

ORIGINAL ARTICLE

Perivascular adipose tissue contains functional catecholamines

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Keywords

Blood pressure, catecholamines, indirect sympathomimetic, perivascular adipose tissue

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Funding Information

This work was funded by National Institutes of Health (NIH) PO1 HL070687.

Received: 27 February 2014; Accepted: 4 March 2014

Pharma Res Per, 2 (3), 2014, e00041, doi:10.1002/prp2.41

doi: 10.1002/prp2.41

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. High-pressure liquid chromatography, 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 norepinephrine (NA), dopamine (DA) and the tryptamine serotonin (5-hydroxytryptamine [5-HT]) from PVAT isolated from both arteries. In both arteries, tyramine-induced concentration-dependent contraction was rightward-shifted and reduced by the noradrenaline transporter inhibitor nisoxetine (1 $\mu\text{mol/L}$), the vesicular monoamine transporter inhibitor tetrabenazine (10 $\mu\text{mol/L}$), and abolished by the α adrenoreceptor antagonist prazosin (100 nmol/L). 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 NA 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.

Abbreviations

TTX, tetrodotoxin; EFS, electric field stimulation; PSS, physiological salt solution; A, adrenaline; DA, dopamine; CGX, celiac ganglionectomy; NET, noradrenaline transporter; PBS, phosphate buffered saline; PE, phenylephrine; PVAT, perivascular adipose tissue; RA, rat aorta; RMA, rat mesenteric artery; SGx, sham ganglionectomy; VMAT, vesicular monoamine transporter; NA, norepinephrine.

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 (Gollasch and

Dubrovskaya 2004; Brandes 2007; Chaldakov et al. 2007; Aghamohammadzadeh and Heagerty 2012; Aghamohammadzadeh et al. 2012; 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 (Gollasch and Dubrovskaya 2004; Fesus *et al.* 2007). 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 (Gao *et al.* 2006; Galvez-Prieto *et al.* 2008; Lee *et al.* 2009; Huang *et al.* 2010; Payne *et al.* 2010; Owen *et al.* 2013), but the focus has largely been on the anticontractile factors. While studying the role of kynurenes 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 (NA), and adrenaline (A). 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 (Burn and Rand 1958; Nasmyth 1962; Broadley 2010), given its ability to be taken up by the noradrenaline 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. High-pressure liquid chromatography (HPLC) measures of catecholamine content and release, imaging of NA, 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.

Materials and Methods

Animal model

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

High-pressure liquid chromatography

Catecholamine, 5-hydroxytryptamine (5-HT), and tyramine measurements were made by homogenizing the tissue in four times their weight of 0.1 mol/L perchloric 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°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⁻¹ for 5-HT.

Glyoxylic acid staining

Sections (5 mm × 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) for 5 min. The solutions were emptied from the dishes and the sections blown dry with N₂ gas. The dried sections then were placed on glass slides and heated to 120°C for 5 min. 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 (Melville, NY) using a 4',6-diamidino-2-phenylindole 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). Sections were incubated for 24 h with an NA-specific antibody (ab887; Abcam, Cambridge, MA) or no primary antibody at 4°C. Sections were developed using a DAB (3,3-diaminobenzidine) developing solution (Vector Laboratories, Burlingame, CA). Slides were counterstained with Vector Hematoxylin (30 sec). Sections were photographed on a Nikon TE2000 inverted microscope using MMI[®] Cellcut Software (MMI, Haslett, MI).

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 into half. All blood clots on the face of PVAT were removed. In a microfuge tube, PVATs were incubated in 500 μ L physiological salt solution (PSS in mmol/L; NaCl 130; KCl 4.7; KH₂PO₄ 1.8; MgSO₄ \times 7H₂O 1.7; NaHCO₃ 14.8; dextrose 5.5; CaNa₂ ethylenediaminetetraacetic acid 0.03, CaCl₂ 1.6 [pH 7.2]) containing 1 μ mol/L of the monoamine oxidase inhibitor pargyline (Sigma Chemical Company) and vehicle or nisoxetine (1 μ mol/L) for 30 min at 37°C prior to the addition of either vehicle (water) or tyramine (10⁻⁴ mol/L; Sigma Chemical Co.). Tissues incubated for 1 h at 37°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). 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°C by a heating circulator, were filled with oxygenated PSS. Rings were placed under optimum resting tension (4 g for rat thoracic aorta, 1.2 g for rat superior mesenteric artery) and equilibrated for 1 h. An initial concentration of 10 μ mol/L phenylephrine-tested (PE) arterial viability

