Takemori, K., Su, L.-Y. Y., An, W.-S. S., Lu, C., Sharma, A. M., et al. (2006). Perivascular adipose tissue promotes vasoconstriction: the role of superoxide anion. *Cardiovasc Res* 71, 363–373. doi:10.1016/j.cardiores.2006.03.013.

Gutteridge, J. M., and Stocks, J. (1981). Caeruloplasmin: physiological and pathological perspectives. *Crit Rev Clin Lab Sci* 14, 257–329. doi:10.3109/10408368109105866.

El Hadri, K., Moldes, M., Mercier, N., Andreani, M., Pairault, J., and Feve, B. (2002). Semicarbazide-sensitive amine oxidase in vascular smooth muscle cells: differentiation-dependent expression and role in glucose uptake. *Arterioscler Thromb Vasc Biol* 22, 89–94.

Hall, J. E., da Silva, A. A., do Carmo, J. M., Dubinion, J., Hamza, S., Munusamy, S., et al. (2010). Obesity-induced hypertension: role of sympathetic nervous system, leptin, and melanocortins. *J Biol Chem* 285, 17271–6. doi:10.1074/jbc.R110.113175.

Jahng, J. W., Houpt, T. A., Wessel, T. C., Chen, K., Shih, J. C., and Joh, T. H. (1997). Localization of monoamine oxidase A and B mRNA in the rat brain by in situ hybridization. *Synapse* 25, 30–6. doi:10.1002/(SICI)1098-2396(199701)25:1<30::AID-SYN4>3.0.CO;2-G.

Kalaria, R. N., Mitchell, M. J., and Harik, S. I. (1988). Monoamine oxidases of the

human brain and liver. *Brain*, 1441–51.

Karadi, I., Meszaros, Z., Csanyi, A., Szombathy, T., Hosszufalusi, N., Romics, L., et al. (2002). Serum semicarbazide-sensitive amine oxidase (SSAO) activity is an independent marker of carotid atherosclerosis. *Clin Chim Acta* 323, 139–146.

Karhunen, T., Tilgmann, C., Ulmanen, I., Julkunen, I., and Panula, P. (1994). Distribution of catechol-O-methyltransferase enzyme in rat tissues. *J Histochem Cytochem* 42, 1079–1090. doi:10.1177/42.8.8027527.

Kotchen, T. A. (2010). Obesity-Related Hypertension: Epidemiology, Pathophysiology, and Clinical Management. *Am J Hypertens* 23, 1170–1178. doi:10.1038/ajh.2010.172.

Linder, A. E., Ni, W., Szasz, T., Burnett, R., Diaz, J., Geddes, T. J., et al. (2008). A serotonergic system in veins: serotonin transporter-independent uptake. *J Pharmacol Exp Ther* 325, 714–22. doi:10.1124/jpet.107.135699.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402–408. doi:10.1006/meth.2001.1262.

Lizcano, J. M., Fernandez de Arriba, A., Tipton, K. F., Unzeta, M., de Arriba, A. F., Tipton, K. F., et al. (1996). Inhibition of bovine lung semicarbazide-sensitive amine oxidase (SSAO) by some hydrazine derivatives. *Biochem Pharmacol* 52, 187–195. doi:10.1016/0006-2952(96)00132-3.

Löhn, M., Dubrovska, G., Lauterbach, B., Luft, F. C., Gollasch, M., Sharma, A. M., et al. (2002). Periadventitial fat releases a vascular relaxing factor. *FASEB J* 16, 1057–1063. doi:10.1096/fj.02-0024com.

Mercier, N., Moldes, M., El Hadri, K., and Fève, B. (2001). Semicarbazide-sensitive amine oxidase activation promotes adipose conversion of 3T3-L1 cells. *Biochem J* 358, 335–342.

Mészáros, Z., Karádi, I., Csányi, A., Szombathy, T., Romics, L., and Magyar, K. (1999). Determination of human serum semicarbazide-sensitive amine oxidase activity: a possible clinical marker of atherosclerosis. *Eur J Drug Metab Pharmacokinet* 24, 299–302.

Myöhänen, T. T., Schendzielorz, N., and Männistö, P. T. (2010). Distribution of catechol-O-methyltransferase (COMT) proteins and enzymatic activities in wild-type and soluble COMT deficient mice. *J Neurochem* 113, 1632–43. doi:10.1111/j.1471-4159.2010.06723.x.

Ozaita, A., Olmos, G., Boronat, M. A., Lizcano, J. M., Unzeta, M., and García-Sevilla, J. A. (1997). Inhibition of monoamine oxidase A and B activities by imidazol(ine)/guanidine drugs, nature of the interaction and distinction from I2-imidazoline receptors in rat liver. *Br J Pharmacol* 121, 901–12. doi:10.1038/sj.bjp.0701214.

Pizzinat, N., Marti, L., Remaury, A., Leger, F., Langin, D., Lafontan, M., et al. (1999). High expression of monoamine oxidases in human white adipose tissue: evidence for their involvement in noradrenaline clearance. *Biochem Pharmacol* 58, 1735–1742.

Poon, C. C. W., Seto, S. W., Au, A. L. S., Zhang, Q., Li, R. W. S., Lee, W. Y. W., et al. (2010). Mitochondrial monoamine oxidase-A-mediated hydrogen peroxide generation enhances 5-hydroxytryptamine-induced contraction of rat basilar artery. *Br J Pharmacol* 161, 1086–98. doi:10.1111/j.1476-5381.2010.00941.x.

Precious, E., and Lyles, G. A. (1988). Properties of a Semicarbazide-Sensitive Amine Oxidase in Human Umbilical Artery. *J Pharm Pharmacol* 40, 627–633. doi:10.1111/j.2042-7158.1988.tb05322.x.

Repessé, X., Moldes, M., Muscat, A., Vatier, C., Chetrite, G., Gille, T., et al. (2015). Hypoxia inhibits semicarbazide-sensitive amine oxidase activity in adipocytes. *Mol Cell Endocrinol* 411, 58–66. doi:10.1016/j.mce.2015.04.011.

Soltis, E. E., and Cassis, L. A. (1991). Influence of perivascular adipose tissue on rat aortic smooth muscle responsiveness. *Clin Exp Hypertens A* 13, 277–296.

Stock, K., and Westermann, E. O. (1963). Concentration of norepinephrine, serotonin, and histamine, and of amine-metabolizing enzymes. *J Lipid Res* 4, 297–304. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14168167 [Accessed April 25, 2016].

Sturza, A., Leisegang, M. S., Babelova, A., Schröder, K., Benkhoff, S., Loot, A. E., et al. (2013). Monoamine oxidases are mediators of endothelial dysfunction in the mouse aorta. *Hypertension* 62, 140–146. doi:10.1161/HYPERTENSIONAHA.113.01314.

Visentin, V., Boucher, J., Bour, S., Prevot, D., Castan, I., Carpene, C., et al. (2005). Influence of high-fat diet on amine oxidase activity in white adipose tissue of mice prone or resistant to diet-induced obesity. *J Physiol Biochem* 61, 343–352.

Visentin, V., Prévot, D., De Saint Front, V., Durand, Morin-Cussac, N., Thalamas, C., et al. (2004). Alteration of amine oxidase activity in the adipose tissue of obese subjects. *Obes Res* 12, 547–55. doi:10.1038/oby.2004.62.

Wanecq, E., Bour, S., Verwaerde, P., Smih, F., Valet, P., and Carpéné, C. (2006). Increased monoamine oxidase and semicarbazide-sensitive amine oxidase activities in white adipose tissue of obese dogs fed a high-fat diet. *J Physiol Biochem* 62, 113–23.

Zhao, B., Summers, F. A., and Mason, R. P. (2012). Photooxidation of Amplex Red to resorufin: implications of exposing the Amplex Red assay to light. *Free Radic Biol Med* 53, 1080–7. doi:10.1016/j.freeradbiomed.2012.06.034.

CHAPTER 5

NE transport in PVAT contributes to PVAT's anti-contractile effect on mesenteric resistance arteries exposed to NE

Abstract

PVAT exerts an anti-contractile effect on arteries exposed to NE. Functional studies to assess the ability of NE transport in PVAT to shift the concentration-response to NE have yet to be done. We hypothesized that inhibiting NE transport in mesenteric PVAT would decrease PVAT's anti-contractile effect on arteries exposed to NE. We mounted isolated mesenteric resistance arteries with and without PVAT from male Sprague-Dawley rats into a four-channel myograph and recorded the force of contraction continuously. Arteries with and without PVAT were incubated with vehicle or an inhibitor of transport for one hour prior to adding NE in a cumulative fashion. Incubation of arteries with PVAT (+PVAT) with nisoxetine (to inhibit the norepinephrine transporter [NET]), but not citalopram (inhibits the serotonin transporter [SERT]) or corticosterone (inhibits the organic cation transporter 3 [OCT3]), shifted the NE-response curve to the left. No effect was observed in arteries without PVAT (-PVAT). Citalopram shifted the concentration-response curve of +PVAT arteries, but not -PVAT arteries, exposed to 5-hydroxytryptamine (5-HT). Moreover, inhibition of SERT and OCT3 together did not affect PVAT's anti-contractile effect to NE. We also performed experiments to measure the effect of transport and metabolism in arteries with and without PVAT on the contraction to NE. Incubation with SPN (SPN= 1 mM semicarbazide to inhibit the semicarbazide sensitive amine oxidase [SSAO], 10 μM pargyline to inhibit monoamine oxidase A and B [MAO-A/B] and 1 μM nisoxetine to inhibit NET) but not SPC (SPC= 1 mM semicarbazide, 10 μM pargyline and 100 μM corticosterone to inhibit OCT3), shifted the NE-response curve of +PVAT arteries, but not -PVAT arteries, to the left. These data support a role for NET in PVAT's anti-contractile effect on mesenteric resistance arteries exposed to NE

and SERT to 5-HT. By contrast, OCT3 and SERT were not required for NE transport or metabolism to alter arterial contraction. These data support the conclusion PVAT has an important function in NE removal to reduce exposure of arteries to NE and decrease contraction.

Introduction

Before the discovery that PVAT reduces contraction of the aorta to NE, PVAT's role in influencing blood vessel function had not been appreciated (Soltis and Cassis, 1991). We added more evidence that calls attention to PVAT's interaction with the vasculature, including the finding that mesenteric PVAT accumulates exogenously applied NE in a transporter-dependent manner (Chapter 3). In Chapter 3, we described experiments demonstrating mesenteric PVAT could take up NE. NE uptake measured by HPLC in mesenteric PVAT was reduced upon inhibition of NET. Inhibiting both OCT3 and SERT together also reduced NE uptake as well as inhibiting all three transporters, NET, SERT, and OCT3 together. Inhibition of SERT or OCT3 individually with the addition of citalopram (100 nM) or corticosterone (100 μM) did not reduce uptake. These findings suggested that NET, OCT3 and SERT have cooperative roles in uptake of NE into whole mesenteric PVAT. Focusing in on the adipocyte, we found that uptake of the NE transporter dye ASP^+ was reduced by inhibiting NET alone and in contrast to what we observe with whole PVAT NE uptake, the inhibition of OCT3 or SERT alone was sufficient to block ASP^+ uptake into the adipocytes. Thus, adipocyte uptake and whole PVAT uptake are different in the transporters involved that can take up NE, but similar in that there are multiple ways that NE can be taken up. Low presence of NET was observed in whole mesenteric PVAT and the presence of NET in isolated adipocytes and the SVF was below our level of detection. OCT3, on the other hand, was highly expressed in PVAT adipocytes, especially those of the aortic PVAT. Aortic PVAT uptake of NE was significantly reduced (by ~40%) with inhibition of OCT3 alone by corticosterone (100 μM). Thus, OCT3 was a candidate to take up NE into adipocytes.