and validation of an intact endothelial cell layer was tested by acetylcholine-induced (1 μ mol/L) 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 min) necessary for a response to plateau prior to the next addition. In some experiments, either vehicle (water, 0.1% dimethylsulfoxide [DMSO]) or inhibitor was added for 1 h 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 msec, frequency 20 Hz, voltage 120 V). If electric field stimulation (EFS)-induced contraction was observed, tetrodotoxin (TTX), a fast sodium channel inhibitor, was incubated for 30 min 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 celiac ganglionectomy (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 sham ganglionectomy (SGx) underwent a sham operation that was performed by accessing and exposing the celiac plexus only. All rats were given an intramuscular injection of piperacillin. Animals were used after a recovery period of 5 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°C prior to isolation of amines. In some experiments, the superior mesenteric artery was used for measurement of isometric contraction.

Materials

Phenylephrine hydrochloride, acetylcholine hydrochloride, tyramine hydrochloride, and inhibitors were purchased

from Sigma Chemical Company (citalopram hydrochloride, desipramine hydrochloride, fluoxetine hydrochloride, nisoxetine hydrochloride, prazosin hydrochloride, tetrabenazine) or Tocris Bioscience (part of R&D Systems, Minneapolis, MN; LY53857, GBR 12935, Sibutramine metabolite 2 BTS 54-505).

Statistical analyses

Data are reported as mean \pm SEM for number of animals indicated in parentheses (*N*). HPLC are reported as ng gram tissue⁻¹. Histochemical and immunohistochemical images depict sections incubated with and without primary antibody, and are representative of a minimum of four (4) 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 mean \pm SEM as force (mg) or as a percentage of the initial contraction to a maximal concentration of PE, 10 μ mol/L. Potency values ($-\log EC_{50}$, mol/L) 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 analysis of variance (ANOVA) was performed after confirming the normality of data distribution. Equality of data variances was tested using the *F*-test (Stat-Plus/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, NA, and A in fat that surrounds the aorta (aortic PVAT), the brown fat pad (interscapular), fat that surrounds the superior mesenteric artery (Mes PVAT), and the fat directly behind the left kidney (a white adipose tissue, retroperitoneal). Figure 1A demonstrates that PVATs (aortic and mesenteric) contain significant levels of NA relative to the well-known, sympathetically dependent brown fat pad (scapular). Importantly, both DA and A could be detected in all tissues, but most prominently in the mesenteric PVAT. The NA measured in the mesenteric PVAT was visualized in the cytoplasm of the adipocyte through glyoxylic acid histochemistry (Fig. 1B); aortic PVAT was too dense to visualize. NA was also detected immunohistochemically in mesenteric PVAT (Fig. 1C), observed primarily in what appears to be adipocyte cytoplasm and consistent with glyoxylic acid staining.

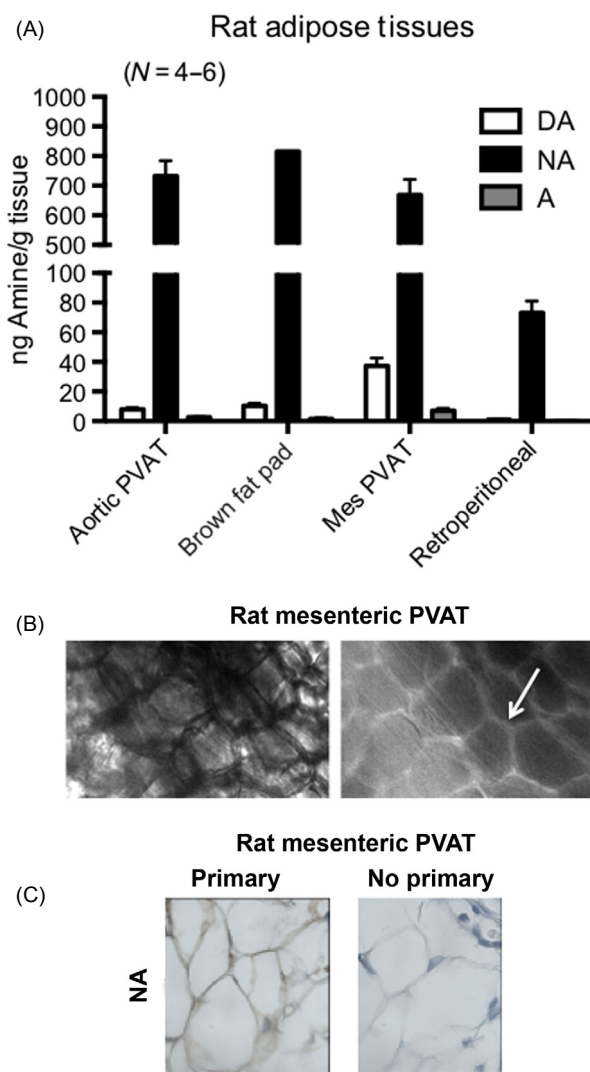


Figure 1. (A) HPLC measures of catecholamine content (ng g tissue⁻¹) in aortic PVAT, brown fat pad (interscapular), mesenteric PVAT, and retroperitoneal fat from the same rats. Bars represent mean \pm SEM for number of animals in parentheses. (B) Representative image of glyoxylic acid staining of rat mesenteric PVAT where the left hand side is bright field picture, right hand side fluorescent glyoxylic acid image. Representative of four number of animals. (C) Detection of NA in the superior mesenteric PVAT. Representative of four individual animals. Left panel are images from sections exposed with primary antibody (primary), 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} mol/L) stimulated release of NA, DA, and 5-HT from the mesenteric (Fig. 2A) and aortic (Fig. 2B) PVATs into the surrounding buffer. Tyramine

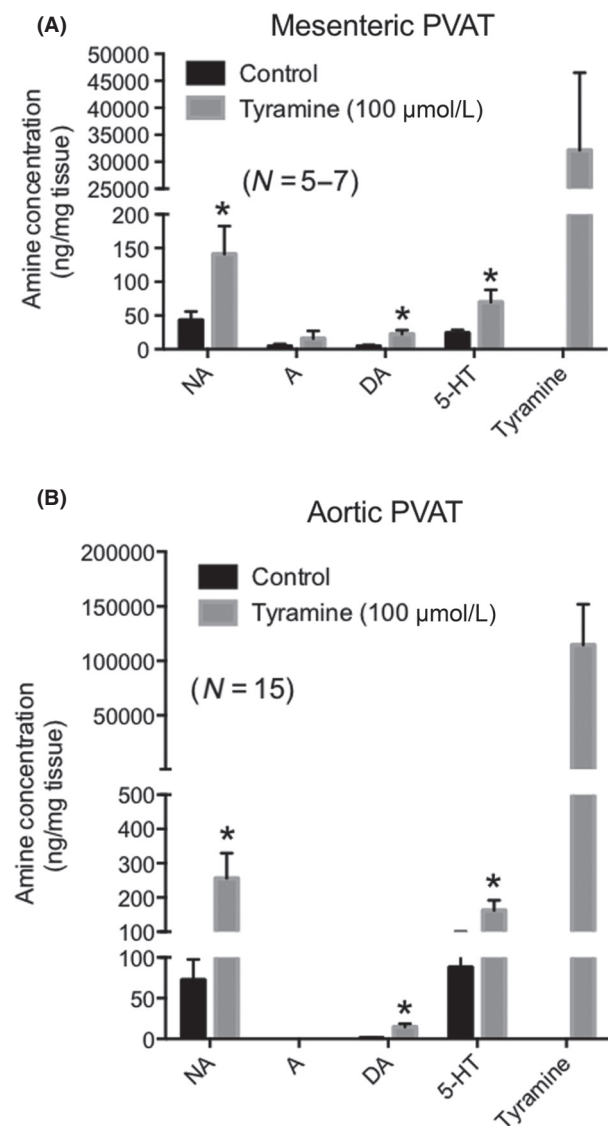


Figure 2. Tyramine-released catecholamines from mesenteric (A) and aortic (B) PVAT as measured through HPLC. Bars represent mean \pm SEM for number of animals in parentheses. Solid bars are values for samples incubated with normal PSS, gray bars for samples incubated with 10^{-4} mol/L Tyramine. *Statistically significant increase ($P < 0.05$) versus appropriate control.