It remained to be investigated whether transport of NE into PVAT decreases contraction of mesenteric resistance arteries to NE. To test our hypothesis that transport of NE contributes to the anti-contractile effect of PVAT on arteries exposed to NE, mesenteric resistance arteries from normal male Sprague-Dawley rats were mounted into a four-channel myograph and incubated with inhibitors of OCT3, NET or SERT (100 μM corticosterone, 100 nM citalopram, or 1 μM nisoxetine, respectively), or in combination, before adding the agonist in a cumulative fashion. The force of contraction was recorded continuously.

White adipose tissue is a home for enzymes that can metabolize NE (Abella et al., 2004; Pizzinat et al., 1999). In Chapter 4, we described mesenteric PVAT as having high amine oxidase activity. The enzymes semicarbazide-sensitive amine oxidase (SSAO) and monoamine oxidase A (MAO-A) contribute to metabolism of NE and PVAT's anti-contractile effect. To test whether NE metabolism and transport worked cooperatively to produce the anti-contractile effect of PVAT, arteries with or without PVAT were incubated with inhibitors of transport *and* metabolism before addition of NE.

The ability of PVAT to transport and metabolize NE is a new function of PVAT to include in the growing list of how PVAT can influence blood vessel function. We look for the first time at the effects of the adrenergic system in PVAT's role in NE removal to reduce contraction to NE.

Methods

Chemicals

Norepinephrine hydrochloride, nisoxetine hydrochloride and semicarbazide hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO USA). Corticosterone and citalopram were purchased from Tocris (UK). Pargyline hydrochloride was purchased from Cayman Chemical (Ann Arbor, MI USA).

Animals

Male Sprague-Dawley rats (225-275 gram or ~8-10 weeks of age, Charles River, Indianapolis, IN USA) were used. All protocols were approved by the MSU Institutional Animal Care and Use Committee and follow the "Guide for the Care and Use of Laboratory Animals", Eighth edition, 2011. Rats were anesthetized with sodium pentobarbital (60-80 mg/kg, IP). Anesthesia was verified by lack of paw pinch and eye blink reflexes. Death was assured by pneumothorax and exsanguination after which tissues were removed for one of the following protocols.

Tissue Dissection

The mesentery was collected in physiological salt solution (PSS); in mM; 130 NaCl; 4.7 KCl; 1.8 KH_2PO_4; 1.7 $MgSO_4 \cdot 7H_2O$; 14.8 $NaHCO_3$; 5.5 dextrose; 0.03 $CaNa_2$ ethylenediaminetetraacetic acid, 1.6 $CaCl_2$ (pH 7.2). Mesenteric resistance arteries (~2 mm in length and 150-250 μm in diameter) with or without PVAT for the use in isometric contraction experiments were dissected out out in a Sylgard®-coated petri dish in PSS with the aid of a stereomicroscope.

Isometric Contraction

Rat third-order mesenteric resistance arteries cleaned of fat (-PVAT) or with fat intact (+PVAT) were mounted into a Multi Wire Myograph System 620M (Danish Myo Technology, Denmark). Data were acquired using a PowerLab Data Acquisitions unit (ADInstruments, Colorado Springs, CO USA). Baths contained warmed, oxygenated PSS. Rings were pulled to optimum resting tension (13.3 kPa) with the aid of the normalization module (Danish Myo Technology) and equilibrated for one hour with washes every 20 minutes. The arteries were exposed to an initial concentration of 60 mM KCl to test viability. Tissues were washed and tone returned to baseline and exposed to another concentration of 60 mM KCl. The contraction response to the second exposure to 60 mM KCl was used as the maximum contraction value to which the following NE curve was normalized to. Tissues were then washed and returned to baseline. Either vehicle or inhibitor was added for one hour without washing before construction of the NE concentration response curve. NE was added in a cumulative fashion, with significant time necessary for a response to plateau prior to the next addition. Tissues were washed and a final 60 mM KCl addition was performed to test for tissue viability at the end of the experiment.

Statistical Analysis

Data are reported as means±SEM for number of animals indicated by N within the graphs. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA). Contraction was reported as means±SEM as a percentage of the initial contraction to 60 mM KCl. Potency means (-log EC_{50}, M) were calculated

using GraphPad Prism 6.0 as concentrations necessary to cause a half-maximal effect. Where a maximum was not achieved, the values are estimated and true potencies equal or greater than that reported. Unpaired Student's t-tests were performed to the compare $-\log EC_{50}$ values between tissues with PVAT (+PVAT) and vehicle vs. +PVAT and inhibitor. Maximum contraction means were compared with a one-way ANOVA. The Holm-Sidak's multiple comparisons test was used to compare the means. $P<0.05$ was considered statistically significant.

Results

Pharmacological inhibition of OCT3 does not increase the contraction of arteries with PVAT to NE

To inhibit OCT3, we incubated mesenteric resistance arteries with (+) and without (-) PVAT with 100 μM corticosterone before construction of a NE-response curve. The NE-response curves for arteries + and -PVAT incubated with vehicle were shifted to the left from the curves for arteries incubated with vehicle (DMSO). However, the -logEC_{50} calculated from the two curves were not statistically different (-PVAT with vehicle *vs.* -PVAT with corticosterone p= 0.14, +PVAT with vehicle vs. +PVAT with corticosterone p=0.33, N=4). The -logEC_{50} and maximum contraction values are listed in Table 3. We conclude that inhibition of OCT3 uptake of NE into mesenteric PVAT may not affect the mesenteric arteries' contraction to NE.

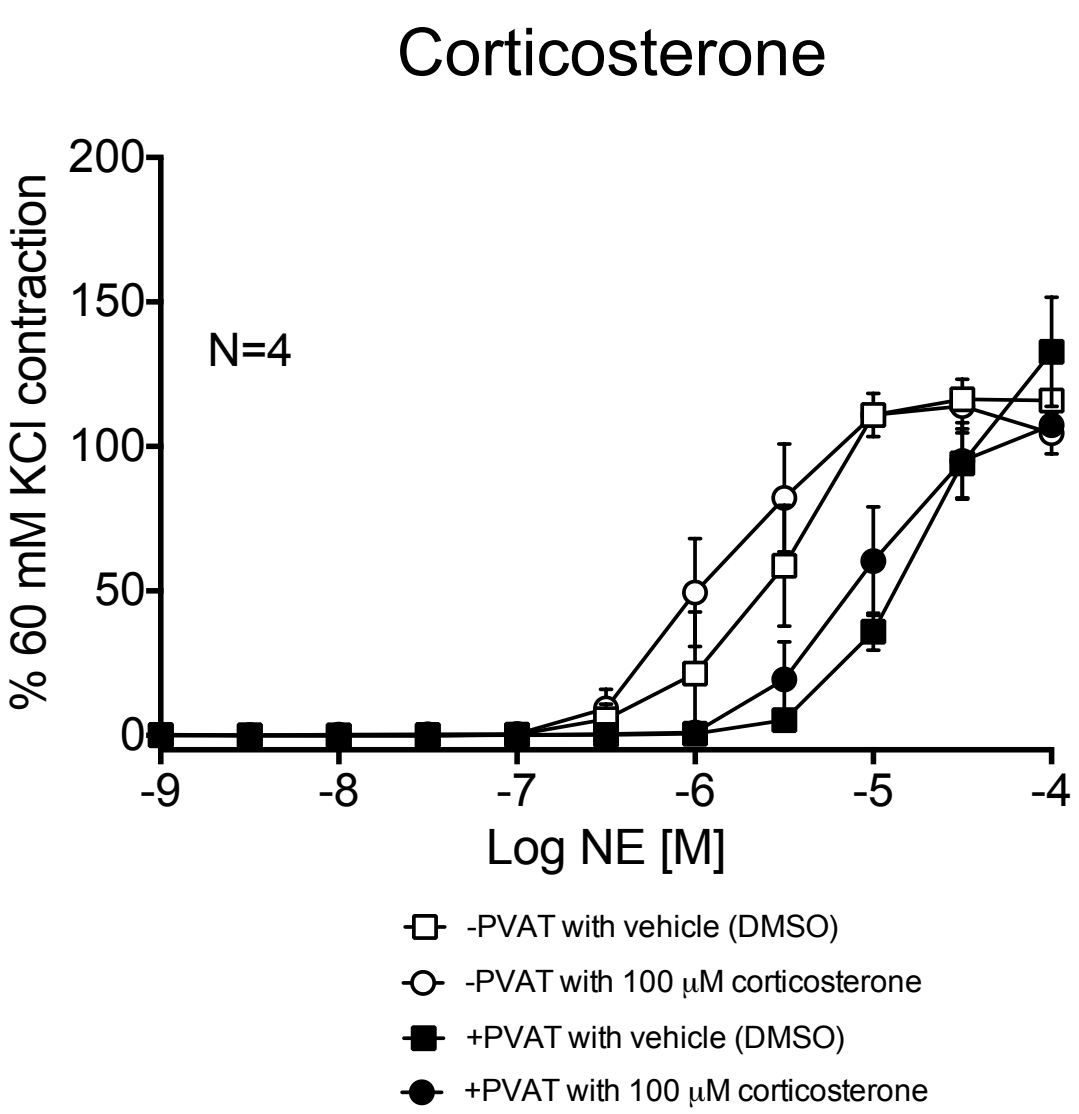


Figure 28. NE-induced contraction of mesenteric resistance arteries with and without PVAT incubated with corticosterone.

NE-induced contraction of the rat mesenteric resistance arteries + or -PVAT, with 100 μM corticosterone (inhibits OCT3) or vehicle (H_2O). The force of contraction was normalized to the percent of the 60 mM KCl contraction. Bars represent means±SEM. N= the number of animals used in each group.

Inhibition of SERT increases the potency of 5-HT, but not NE, on arteries with PVAT

To test whether SERT contributes to PVAT's anti-contractile effect in arteries, mesenteric resistance arteries with (+) and without (-) PVAT were incubated for one hour with either vehicle (H_2O) or 100 nM citalopram prior to the cumulative addition of NE. We did not observe a difference in the -logEC_{50} of the curve for arteries incubated with vehicle *vs.* those incubated with 100 nM citalopram (Figure 29A, N=4). We then conducted a similar experiment, but with 5-HT as the agonist instead of NE. Mesenteric resistance arteries + and -PVAT were incubated with vehicle (H_2O) or 100 nM citalopram, after which a 5-HT concentration-response curve was performed. The +PVAT arteries exhibited a right-shift in the 5-HT contraction curve (-PVAT arteries with vehicle -logEC_{50}=6.27±0.07 *vs.* +PVAT arteries with vehicle -logEC_{50}= 5.09±0.13) (Figure 29B, N=4). The curve for the +PVAT arteries incubated with 100 nM citalopram was shifted to the left and resulted in a lower -logEC_{50} than the +PVAT arteries incubated with vehicle (Figure 29B). We conclude that mesenteric PVAT decreases contraction of arteries to 5-HT in addition to NE. PVAT's anti-contractile effect to 5-HT, but not NE, on mesenteric resistance arteries is due to uptake through SERT.