was detected in HPLC such that absence or addition of tyramine to samples could be appropriately validated. In separate experiments, nisoxetine ($1 \mu\text{mol/L}$ preincubation) reduced tyramine-induced NA release in aortic PVAT (tyramine = 623 ± 144 ng gram tissue $^{-1}$, tyramine and nisoxetine = 403 ± 38 ng gram tissue $^{-1}$, $P < 0.05$). Tyramine caused a concentration-dependent contraction of the isolated thoracic aorta (rat aorta; RA) and superior mesenteric artery (rat mesenteric artery; RMA) in arteries with intact PVAT and minimal contraction in arteries with

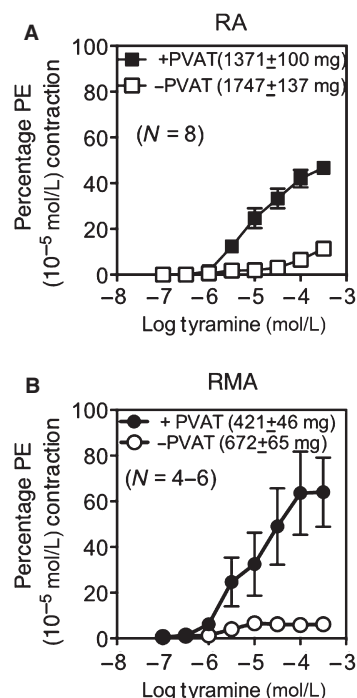


Figure 3. Tyramine-induced contraction in the isolated RA (A) and RMA (B) of the normal Sprague-Dawley rat. Points represent mean \pm SEM for the number of animals in parentheses. Values in key legend are the response in milligrams to 10^{-5} mol/L PE.

PVAT removed (Fig. 3A and B, respectively). All tissues without PVAT possessed a robust contraction to a maximum concentration of the α_1 adrenoceptor agonist PE (values in parentheses in key). A representative tracing of tyramine-induced contraction in the RMA is shown in Figure 4A, left. Contraction was relatively slow to develop. The α_1 adrenoceptor antagonist prazosin ($1 \mu\text{mol/L}$) significantly reduced tyramine-induced contraction when added directly, illustrated in Figure 4A. This was quantified in both arteries when using a 10-fold lower concentration of prazosin (100 nmol/L) (Fig. 4A, right). These data raised the possibility that sympathetic nerve fibers within PVAT could be the source of catecholamines.

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 (Fig. 4B). 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 nmol/L) and by prazosin (100 nmol/L), indicating that EFS-induced contraction was mediated primarily by sympathetic nerves and stimulation of adrenoceptors.