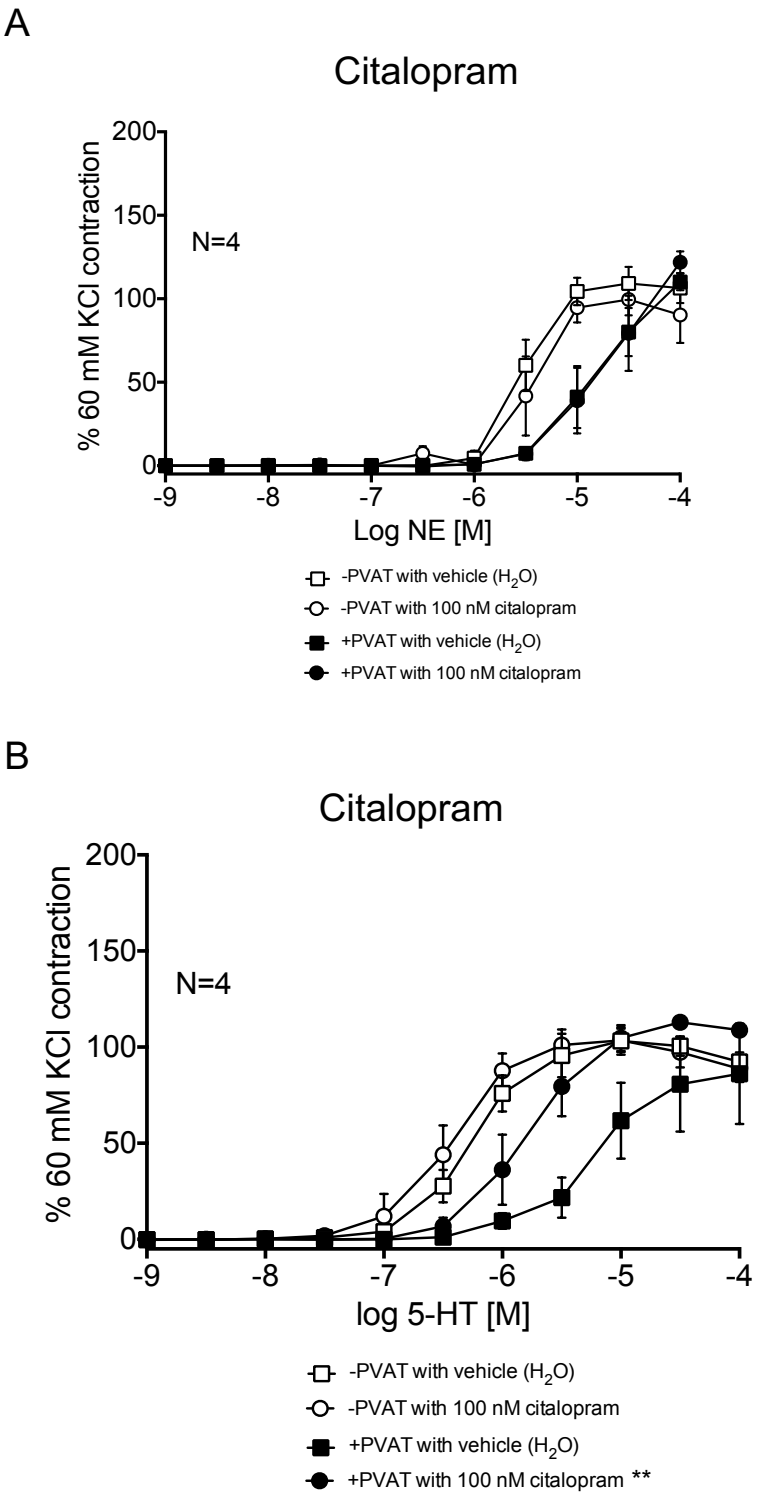


Figure 29. NE and 5-HT-induced contraction of mesenteric resistance arteries with and without PVAT incubated with citalopram.

Figure 29. (cont'd)

(A) NE-induced contraction of the rat mesenteric resistance arteries + or - PVAT, with 100 nM citalopram (inhibits SERT) or vehicle (H_2O). (B) 5-HT-induced contraction of the rat mesenteric arteries + or -PVAT, with 100 nM citalopram or vehicle (H_2O). The force of contraction was normalized to the percent of 60 mM KCl contraction. Bars represent means±SEM. N= the number of animals used in each group. **p<0.01 potency *vs*. +PVAT with vehicle.

Inhibition of NET increases the potency of NE at arteries with PVAT

Next, we explored the role of NET in PVAT's anti-contractile effect on arteries exposed to NE. To test whether NET's role in the anti-contractile effect of PVAT, we incubated mesenteric resistance arteries + and -PVAT with 1 μM nisoxetine for one hour prior to the construction of a NE-response curve. Nisoxetine caused a leftward shift in the curve of +PVAT arteries compared to +PVAT arteries incubated with vehicle (H_2O; Figure 30) reflected by an increase in the potency of NE ($p<0.05$, Table 3; N=4). We conclude that uptake of NE through NET in PVAT may contribute to PVAT's anti-contractile effect on mesenteric resistance arteries exposed to NE.

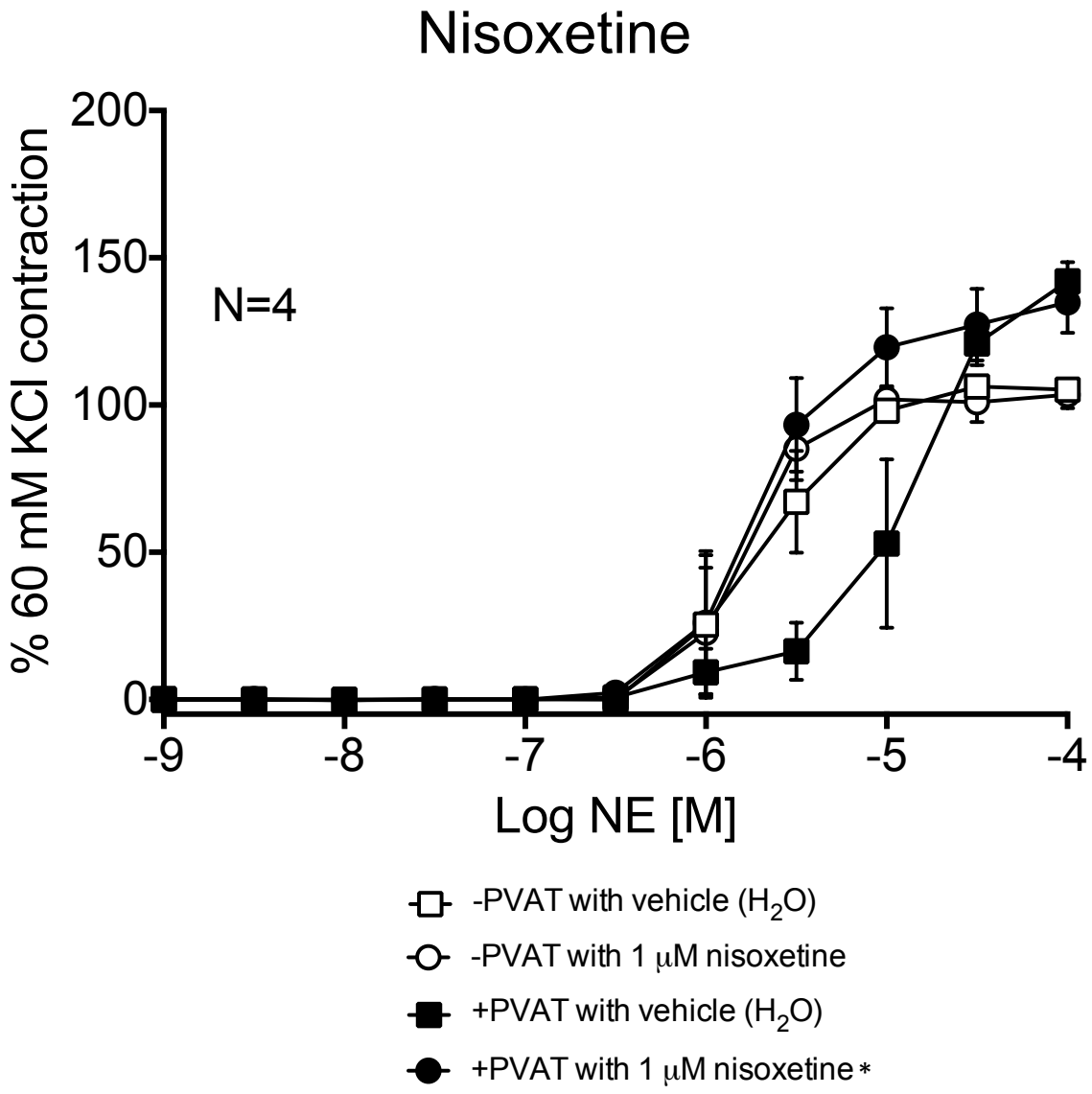


Figure 30. NE-induced contraction of mesenteric resistance arteries with and without PVAT incubated with nisoxetine.

NE-induced contraction of the rat mesenteric resistance arteries + or -PVAT, incubated with 1 μM nisoxetine (inhibits NET) or vehicle (H_2O). The force of contraction was normalized to the percent of 60 mM KCl contraction. Bars represent means±SEM. N= the number of animals used in each group. *$p<0.05$ potency vs. +PVAT with vehicle.

Inhibition of uptake through SERT and OCT3 does not affect the potency of NE at arteries with or without PVAT

Both SERT and OCT3 are expressed on adipocytes (Pizzinat et al., 1999; Stunes et al., 2011). It is possible that multiple transporters may be involved with taking up NE in PVAT. We tested whether dual inhibition of SERT and OCT3 was also necessary for PVAT's anti-contractile effect in our contractility assay. Mesenteric resistance arteries + or -PVAT were incubated with 100 nM citalopram *and* 100 μM corticosterone for one hour (to inhibit both SERT and OCT, respectively) before adding increasing concentrations of NE. The presence of both inhibitors did not alter the NE-response curves of any of the tissues (+ or -PVAT, vehicle *vs*. inhibitors) (Figure 31; N=4). Thus, NE uptake through SERT and OCT3 is not required for PVAT to reduce the contraction of arteries to NE.

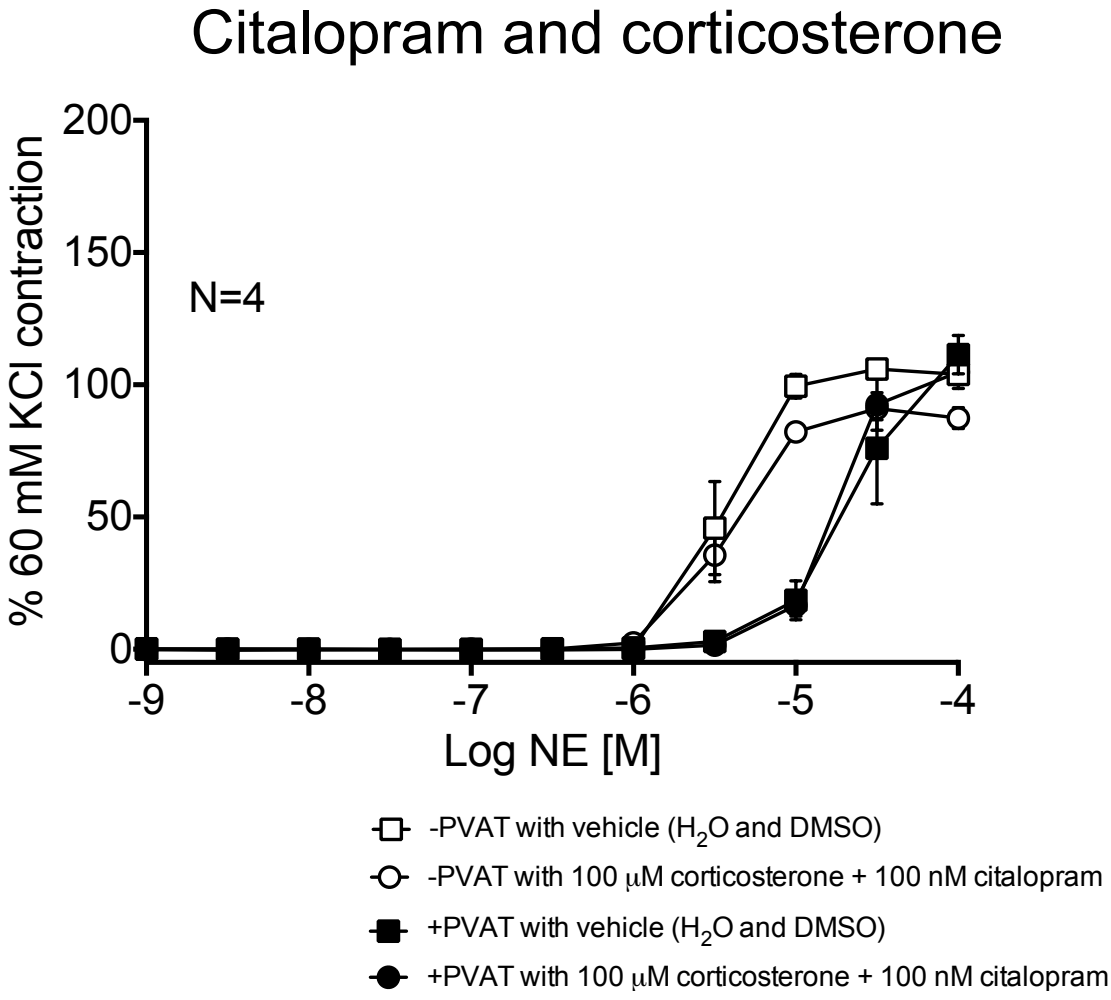


Figure 31. NE-induced contraction of mesenteric resistance arteries with and without PVAT incubated with citalopram and corticosterone.

NE-induced contraction of the rat mesenteric resistance arteries with or without PVAT and incubated for one hour with or without (vehicle) the inhibitors 100 nM citalopram (inhibits SERT) and 100 μM corticosterone (inhibits OCT3). The force of contraction was normalized to the percent of 60 mM KCl contraction. Bars represent means±SEM. N= the number of animals used in each group.

Inhibition of uptake through OCT3 and metabolism through SSAO and MAO does not decrease PVAT's anti-contractile effect on arteries to NE

We discovered that inhibition of metabolism of NE in PVAT caused a reduction in PVAT's anti-contractile effect on arteries exposed to NE (Chapter 4). We then tested whether transport of NE potentiated metabolism of NE in PVAT. Inhibition of SSAO (with 1 mM semicarbazide), MAO-A and B (with 10 μM pargyline), and OCT3 (with 100 μM corticosterone; SPC) surprisingly did not alter PVAT's anti-contractile effect when compared to vehicle (H_2O as the vehicle for pargyline and semicarbazide, DMSO as the vehicle for corticosterone; Figure 32A, N=4-5). The curve for the arteries +PVAT that were incubated with inhibitors was shifted slightly to the left from arteries +PVAT with vehicle but this shift did not translate into a significant change in the -logEC_{50} (Table 3). It appears that inhibiting the entry of NE through OCT3 does not decrease NE removal. By contrast, inhibition of metabolism and uptake through NET by SPN (SPN= 1 mM semicarbazide, 10 μM pargyline and 1 μM nisoxetine) shifted the +PVAT curve to the left (Figure 32B; N=4) reflected by an increase in potency of NE on +PVAT arteries.

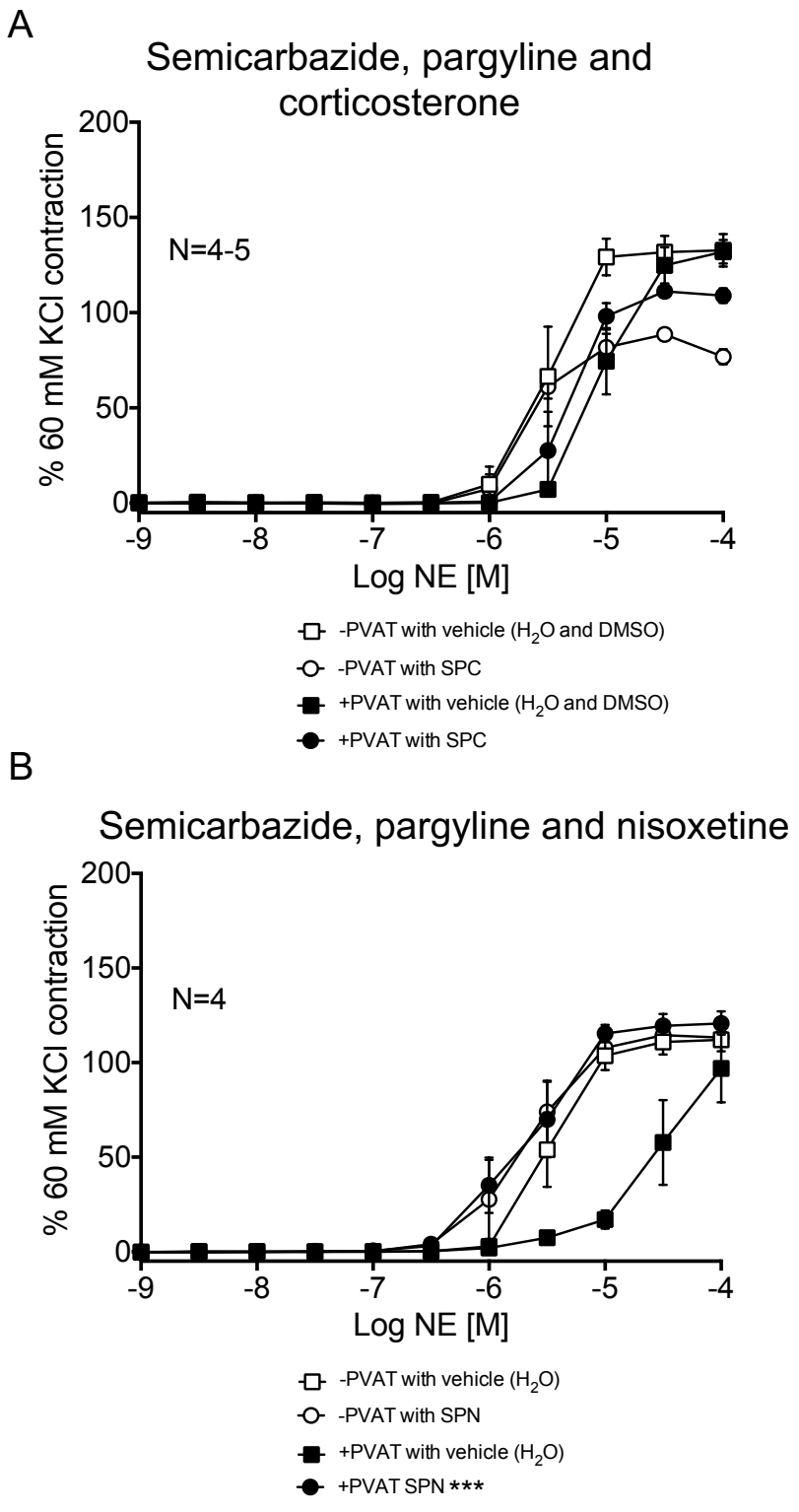


Figure 32. NE-induced contraction of mesenteric resistance arteries with and without PVAT incubated with inhibitors of metabolism and transport.

Figure 32. (cont'd)

NE-induced contraction of the rat mesenteric resistance arteries + or -PVAT incubated inhibitors of NE transport and metabolism or vehicle (H_2O for semicarbazide and pargyline, and DMSO for corticosterone). NE curves after an hour incubation with (A) SPC= 1 mM semicarbazide, 10 μM pargyline, and 100 μM corticosterone (used to inhibit SSAO, MAO-A/B and OCT3), and (B) SPN= 1 mM semicarbazide, 10 μM pargyline and 1 μM nisoxetine (used to inhibit SSAO, MAO-A/B, and NET). The force of contraction was normalized to the percent of 60 mM KCl contraction. Bars represent means±SEM. N= the number of animals used in each group. ***$p<0.001$ vs. +PVAT with vehicle control (no inhibition).

PVAT	Inhibitor	Agonist	-logEC_{50} (M)		Max Contraction % of 60 mM KCl
-	vehicle	NE	5.72±0.12		116.32±0.57
-	100 µM corticosterone	NE	5.99±0.11		113.98±9.26
+	vehicle	NE	4.98±0.12		132.78±18.89
+	100 µM corticosterone	NE	5.14±0.09		107.38±3.39
-	vehicle	NE	5.60±0.10		109.17±9.91
-	100 nM citalopram	NE	NC		99.59±9.55
+	vehicle	NE	4.91±0.09		121.84±6.54
+	100 nM citalopram	NE	4.92±0.11		110.03±4.88
-	vehicle	5-HT	6.27±0.07		103.20±7.16
-	100 nM citalopram	5-HT	6.47±0.09		103.56±6.10
+	vehicle	5-HT	5.09±0.13		86.10±26.15
+	100 nM citalopram	5-HT	5.88±0.09	**	112.89±2.23
-	vehicle	NE	5.71±0.10		106.32±3.33
-	1 µM nisoxetine	NE	5.80±0.10		103.55±4.56
+	vehicle	NE	5.23±0.15		141.99±6.59
+	1 µM nisoxetine	NE	5.92±0.16	*	134.94±10.36
-	vehicle	NE	5.49±0.09		106.03±2.20
-	100 nM citalopram and 100 µM corticosterone	NE	5.32±0.06		91.03±4.27
+	vehicle	NE	4.76±0.10		111.46±7.32
+	100 nM citalopram and 100 µM corticosterone	NE	4.81±0.08		104.76±6.25
-	vehicle	NE	5.76±0.15		131.05±8.60
-	SPC	NE	5.46±0.12		88.31±2.34#
+	vehicle	NE	5.27±0.13		130.02±6.19
+	SPC	NE	5.41±0.10		108.17±3.63
-	vehicle	NE	5.57±0.11		112.05±6.07
-	SPN	NE	5.81±0.11		114.56±3.15
+	vehicle	NE	4.66±0.10		114.091±3.44
+	SPN	NE	5.86±0.12	***	120.69±6.43

Table 3. Pharmacological parameters of NE and 5-HT on isolated mesenteric resistance arteries with (+) or without (-) PVAT.

Table 3. (cont'd)

Means ±SEM were calculated from figures presented in Figures 28-32. SPC= 10 μM pargyline, and 100 μM corticosterone. SPN= 1 mM semicarbazide, 10 μM pargyline and 1 μM nisoxetine. NC= not convergent. *p<0.05, **p<0.01, ***p<0.001 *vs.* +PVAT with vehicle response.

Discussion

NE uptake by OCT3 alone does not reduce the contraction of arteries with PVAT to NE

Since the NE transporter OCT3 is highly expressed in PVAT (Figure 15), we tested whether inhibiting OCT3 would decrease PVAT's anti-contractile effect on mesenteric resistance arteries exposed to NE (Figure 28). In our contractility assays, inhibiting OCT3 mediate NE uptake did not significantly shift the NE-response curves of arteries with or without PVAT. This is consistent with the inability of corticosterone (100 μM) to significantly reduce NE-uptake (Figure 10) in isolated mesenteric PVAT. This is different from uptake of NE in the PVAT of the aorta, which was significantly reduced with corticosterone (Figure 17). We conclude that OCT3 uptake of NE in mesenteric PVAT does not contribute significantly to PVAT's anti-contractile effect on arteries exposed to NE. However, the role of OCT3 in aortic PVAT's uptake of NE to reduce contraction of the aorta may be significant and warrants further investigation.