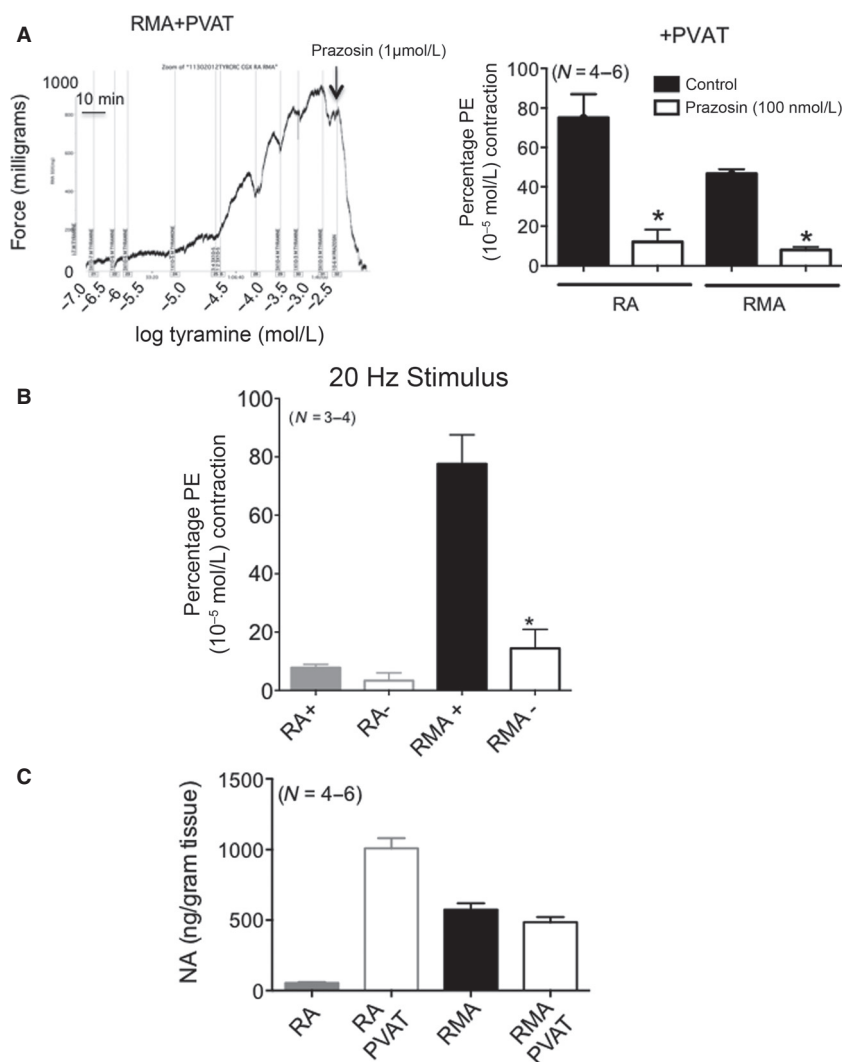


Figure 4. (A) Left: Inhibition of tyramine-induced maximum contraction by the α_1 adrenoceptor antagonist prazosin (1 $\mu\text{mol/L}$). Right: Quantification of inhibition of tyramine-induced contraction by a 10-fold lower concentration of prazosin. Bars represent mean \pm SEM for number of animals in parentheses. *Significant difference versus appropriate control values ($P < 0.05$). (B) Contraction of isolated RA and RMA +/-PVAT to maximum electrical field stimulation (20 Hz). (C) NA content in artery proper and PVAT around artery for the RA and RMA. Bars represent mean \pm SEM for number of animals in parentheses. *Statistically significant increase ($P < 0.05$) versus +PVAT values.

TTX (300 nmol/L) did not modify tyramine-induced contraction itself. Importantly, all tissues used in EFS experiments contracted to a maximal concentration of PE (10 $\mu\text{mol/L}$, mg: RA + PVAT = 1229 ± 264 ; RA - PVAT: 1911 ± 365 ; RMA + PVAT = 732 ± 116 ; RMA - PVAT = 1154 ± 343). The relative NA content of the artery and arterial PVAT is compared in Figure 4C. NA content was significantly higher in aortic PVAT versus the aorta, while NA 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. CGX reduced the content of NA in the superior mesenteric artery PVAT by 38%; A and 3,4-dihydroxyphenylalanine were similarly reduced when compared to SGx rats (Fig. 5A). These values are quantitatively lower than those shown in Figure 1. We attribute this to the fact that the tissues from the animals used to generate data in Figure 5 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

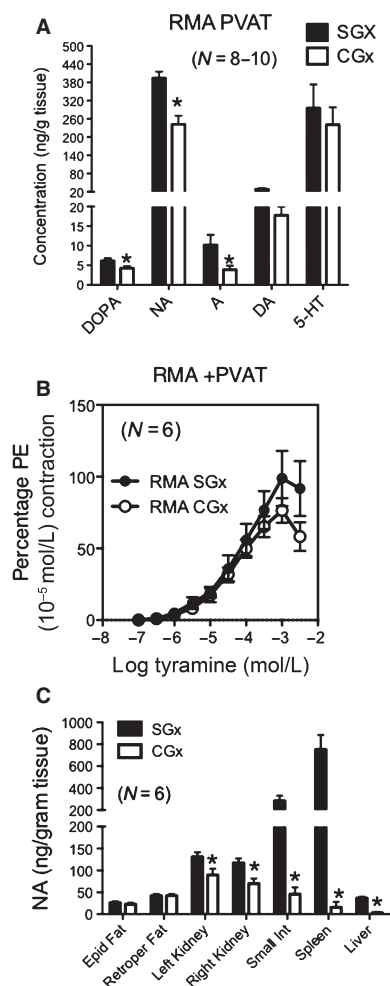


Figure 5. (A) Quantitation of RMA PVAT catecholamines in animals with a sham (black; SGx) or (white; CGx) celiac ganglionectomy. (B) Tyramine-induced RMA + PVAT contraction in animals with SGx or CGx surgery. (C) HPLC validation of CGX through reduced NA content of those tissues known to be innervated by the celiac ganglion (liver, small intestine and spleen). Bars represent mean \pm SEM for number of animals in parentheses. *Statistically significant increase ($P < 0.05$) versus SGx values.