Inhibition of SERT uptake increases the potency of 5-HT on arteries with PVAT

Adipocytes from rat visceral adipose tissue express SERT (Stunes et al., 2011). Although, SERT is mainly known for transporting 5-HT, amine transporters are promiscuous in their substrates (Daws, 2009). Thus, we needed to test whether SERT in mesenteric PVAT had a role in NE uptake. Inhibiting SERT did not reduce NE uptake in experiments using isolated PVAT (Figure 10). Furthermore, tyramine-dependent induced contraction of the thoracic rat aorta with PVAT was not reduced by citalopram (1 μM) (Figure 8). However, uptake of ASP^+, a NE transporter substrate dye, into mesenteric PVAT adipocytes was decreased by inhibiting SERT with 100 nM citalopram (Figure 13).

In isolated mesenteric resistance arteries with or without PVAT, inhibiting SERT with citalopram (100 nM) did not effect contraction to NE. Thus, even though SERT is present on adipocytes and can transport ASP^+, SERT uptake does not account for PVAT's anti-contractile effect on arteries exposed to NE. We also tested whether PVAT was anti-contractile to SERT's preferred substrate 5-HT. PVAT exhibited an anti-contractile effect on arteries exposed to 5-HT, which was reduced by SERT inhibition. While this dissertation set out to identify and describe PVAT's adrenergic system, these data provide evidence for a serotonergic system within PVAT important for it's anti-contractile effect to 5-HT.

NET uptake contributes to PVAT's anti-contractile effect to NE

NE uptake's role in the anti-contractile effect of PVAT on rat thoracic aorta was discovered by Soltis and Cassis (1991). The authors discovered that PVAT reduced contraction of the aorta to NE and by blocking NE transport with deoxycorticosterone and desipramine they were able to remove this anti-contractile effect of PVAT. Desipramine is an inhibitor of NET and deoxycorticosterone is an inhibitor of OCT3. The authors did not determine to which transporter(s) (NET, SERT, OCT3, or a combination) this effect was attributed. In our studies, inhibiting NET with 1 μM nisoxetine or 1 μM desipramine reduced tyramine's potency on rat aortae and superior mesenteric arteries with PVAT compared to those incubated with the vehicle for the inhibitor, suggesting the presence and function of NET in PVAT (Figure 8). Furthermore, nisoxetine (a more specific inhibitor for NET than desipramine) was able to reduce both NE and ASP^+ uptake in mesenteric PVAT (Figure 10 and 13). Thus, we needed to test whether NET had a role in PVAT's

anti-contractile effect. Mesenteric resistance arteries with PVAT that were incubated with nisoxetine, had a NE-response curve that was shifted to the left. Nisoxetine addition did not have an effect on arteries without PVAT, supporting the conclusion that the anti-contractile effect of PVAT was reversed by inhibiting NET with nisoxetine.

Inhibition of SERT and OCT3 does not affect PVAT's anti-contractile effect to NE

The experiments performed by Soltis and Cassis (1991) to establish PVAT's anti-contractile effect on the aorta exposed to NE included the use of two transporter inhibitors, deoxycorticosterone and desipramine. Deoxycorticosterone was used as an OCT3 inhibitor and desipramine was used to inhibit NET [Ki=7.36 nM (Paczkowski et al., 1999)]. However, desipramine also inhibits SERT at higher concentrations; Ki 129.00 nM (Owens et al., 1997). The two inhibitors applied together reversed PVAT's anti-contractile effect (Soltis and Cassis, 1991). Since it is possible that desipramine (used at 100 nM in the study) could inhibit a portion of the SERTs present in PVAT, it was necessary to test whether SERT and OCT3 were involved in NE uptake. Citalopram (100 nM) or corticosterone (100 μM) applied individually did not reduce NE uptake in mesenteric PVAT (Figure 10). However, incubating the mesenteric PVAT with both of the inhibitors together reduced NE uptake into PVAT (Figure 10). In our contractility assay, citalopram and corticosterone did not have an effect on the contraction of mesenteric resistance arteries with or without PVAT to NE. A possible explanation for this discrepancy in findings is that although SERT and OCT3 can take up NE into PVAT that has been incubated with the PVAT for 30 minutes, the amount that they take up is not sufficient enough or the rate of uptake not fast enough to affect contraction of arteries to exogenously added NE.

Inhibition of OCT3 and metabolism through SSAO and MAO does not decrease PVAT's anti-contractile effect on arteries to NE

Inhibition of metabolism of NE in PVAT caused a reduction in PVAT's anti-contractile effect on arteries exposed to NE (Chapter 4). Thus, we next tested whether transport of NE could potentiate metabolism of NE in PVAT by assisting in the relocation of NE into the intracellular environment. Studies by (Pizzinat et al., 1999) demonstrated the presence of OCT3 on adipocytes as well as the NE metabolizer monoamine oxidase. The incubation of arteries with corticosterone, semicarbazide and pargyline did not shift the NE-response curve of +PVAT or -PVAT arteries. Incubating arteries with nisoxetine, semicarbazide and pargyline shifted the +PVAT NE-response curve to the left. However, nisoxetine alone (Figure 30) was also able to shift the +PVAT NE-response curve to the left. NE metabolism may not be required for PVAT's anti-contractile effect. NE uptake is more functionally important in reducing arterial contraction.

Limitations

A limitation in our experiment is that we added in exogenous NE to the baths to model what we think is occurring in the body. However, adding NE in this way exposes the entire artery (including the lumen) to NE. This would be different from the physiological situation where NE released within and near PVAT (away from the luminal side of the artery) would come in contact with PVAT to be taken up and metabolized.

Conclusion

PVAT's role in modulating vascular response to NE is dysfunctional in disease (Van de Voorde et al., 2014). In one model of hypertension, the anti-contractile effect of PVAT on mesenteric arteries from spontaneously hypertensive rats (SHR) exposed to exogenous NE was reduced compared to that of normotensive Wistar-Kyoto rats (Török et al., 2016). While various mechanisms are proposed to exist that contribute to the anti-contractile effect of PVAT, the mechanisms that are responsible for NE removal in PVAT are not completely understood.

NE uptake through NET is important in PVAT's anti-contractile effect on arteries exposed to NE. PVAT also exhibits an anti-contractile effect on arteries exposed to 5-HT, which is mediated through uptake by SERT. These data round out our understanding of how an adrenergic system in PVAT effects the contraction of mesenteric resistance arteries. Investigation of PVAT NET and SERT uptake in tissues from rodent models of disease (hypertension, obesity, and obesity-related hypertension) is now necessary to investigate whether alterations in PVAT NE uptake contribute to increased vascular reactivity to NE in disease.

REFERENCES

REFERENCES

Abella, A., Garcia-Vicente, S., Viguerie, N., Ros-Baro, A., Camps, M., Palacin, M., et al. (2004). Adipocytes release a soluble form of VAP-1/SSAO by a metalloprotease-dependent process and in a regulated manner. *Diabetologia* 47, 429–438.

Daws, L. C. (2009). Unfaithful neurotransmitter transporters: focus on serotonin uptake and implications for antidepressant efficacy. *Pharmacol Ther* 121, 89–99. doi:10.1016/j.pharmthera.2008.10.004.

Owens, M. J., Morgan, W. N., Plott, S. J., and Nemeroff, C. B. (1997). Neurotransmitter Receptor and Transporter Binding Profile of Antidepressants and Their Metabolites 1. 283, 1305–1322.

Paczkowski, F. A., Bryan-lluka, L. J., Pörzgen, P., Brüss, M., and Bönisch, H. (1999). Comparison of the Pharmacological Properties of Cloned Rat , Human , and Bovine Norepinephrine Transporters 1. *J Pharmacol Exp Ther* 290, 761–767.

Pizzinat, N., Marti, L., Remaury, A., Leger, F., Langin, D., Lafontan, M., et al. (1999). High expression of monoamine oxidases in human white adipose tissue: evidence for their involvement in noradrenaline clearance. *Biochem Pharmacol* 58, 1735–1742.

Soltis, E. E., and Cassis, L. A. (1991). Influence of perivascular adipose tissue on rat aortic smooth muscle responsiveness. *Clin Exp Hypertens A* 13, 277–296.

Stunes, A. K., Reseland, J. E., Hauso, O., Kidd, M., Tommeras, K., Waldum, H. L., et al. (2011). Adipocytes express a functional system for serotonin synthesis, reuptake and receptor activation. *Diabetes Obes Metab* 13, 551–558. doi:10.1111/j.1463-1326.2011.01378.x.

Török, J., Zemančíková, A., and Kocianová, Z. (2016). Interaction of perivascular adipose tissue and sympathetic nerves in arteries from normotensive and hypertensive rats. *Physiol Res* 65, S391–S399.

Van de Voorde, J., Boydens, C., Pauwels, B., and Decaluwe, K. (2014). Perivascular adipose tissue, inflammation and vascular dysfunction in obesity. *Curr Vasc Pharmacol* 12, 403–411.

CHAPTER 6

Discussion

Introduction

This dissertation introduced the idea and provided supporting evidence that perivascular adipose tissue (PVAT) contains an adrenergic system, which has a role in the release, uptake and metabolism of norepinephrine (NE). The goal of this research was to examine the function of an adrenergic system in PVAT of normal animals (non-obese and non-hypertensive). Several sites for PVAT have been used in PVAT research for example, PVAT around the mesenteric artery (Török et al., 2016), mesenteric resistance arteries (Mann et al., 2014), aorta (Soltis and Cassis, 1991), coronary artery (Owen et al., 2013), saphenous vein, and renal sinus (Gil-Ortega et al., 2015). For the purpose of answering the questions posed in this dissertation, we focused on the PVAT of the mesentery because of the strong association of increased visceral fat with cardiovascular risk (Kotchen, 2010). We focused on PVAT around the superior mesenteric artery the mesenteric resistance arteries for most of the studies but also included the aortic PVAT when it was necessary to represent another distinct PVAT depot, as mesenteric PVAT is similar to white adipose tissue (WAT) and aortic-PVAT is similar to brown adipose tissue (BAT) and is considered a mixed-PVAT (Gao, 2007). For a holistic view of PVAT, we examined the PVAT stromal vascular fraction (SVF) and/or adipocytes in regards to NE content (Figure 9), organic cation transporter 3 (OCT3) presence (Figure 15) and amine oxidase activity (Figures 22-26). Our careful examination of PVAT and its components revealed a functional adrenergic system. Before a discussion of future directions of the research, a summary of the findings along with a synthesis of how the findings fit together (including any inconsistences found) in the results will be provided.

In Chapter 1, we learned about just how little is known about the adrenergic components in PVAT. From studies done in white and brown adipose tissue, we know that adipose tissue (from non-PVAT depots) is innervated by the sympathetic nervous system (Bartness et al., 2010; Bartness and Song, 2007). Innervation by parasympathetic nerves is controversial and not widely supported (Bartness et al., 2014; Giordano et al., 2006; Kreier et al., 2002). We found that PVAT contains catecholamines, of which NE is present in the highest concentration (Figure 2). These findings prompted us to ask the question of whether catecholamines could be released from PVAT and cause contraction of the underlying vessels. Addition of the sympathomimetic tyramine caused the release of NE, 5-HT, and dopamine (Figure 3). Tyramine added to the mesenteric artery or the aorta caused a PVAT-dependent contraction supporting that the contractant released by tyramine originated from the PVAT (Figure 4). Furthermore, celiac ganglionectomy to remove the nerve innervation from PVAT did not reduce the contraction of the mesenteric artery to tyramine suggesting that the contractile substance(s) released from PVAT were present independent of the nerve (Figure 7). In subsequent experiments, we measured the concentration of NE in the adipocytes separated from PVAT. We made the discovery that adipocytes isolated from PVAT maintained NE, the concentration of NE in adipocytes not being statistically different from the concentration of NE found in whole PVAT (Figure 9).