RMA + PVAT from CGx versus SGx rats (~22% reduction; Fig. 5B). Tyramine potency was not different between the two groups. The dramatic reduction in the liver, small intestine, and splenic content of NA when comparing ganglionectomized to control (Fig. 5C) supports successful ganglionectomy.

Tyramine-induced PVAT-dependent contraction depends on the NET

As 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 6, separated by the vehicle used in each experiment (A: water; B: 0.1% DMSO). Table 1 reports pharmacological parameters ($-\log EC_{50}$ as potency values, maximum contraction) for the data presented in Figure 6 and, for some interventions, the RMA under the conditions described below (shown in Fig. 6). Findings using LY53857, GBR 12935, and fluoxetine in the RA are shown only in Table 1 given that they were negative in outcome. Statistical comparisons are not shown in Figure 6 for the sake of 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 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 metabolite 2 of the NET/SERT inhibitor sibutramine, BTS 54-505. Inhibition of the DA transporter inhibitor by GBR 12935 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 (Fig. 6A). 5-HT, though released by tyramine in the RA PVAT, does not participate in tyramine-induced contraction, as the 5-HT₂ receptor antagonist LY53857 did not modify tyramine-induced contraction (Table 1). Finally, the VMAT inhibitor tetrabenazine shifted tyramine-induced contraction modestly to the right and reduced maximum contraction compared to vehicle (Fig. 6B).

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 RA. Tyramine-induced contraction was also inhibited by nisoxetine (1 μ mol/L), but the maximum contraction recovered to a greater extent in the RMA versus RA.

Discussion and Conclusions

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 (Gollasch and Dubrovskaya 2004; Brandes 2007; Chaldakov et al. 2007; Aghamohammadzadeh and Heagerty 2012; Aghamohammadzadeh et al. 2012; Szasz

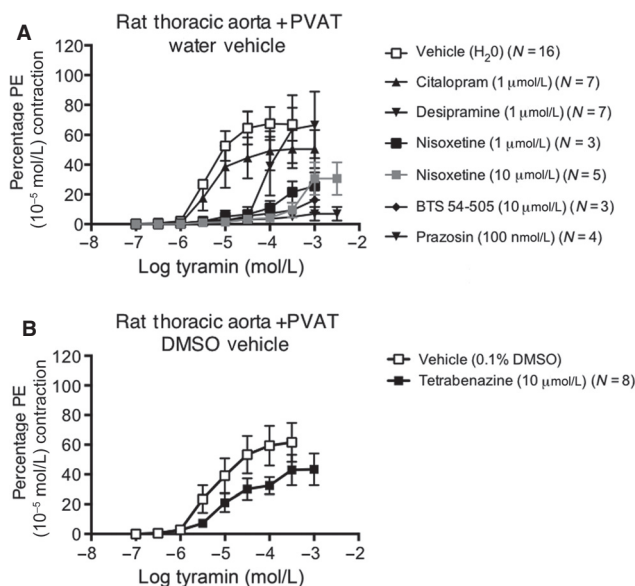


Figure 6. (A) Tyramine-induced contraction in the RA +PVAT in the presence of a series of inhibitors compared to water-based vehicle. (B) Tyramine-induced contraction in the RA + PVAT in the presence of the VMAT inhibitor tetrabenazine or DMSO vehicle. Points represent mean \pm SEM for the number of animals in parentheses.

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 (Gao et al. 2006; Galvez-Prieto et al. 2008; Lee et al. 2009; Huang et al. 2010; Payne et al. 2010; Owen et al. 2013). This study identifies catecholamines as substances that have the potential to participate in the influence of PVAT on arterial function. NA 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 adrenoceptor-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.