One of our major findings was that uptake of NE by OCT3 and NET in PVAT may decrease exposure of arteries to NE in the rat (Chapters 3 and 5). We identified mRNA for OCT3 to be highly expressed in PVAT adipocytes (Figure 15) and that OCT3 contributes to uptake of NE. However, in assays to assess contraction of arteries to NE,

inhibition of OCT3 did not reduce the anti-contractile effect of PVAT on arteries exposed to NE (Figure 28). A Western blot signal for NET in mesenteric PVAT was observed but a NET signal in isolated adipocytes was not detected (Figure 14). By contrast, inhibition of the NET was the key to reducing the anti-contractile effect of PVAT on mesenteric resistance arteries exposed to NE (Figure 30). In the PVAT uptake experiments (Chapter 3), NE was incubated with the tissues for 30 minutes. However, in the contractility experiments, NE was directly applied to arteries with PVAT and contraction was measured in real-time. Thus, it is possible that uptake through OCT3 in mesenteric PVAT does not happen at a rate fast enough to influence contractility to NE.

Contraction of arteries (aorta and superior mesenteric artery) with PVAT to tyramine was reduced by incubating these tissues with the NET inhibitor nisoxetine (1 μM). Both the -logEC_{50} and the percent of maximum contraction to tyramine were reduced with pre-incubation nisoxetine compared to vehicle. These results support the presence of NET in PVAT and NET's role in tyramine-mediated contraction of arteries with PVAT. Thus, these findings are in accordance with our later findings that NET is also important in PVAT's anti-contractile effect to NE (Figure 30).

We also discovered the potential for NE metabolism in PVAT and identified high activity of SSAO in PVAT adipocytes. In isolated mesenteric resistance arteries with or without PVAT, inhibition of SSAO alone did not effect contraction to NE. Inhibition of both SSAO and MAO was required to shift the NE-response curve to the left in the presence of PVAT indicating that a redundancy of oxidases that can metabolize NE exists (Figure 26). In Figure 32 however, inhibition of SSAO, MAO and OCT3 did not shift the curve of the NE-response of +PVAT arteries. To explain this difference we can consider that in

experiments where the metabolism inhibitors, semicarbazide and pargyline, were added, the anti-contractile effect of PVAT was more robust ($-\log EC_{50}$ of -PVAT/vehicle=5.55±0.10 vs. +PVAT/vehicle= 4.73±0.11) than in the experiments where the metabolism inhibitors and the OCT3 inhibitor, corticosterone were used ($-\log EC_{50}$ of -PVAT/vehicle=5.76±0.15 and +PVAT/vehicle=5.27±0.13). The difference in the anti-contractile effect between these two groups of animals used could have been the reason why we did not observe an effect with the metabolism inhibitors in Figure 32. The presence of PVAT consistently reduced the potency of NE (Figure 26A). However, variability in the magnitude of this effect exists between groups (Table 3). This type of variability may be due to the level of innervation in the particular tissue selected, the age of the rats, and the amount of PVAT.

One may ask how it is possible for NE to be detected in PVAT, if PVAT degrades NE readily. There are several explanations for this. NE could be present in a location(s) that is separate and isolated from amine metabolizing enzymes (i.e. a different cell type). This is certainly possible. We presented evidence for the presence of NE in adipocytes (Figure 9) and for high levels of amine oxidase activity in the adipocyte fraction of mesenteric PVAT (Figures 22 and 24). MAO activity in PVAT was low and below the level of detection. When the SVF was enriched, low MAO activity was detected (Figures 23D and 25D). A reason why NE could still be present in PVAT is that NE may be a poor substrate for SSAO and thus is protected from metabolism within the adipocyte. Another possibility is that NE is stored in PVAT in a location protected by metabolizing enzymes (i.e. a vesicle). This explanation is also feasible as we observed a reduction in the contraction to tyramine by rat aorta +PVAT or SMA +PVAT when the tissues have been

incubated with the vesicular monoamine transporter (VMAT) inhibitor tetrabenazine (10 μM). A third possibility is that NE is being synthesized in PVAT and thus synthesis replenishes stores of NE in PVAT compensating for any NE loss through its metabolism. Previous research has implicated visceral white adipose tissue adipocytes as producers catecholamines (Kvetnansky et al., 2012; Vargovic et al., 2011, 2013). Other cell types that can be found in PVAT such as lymphocytes and macrophages also produce catecholamines (Brown et al., 2003; Josefsson et al., 1996). These possibilities are not mutually exclusive and thus it is possible that synthesis and storage of NE occurs in PVAT. However, this hypothesis remains to be tested.

Discrepant results and other hypotheses

The NE uptake experiments informed us of the potential of PVAT to take up NE and ASP^+. This effect was inhibited by nisoxetine suggesting a role for NET. Incubating the mesenteric PVAT with both citalopram (100 nM) or corticosterone (100 μM) significantly reduced NE uptake (Figure 11) and ASP^+ uptake (Figure 13). Inhibition of ASP^+ uptake was accomplished by inhibiting NET, OCT3, or SERT (Figure 13). However, the inhibitors applied individually did not reduce NE uptake in mesenteric PVAT (Figure 11). While the pharmacological profile of uptake of ASP^+ is similar to that of [^{3}H]NE , ASP^+ pharmacology is not identical to that of NE (Haunso and Buchanan, 2007). NE is over three times more potent at inhibiting NET uptake of ASP^+ (Ki=1261 nM) vs. uptake of [^{3}H]NE (Ki=4255 nM) (Haunso and Buchanan, 2007). ASP^+ is more easily inhibited by transporter inhibitors than NE and this could explain why sole inhibition of SERT or OCT3 was sufficient to reduce ASP^+ uptake but not for NE uptake.. Our data also suggest that NE uptake could occur through multiple different transporters since both SERT and OCT3 required inhibition to reduce uptake. In assessing the contraction of the mesenteric resistance arteries with or without PVAT to NE, incubation with 100 nM citalopram and 100 μM corticosterone did not have an effect. A hypothesis that would explain this discrepancy in findings is that although SERT and OCT3 can take up a saturating concentration of NE (10 μM) into PVAT that has been incubated with it for 30 minutes, the rate of uptake through these transporters is not fast enough to affect contraction of arteries to exogenously added NE in our myograph system where the contraction is recorded in real-time. The kinetics of each transporter will dictate which one will be the

most important in NE uptake depending on the timing of the application and concentration of NE.

Another hypothesis that emerges is that NE metabolism produces H_2O_2, which plays a role in reducing contraction of arteries with PVAT to NE. Both actions of PVAT, the metabolism and thereby removal of NE *and* the production of H_2O_2 may be important in reducing contraction to NE. However, the role of H_2O_2 in PVAT's anti-contractile effect to NE was not tested.

Future directions

NE synthesis and release

We opened several avenues of investigation with the discovery that PVAT has a releasable store of catecholamines (Chapter 2). However, more work needs to be done to determine where these catecholamines originate from. Innervation of PVAT by nerves containing NE could contribute to NE stored in PVAT. Bulloch and Daly demonstrated that nerves (they did not distinguish whether they were sympathetic or not) traverse through PVAT (Bulloch and Daly, 2014). A more comprehensive study of nerves that innervate different PVATs and their type (sympathetic, PNS, or sensory) is necessary to get an idea of the number of nerves that are in PVAT and the neurotransmitters the nerves contribute to PVAT. Bartness' group traced the origin of nerves from BAT and WAT depots to areas of the CNS with the use of pseudorabies virus neural tracers (Bartness et al., 2010; Bartness and Song, 2007). We propose that similar studies be performed to trace the nerves in PVAT to locate their origin in the CNS to reveal the networks that participate in the neurohormonal control of PVAT and the underlying vasculature. Another technique that may be employed to investigate sympathetic innervation in PVAT is electron microscopy (EM). By EM, we can examine sympathetic nerves (identified by immunostaining of TH with gold particles) closely and identify the location of any NE storage vesicles.

Another avenue of research is to locate what cell types in PVAT contribute to the catecholamine content of PVAT. Although nerves within PVAT may be an important source of the catecholamines found there, they are not the only source. We learned by removing the celiac ganglion and thus the source of neural innervation to the mesentery,

that NE is present in PVAT independent of nerves (Figure 7). We also observed that when we isolated adipocytes from PVAT, removing the SVF, NE remained (Figure 9). Several cell types found in PVAT have the capability of synthesizing NE including adipocytes, lymphocytes and macrophages (Nguyen et al., 2011; Qiu et al., 2004; Vargovic et al., 2011). Other studies to determine whether other cell types in PVAT contain NE are necessary.

Detection of NE synthesis could be performed by using assays to detect tyrosine hydroxylase (TH) activity, TH protein and expression of mRNA for TH in whole PVAT and its fraction components (adipocytes and the SVF) to find where NE synthesis occurs. Fluorescence activated cell sorting (FACS) of the SVF into preadipocytes (Contreras et al., 2016), macrophages, and lymphocytes prior to analyzing for TH expression or activity would allow for the narrowing in on the cells types containing TH. To identify TH synthesis *in vivo*, experiments can be conducted to test NE turnover (NETO) in specific adipose depots including PVATs (Brito et al., 2008). These assays can be performed after macrophage depletion by clodronate (Bu et al., 2013) to assess the contribution of macrophages to NETO in fat. Inflammation in adipose tissue is observed in obesity (Kang, 2013), thus TH activity should also be assessed in obesity models.

NE uptake

We identified OCT3 as capable of NE uptake. However, OCT3 did not have a role in PVAT's anti-contractile effect to NE in normal mesenteric resistance arteries with PVAT. A question that remains is, "what are the endogenous substrates for OCT3 in adipose tissue?" The endogenous substrates for OCT3 on adipocytes are not currently

known. An interesting avenue of research would be to investigate the physiological role of OCT3 in adipocyte biology and find its endogenous substrates. Adipocyte-specific knock-out of OCT3 would help provide clues on OCT3 function on adipogenesis, blood pressure, lipolysis, insulin sensitivity and adipokine secretion. The commonly used model of adipocytes, the 3T3-L1 cell line could be used in this research as they do not express OCT3, NET or SERT (data not shown). The 3T3-L1 adipocyte model would allow for selective expression of OCT3 in these cells to study their role on adipocyte biology without having the influence of other transporters present that may complicate result interpretation.

Our contractility studies clearly demonstrated a role for NET-mediated uptake in PVAT's anti-contractile effect on mesenteric arteries exposed to NE (Figure 30). Uptake of NE into mesenteric PVAT was reduced around 30% with nisoxetine (Figure 10). However, uptake of ASP^+ into adipocytes was only inhibited by 20% with nisoxetine (Figure 13). Furthermore, NET signal in Western blots was below the level of detection (Figure 14) suggesting that NET is not strongly expressed in PVAT adipocytes. It is possible that NET is present in the nerves that traverse PVAT. Removal of the celiac ganglion prior to collecting tissues for contractility experiments would help test this hypothesis. It would be expected that the removal of the source of innervation to mesenteric PVAT would also remove PVAT's anti-contractile effect to NE through depletion of NET containing nerves. This surgical intervention may then "unmask" any role that the low affinity but high activity transporter OCT3 may play in uptake of NE. Ultimately, *in vivo* experiments to determine the importance of OCT3 and NET on uptake in PVAT are necessary. Infusion of [^{3}H]NE into rats with and without OCT3 or NET

inhibition could inform us which tissues can accumulate circulating NE and whether the mechanism is OCT3- or NET-dependent. The limitation to this type of experiment is that it would not reflect uptake directly from NE released from nerve varicosities or cells in PVAT but from the circulation. Similar experiments can be performed on animals lacking functional OCT3 or NET. To examine the presence of NET in cell types other than adipocytes, florescence activated cell sorting (FACS) of the cells in the SVF followed by gene expression and immunostaining analysis for NET mRNA and protein, respectively, could be performed. One of the major challenges in studying NET and NE transporters in general is the high level of homology between different transporters and the lack of high affinity and specific antibodies. To overcome this, multiple approaches should be used to include thoroughly validated antibodies and/or techniques that are not antibody based such as qPCR with RNA isolated from the different cell types in PVAT.

A simple question emerges from our studies. If PVAT uptake of NE is inhibited, does this increase blood pressure? Adipocyte specific KO of OCT3 or NET could help in addressing this question. However, a global deletion of OCT3 or NET in adipose tissue will not be specific to PVAT. Currently, there are no common markers for PVAT to target gene disruption to PVAT opposed to other adipose tissue depots.

Now, that we have uncovered the main players of NE uptake in PVAT, we can investigate how this system is perturbed in disease. Investigating PVAT NE uptake function in disease models of obesity, hypertension, and obesity-related hypertension should be a future avenue of research.

NE metabolism

SSAO has multiple roles including adipogenesis (Bour et al., 2007), glucose transport (McDonald et al., 2007), leukocyte adhesion (Salmi and Jalkanen, 1992) and deamination of amines (Elliott et al., 1989). Therapeutics that target SSAO are being explored for their ability to treat liver disease, diabetes, congestive heart failure and most recently chronic obstructive pulmonary disease (Dunkel et al., 2008; Jarnicki et al., 2016). It is necessary to know the vascular effects of targeting SSAO *in vivo* to inform safety assessments of using therapeutics targeted to SSAO on people that have or are at risk of having high blood pressure. In diseases where SSAO levels are elevated, such as in obesity, SSAO metabolism of NE may become physiologically relevant. The effects of chronically high SSAO activity in PVAT have not been investigated. Over time, high SSAO activity in PVAT may increase the levels of advanced glycation products (AGEs) and oxidative stress around the vessel wall. Future studies measuring amine oxidase activity in tissues from models of obesity, hypertension and obesity-related hypertension could answer some of these questions. Substrate specificity for SSAO differs across species (Lyles, 1995), thus it is important to keep this in mind when making conclusions from rat studies. Studies in human tissues would be useful to determine the SSAO activity levels in human PVAT and whether NE serves as a substrate for human SSAO. Investigators interested in targeting SSAO to alleviate inflammatory disease should take our findings in PVAT adipocytes into account and investigate how vascular function in human subjects may be affected. Additionally, caution may also need to be taken when prescribing SSAO inhibitors to patients taking monoamine oxidase inhibitors. This potential for drug-drug interactions should be investigated.

In summary, future experiments that would inform us on the adrenergic system in PVAT should be to 1) identify nerve innervation of PVAT, 2) locate where NE is being stored in PVAT, 3) use ganglionectomy or macrophage depletion to appreciate the contribution of nerves and macrophages to the adrenergic system in PVAT, respectively, and to 4) identify the cell types within PVAT that contribute to the adrenergic system by cell separation, gene expression and cell type-specific knockout of TH (synthesis), NET (uptake), and SSAO/MAO (metabolism).

Future considerations

Defining PVAT

At the center of research on PVAT lies the question of what is considered PVAT. The most common adipose depots that are considered PVAT have been around the thoracic and abdominal aorta, mesenteric arteries and veins, carotid artery and femoral artery (Brown et al., 2014). Additionally, other PVATs have been examined from skeletal muscle, saphenous vein and the renal sinus (Gil-Ortega et al., 2015). What is defined as PVAT follows the common distinction that PVAT is adipose tissue that surrounds arteries and veins. However, most blood vessels are surrounded by adipose tissue, yet only few representative PVATs have been examined. Studying physiological mechanisms in PVAT becomes complex as we do not have a general consensus as to what is considered PVAT. How far away does the adipose tissue have to be from the blood vessel to no longer be considered PVAT? For example, PVAT of the aorta is clearly separate from the surrounding tissue making removal straightforward and easily reproducible. However, PVAT within the skeletal muscle is without clear delineation. Furthermore, arteries and veins within the vascular network of adipose depots such as subcutaneous WAT and IBAT for example, are surrounded by fat. However, this fat is generally not considered "PVAT." This is why it is crucial that great care be taken by researchers to describe the type of adipose tissue they use and any variables that may affect the experimental outcome to aid in interpretation of study results and allow for other laboratories to reproduce the protocols.

Innovative techniques

Current techniques used to investigate the biology of adipose tissue that could be applied to study the adrenergic system in PVAT include transplantation studies (Manka et al., 2014), genetic tagging (Contreras et al., 2014), and optogenetics (Zeng et al., 2015). Conditioned medium from PVAT applied to other cells and tissues can also be useful in studying the effects of PVAT-secreted factors on the SVF and vascular smooth muscle cells. Knockout (KO) models have also served informative and will continue to in the future. However, because the adrenergic system is essential for life and important in development, it can be challenging to interpret results from these models if up-regulation of compensating mechanisms occurs. Conditional knock out models would allow for probing of adrenergic mechanisms specific to adipocytes and specific cell types of the SVF. For example, adipocyte specific knockout of TH would provide insights on the contribution of TH synthesis in adipose tissue to blood pressure. The challenge here would be to separate findings specific to PVAT *versus* other adipose tissue depots as there is a lack of PVAT specific promoters. Models of PVAT deficiency have been generated that may be useful in PVAT research. The A-ZIP/F1 mice lack WAT and mesenteric PVAT; and have reduced amounts of BAT and aortic PVAT (Moitra et al., 1998; Takemori et al., 2007). A-ZIP/F1 mice exhibit enhance contraction to angiotensin II and an elevated blood pressure compared to wild-type mice (Takemori et al., 2007). Adipose tissue loss in this model is not specific to PVAT. A mouse model deficient in peroxisome-proliferator activated receptor gamma (PPARγ) in smooth muscle cells has been generated ($SM22a^{CreKI/CreKI}$ bred with $PPAR\gamma^{flox/flox}$ mice) that lacks aortic PVAT

(Chang et al., 2012). However, mice models lacking only mesenteric PVAT have not been generated.

A challenge in studying PVAT adipocyte biology is that primary adipocytes can only be cultured for a short time. Developments in protocols for culturing primary adipocytes such as ceiling culture, 3-dimensional culture and tissue explants expand the possibilities of PVAT experiments. Selective electroporation of adipocytes to introduce DNA is another useful technique, which is currently a part of the adipose researcher's tool box that could be used to investigate specific biochemical pathways of the adrenergic system (Granneman et al., 2004). A limitation is that mature adipocytes are terminally differentiated and do not divide further. Thus, they cannot be passed in culture. However, preadipocytes can be and much of what we know about adipocyte biology has come from using primary preadipocytes, expanding and differentiating them into adipocytes in culture. Unfortunately, while there are several standard protocols for differentiation in the literature, these protocols are usually modified by each laboratory based on experimental needs and differences in differentiation response depending on the species and adipose depot. Thus, fair comparisons of findings between study to study in these models are challenging to make. It is up to each investigator to select the most appropriate differentiation protocol, validate successful recapitulation of adipocyte function and report thoroughly on their methods.

Why it is important to know a PVAT adrenergic system exists

Our journey through the adrenergic system in PVAT has opened several avenues of research. One direction leads to how PVAT can promote contraction through the release of NE. The other direction leads to how PVAT promotes relaxation by removing NE. A homeostasis is maintained by a balance between the pro- and anti-contractile actions of PVAT which are illustrated in Figure 33. Obesity is a world wide problem (Obesity and overweight (Fact Sheet 311), 2014) and this research comes at a time when we are pressed to find ways to help people suffering obesity-related diseases. If our aim is to protect from and alleviate obesity related problems such as high blood pressure, we need a profound understanding of PVAT physiology and how the homeostatic functions of PVAT are perturbed in disease.

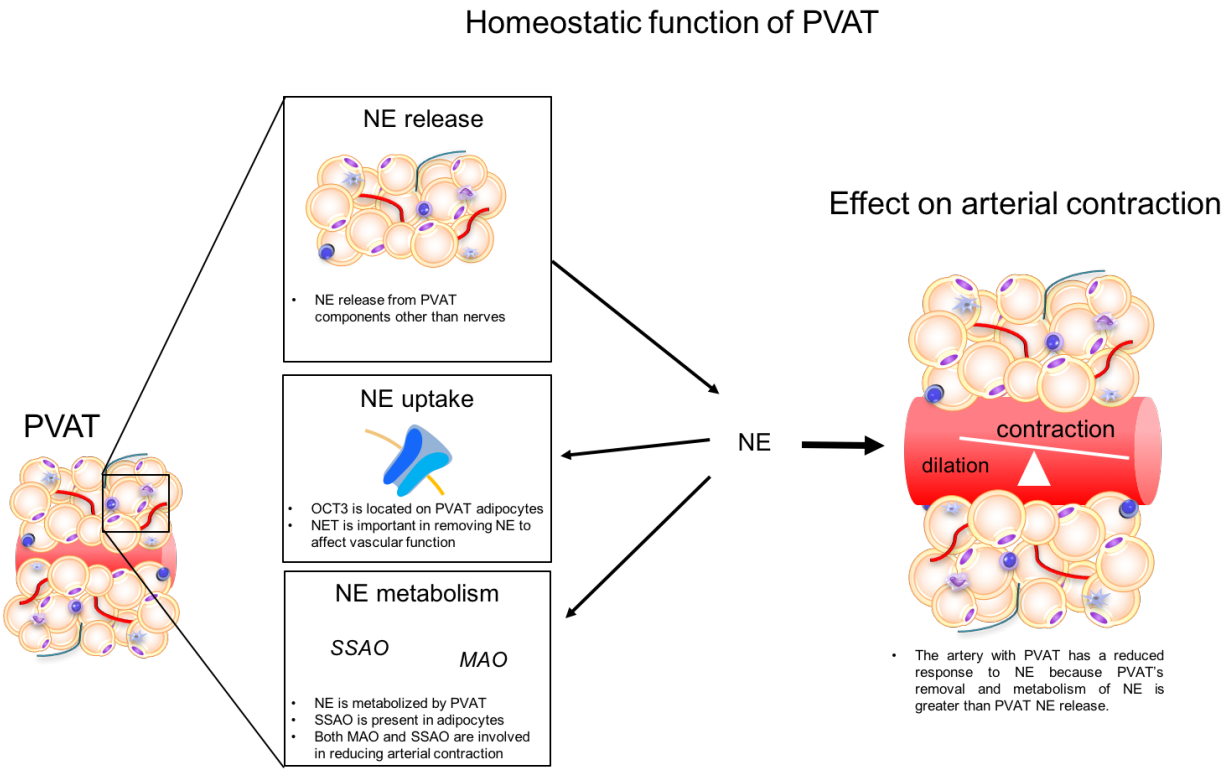


Figure 33. The homeostatic function of PVAT's adrenergic system.

Rat mesenteric PVAT has an adrenergic system that can release, take up and metabolize NE. In health, this system effectively reduces contraction to NE. COMT=catechol-o-methyltransferase, MAO= monoamine oxidase, NE=norepinephrine, SSAO=semicarbazide sensitive amine oxidase, VMAT= vesicle monoamine transporter.