PVAT contains catecholamines

Adipocytes, mesenchymal adipose-derived stem cells, fibroblasts, endothelial cells, small blood vessels, preadipocytes, and blood cells make up PVAT (Kershaw and Flier 2004; Divoux and Clement 2011; Peinado et al. 2012). Immunohistochemical staining, glyoxylic acid staining, and HPLC measurement of NA provide three different measures of the presence of NA in PVAT. The adipocyte cytoplasm appears to be one site of NA localization in PVAT, evidenced by both glyoxylic acid and immunohistochemical staining. Future experiments will likely need to take an electronic microscopic approach to determine whether catecholamines are concentrated vesi-

cularly in adipocytes in the way they are in neurons. Electronic microscopic 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 (Vargovic et al. 2011; Kvetnansky et al. 2012) first published the findings of NA 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 NA, DA, and 5-HT from PVAT from both RA and RMA PVAT, and tyramine-induced contraction was abolished by blockade of α adrenoceptors. 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 NA in adipocytes and the stromal vascular fraction, we did investigate the potential contribution of sympathetic nerves to NA content in PVAT.

Table 1. Pharmacological parameters of tyramine-induced contraction in isolated RA and RMA + PVAT.

Tissue intervention	Tyramine – log EC ₅₀ (mol/L)	Tyramine% PE contraction
RA + PVAT		
Vehicle (water)	5.34 ± 0.06	67.00 ± 11.1
Citalopram (1 μmol/L)	5.36 ± 0.04	50.40 ± 12.8
Desipramine (1 μmol/L)	4.05 ± 0.01*	66.60 ± 22.0
Nisoxetine (1 μmol/L)	3.65 ± 0.34*	25.50 ± 9.3*
Nisoxetine (10 μmol/L)	3.39 ± 0.07*	30.60 ± 11.0*
BTS 54-505 (10 μmol/L)	NC	16.30 ± 8.4*
Prazosin (100 nmol/L)	NC	6.98 ± 4.6*
LY53857 (100 nmol/L)	5.47 ± 0.03	35.00 ± 8.7
GBR 12935 (100 nmol/L)	5.45 ± 0.05	48.90 ± 16.2
Fluoxetine (100 nmol/L)	5.42 ± 0.07	56.20 ± 15.1
Vehicle (0.1% DMSO)	5.26 ± 0.06	61.62 ± 13.1
Tetrabenazine (10 μmol/L)	4.86 ± 0.13	43.44 ± 10.7*
RMA + PVAT		
Vehicle (0.1% DMSO)	4.87 ± 0.04	73.80 ± 20.0
Tetrabenazine (10 μmol/L)	4.38 ± 0.03	41.41 ± 10.5*
Nisoxetine (1 μmol/L)	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

Points represent mean ± SEM for values calculated from figures presented. NC, not convergent.

*Significantly different from vehicle response.

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 NA 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 in 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 NA 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 NA in the PVAT around the RA does not exist in sympathetic nerve terminals and that the PVAT either takes up circulating NA and/or makes NA.

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 CGx, a time during which reinnervation 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 NA content. In the PVAT around the superior mesenteric artery from the same rats, NA content fell from 418 ± 30 to 261 ± 38 ng gram tissue⁻¹ or a 38% fall. Qualitatively, these values were lower than shared in Figure 1, 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% versus 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 NA 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 innervat-

ing 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 NA 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 NA 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; Nasmith 1962) revealed that this NA source in PVAT is dynamic. Tyramine is initially taken up by NET (Goldstein 2008; Broadley 2010). Although 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 (Goldstein 2008; Broadley 2010). Tyramine displacement of NA from storage vesicles is the most commonly cited action of tyramine. The released NA builds up cytoplasmically and leaves the cell through reverse transport by proteins such as NET. In this way, tyramine can be doubly dependent on 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 NA 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 NA 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 PE. 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, DAT inhibitor GBR 12935, 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 C57Bl6 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 versus PVAT-independent function and thus be too nonspecific 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 anticontractile 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 anticontractile factors, typically produced by PVAT, mask the catecholamine pool which might become functional (revealed) when anticontractile factors are lost in disease. This remains to be determined.

Conclusion, Novelty, and Significance

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.

Acknowledgements

This work was funded by National Institutes of Health (NIH) PO1 HL070687.

Disclosures

None declared.

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