Conclusion

Our findings that PVAT contains an adrenergic system deepened our appreciation for PVAT's role in regulating vascular function. This speaks volumes to the necessity of PVAT research. Especially, when we consider that the vasculature has been most commonly studied after removal the layer of PVAT. Our major findings are that PVAT contains a store of catecholamines that can be released to cause contraction, PVAT can release, take up, and metabolize NE. All of the research in this dissertation has been performed in normal male Sprague-Dawley rats. Now, that we know where to look and what key players to follow, we can move to investigate the adrenergic system in diseases such as obesity, and obesity-related hypertension, in females, and in human tissue. These are just some of the many places to go.

REFERENCES

REFERENCES

Bartness, T. J., Liu, Y., Shrestha, Y. B., and Ryu, V. (2014). Neural innervation of white adipose tissue and the control of lipolysis. *Front Neuroendocrinol* 35, 473–493. doi:10.1016/j.yfrne.2014.04.001.

Bartness, T., and Song, C. (2007). Sympathetic and sensory innervation of white adipose tissue. 48, 1655–1672. doi:10.1194/jlr.R700006-JLR200.

Bartness, T., Vaughan, C., and Song, C. (2010). Sympathetic and sensory innervation of brown adipose tissue. *Int J Obes* 34, S36–S42. doi:10.1038/ijo.2010.182.

Bour, S., Daviaud, D., Gres, S., Lefort, C., Prévot, D., Zorzano, A., et al. (2007). Adipogenesis-related increase of semicarbazide-sensitive amine oxidase and monoamine oxidase in human adipocytes. *Biochimie* 89, 916–925. doi:10.1016/j.biochi.2007.02.013.

Brito, N. A., Brito, M. N., and Bartness, T. J. (2008). Differential sympathetic drive to adipose tissues after food deprivation, cold exposure or glucoprivation. *Am J Physiol Regul Integr Comp Physiol* 294, R1445–R1452. doi:10.1152/ajpregu.00068.2008.

Brown, N. K., Zhou, Z., Zhang, J., Zeng, R., Wu, J., Eitzman, D. T., et al. (2014). Perivascular adipose tissue in vascular function and disease: a review of current research and animal models. *Arterioscler Thromb Vasc Biol* 34, 1621–1630. doi:10.1161/ATVBAHA.114.303029.

Brown, S. W., Meyers, R. T., Brennan, K. M., Rumble, J. M., Narasimhachari, N., Perozzi, E. F., et al. (2003). Catecholamines in a macrophage cell line. *J Neuroimmunol* 135, 47–55. doi:10.1016/S0165-5728(02)00435-6.

Bu, L., Gao, M., Qu, S., and Liu, D. (2013). Intraperitoneal injection of clodronate liposomes eliminates visceral adipose macrophages and blocks high-fat diet-induced weight gain and development of insulin resistance. *AAPS J* 15, 1001–11. doi:10.1208/s12248-013-9501-7.

Bulloch, J. M., and Daly, C. J. (2014). Autonomic nerves and perivascular fat: interactive mechanisms. *Pharmacol Ther* 143, 61–73. doi:10.1016/j.pharmthera.2014.02.005.

Chang, L., Villacorta, L., Li, R., Hamblin, M., Xu, W., Dou, C., et al. (2012). Loss of perivascular adipose tissue on peroxisome proliferator-activated receptor-gamma deletion in smooth muscle cells impairs intravascular thermoregulation and enhances atherosclerosis. *Circulation* 126, 1067–1078. doi:10.1161/CIRCULATIONAHA.112.104489.

Contreras, G. A., Lee, Y., Mottillo, E. P., and Granneman, J. G. (2014). Inducible brown adipocytes in subcutaneous inguinal white fat : the role of continuous sympathetic

stimulation. *AJP Endocrinol Metab* 307, E793–E799. doi:10.1152/ajpendo.00033.2014.

Contreras, G. A., Thelen, K., Ayala-Lopez, N., and Watts, S. W. (2016). The distribution and adipogenic potential of perivascular adipose tissue adipocyte progenitors is dependent on sexual dimorphism and vessel location. *Physiol Rep* 4, e12993. doi:10.14814/phy2.12993.

Dunkel, P., Gelain, A., Barlocco, D., Haider, N., Gyires, K., Sperlágh, B., et al. (2008). Semicarbazide-sensitive amine oxidase/vascular adhesion protein 1: recent developments concerning substrates and inhibitors of a promising therapeutic target. *Curr Med Chem* 15, 1827–1839. doi:10.2174/092986708785133022.

Elliott, J., Callingham, B. A., and Sharman, D. F. (1989). Semicarbazide-sensitive amine oxidase (SSAO) of the rat aorta. Interactions with some naturally occurring amines and their structural analogues. *Biochem Pharmacol* 38, 1507–1515.

Gao, Y.-J. J. (2007). Dual modulation of vascular function by perivascular adipose tissue and its potential correlation with adiposity/lipoatrophy-related vascular dysfunction. *Curr Pharm Des* 13, 2185–2192. doi:10.2174/138161207781039634.

Gil-Ortega, M., Somoza, B., Huang, Y., Gollasch, M., and Fernandez-Alfonso, M. S. (2015). Regional differences in perivascular adipose tissue impacting vascular homeostasis. *Trends Endocrinol Metab* 26, 367–375. doi:10.1016/j.tem.2015.04.003.

Giordano, A., Song, C. K., Bowers, R. R., Ehlen, J. C., Frontini, A., Cinti, S., et al. (2006). White adipose tissue lacks significant vagal innervation and immunohistochemical evidence of parasympathetic innervation. *Am J Physiol Regul Integr Comp Physiol* 291, R1243-55. doi:10.1152/ajpregu.00679.2005.

Granneman, J. G., Li, P., Lu, Y., and Tilak, J. (2004). Seeing the trees in the forest: selective electroporation of adipocytes within adipose tissue. *Am J Physiol Endocrinol Metab* 287, E574–E582. doi:10.1152/ajpendo.00567.2003.

Haunso, A., and Buchanan, D. (2007). Pharmacological characterization of a fluorescent uptake assay for the noradrenaline transporter. *J Biomol Screen* 12, 378–384. doi:10.1177/1087057107299524.

Jarnicki, A. G., Schilter, H., Liu, G., Wheeldon, K., Essilfie, A.-T., Foot, J. S., et al. (2016). The inhibitor of semicarbazide-sensitive amine oxidase, PXS-4728A, ameliorates key features of chronic obstructive pulmonary disease in a mouse model. *Br J Pharmacol* 173, 3161–3175. doi:10.1111/bph.13573.

Josefsson, E., Bergquist, J., Ekman, R., and Tarkowski, A. (1996). Catecholamines are synthesized by mouse lymphocytes and regulate function of these cells by induction of apoptosis. *Immunology* 88, 140–6.

Kang, Y. S. (2013). Obesity associated hypertension: new insights into mechanism. *Electrolyte Blood Press* 11, 46–52. doi:10.5049/EBP.2013.11.2.46.

Kotchen, T. A. (2010). Obesity-Related Hypertension: Epidemiology, Pathophysiology, and Clinical Management. *Am J Hypertens* 23, 1170–1178. doi:10.1038/ajh.2010.172.

Kreier, F., Fliers, E., Voshol, P. J., Van Eden, C. G., Havekes, L. M., Kalsbeek, A., et al. (2002). Selective parasympathetic innervation of subcutaneous and intra-abdominal fat--functional implications. *J Clin Invest* 110, 1243–50. doi:10.1172/JCI15736.

Kvetnansky, R., Ukropec, J., Laukova, M., Manz, B., Pacak, K., and Vargovic, P. (2012). Stress stimulates production of catecholamines in rat adipocytes. *Cell Mol Neurobiol* 32, 801–813. doi:10.1007/s10571-012-9822-6.

Lyles, G. A. (1995). Substrate-specificity of mammalian tissue-bound semicarbazide-sensitive amine oxidase. *Prog Brain Res* 106, 293–303.

Manka, D., Chatterjee, T. K., Stoll, L. L., Basford, J. E., Konaniah, E. S., Srinivasan, R., et al. (2014). Transplanted perivascular adipose tissue accelerates injury-induced neointimal hyperplasia: role of monocyte chemoattractant protein-1. *Arter Thromb Vasc Biol* 34, 1723–1730. doi:10.1161/ATVBAHA.114.303983.

Mann, S. E., Maille, N., Clas, D., and Osol, G. (2014). Perivascular Adipose Tissue: A Novel Regulator of Vascular Tone in the Rat Pregnancy. *Reprod Sci*. doi:10.1177/1933719114561556.

McDonald, A., Tipton, K., O'Sullivan, J., Olivieri, A., Davey, G., Coonan, A. M., et al. (2007). Modelling the roles of MAO and SSAO in glucose transport. *J Neural Transm* 114, 783–786. doi:10.1007/s00702-007-0688-6.

Moitra, J., Mason, M. M., Olive, M., Moitra, J., Mason, M. M., Olive, M., et al. (1998). Life without white fat : a transgenic mouse Life without white fat : a transgenic mouse. 3168–3181. doi:10.1101/gad.12.20.3168.

Nguyen, K. D., Qiu, Y., Cui, X., Goh, Y. P. S., Mwangi, J., David, T., et al. (2011). Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis. *Nature* 480, 104–108. doi:10.1038/nature10653.

Obesity and overweight (Fact Sheet 311) (2014). 2015. Available at: http://www.who.int/mediacentre/factsheets/fs311/en/.

Owen, M. K., Witzmann, F. A., Mckenney, M. L., Lai, X., Berwick, Z. C., Moberly, S. P., et al. (2013). Perivascular adipose tissue potentiates contraction of coronary vascular smooth muscle: Influence of obesity. *Circulation* 128, 9–18. doi:10.1161/CIRCULATIONAHA.112.001238.

Qiu, Y. H., Peng, Y. P., Jiang, J. M., and Wang, J. J. (2004). Expression of Tyrosine Hydroxylase in Lymphocytes and Effect of Endogenous Catecholamines on Lymphocyte Function. *Neuroimmunomodulation* 11, 75–83. doi:10.1159/000075316.

Salmi, M., and Jalkanen, S. (1992). A 90-kilodalton endothelial cell molecule mediating

lymphocyte binding in humans. *Science* 257, 1407–9.

Soltis, E. E., and Cassis, L. A. (1991). Influence of perivascular adipose tissue on rat aortic smooth muscle responsiveness. *Clin Exp Hypertens A* 13, 277–296.

Takemori, K., Gao, Y. J., Ding, L., Lu, C., Su, L. Y., An, W. S., et al. (2007). Elevated blood pressure in transgenic lipoatrophic mice and altered vascular function. *Hypertension* 49, 365–372. doi:10.1161/01.HYP.0000255576.16089.b9.

Török, J., Zemančíková, A., and Kocianová, Z. (2016). Interaction of perivascular adipose tissue and sympathetic nerves in arteries from normotensive and hypertensive rats. *Physiol Res* 65, S391–S399.

Vargovic, P., Ukropec, J., Laukova, M., Cleary, S., Manz, B., Pacak, K., et al. (2011). Adipocytes as a new source of catecholamine production. *FEBS Lett* 585, 2279–2284. doi:10.1016/j.febslet.2011.06.001.

Vargovic, P., Ukropec, J., Laukova, M., Kurdiova, T., Balaz, M., Manz, B., et al. (2013). Repeated immobilization stress induces catecholamine production in rat mesenteric adipocytes. *Stress* 16, 340–352. doi:10.3109/10253890.2012.736046.

Zeng, W., Pirzgalska, R. M., Pereira, M. M. A., Kubasova, N., Barateiro, A., Seixas, E., et al. (2015). Sympathetic neuro-adipose connections mediate leptin-driven lipolysis. *Cell* 163, 84–94. doi:10.1016/j.cell.2015.08.055.