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

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A dynamic adrenergic system that affects blood vessel contraction exists in PVAT (5). Catecholamines are released from PVAT upon the addition of tyramine, a sympathomimetic drug, leading to contraction of the rat aorta and superior mesenteric artery (5). Moreover, pharmacological inhibition of NE transporters (NET) reduces the PVAT-dependent vascular contraction to tyramine (5). Soltis and Cassis (64) discovered that inhibition of NE uptake in the rat aorta abolished the anticontractile effect of PVAT. Collectively, this work led us to investigate the presence of a NE uptake system in PVAT. To test the hypothesis that PVAT has a NE uptake mechanism, functional NE uptake assays, fluorescence imaging, and PCR of mesenteric and aortic PVAT were performed. The presence of PVAT on blood vessels generally reduces vessel contraction in response to various agonists, including norepinephrine (NE) (64). Knowledge on how these mechanisms interact to influence the anticontractile properties of PVAT in NE-induced contraction is not complete (36).

The anticontractile effect of PVAT is lost in obesity and hypertension, implicating PVAT as an integral link between both of these diseases (3). Over one-third of all adults in the United States are hypertensive (13), a condition that significantly increases the risk of death from myocardial infarction or stroke (42). A major risk factor for hypertension is obesity (29). Globally, 13% of adults are obese (have a body mass index of $\geq 30$ (73a), and in the United States, the number is higher, with 34.9% of adults classified as obese (53). In obesity, dysfunction of the anticontractile effect of PVAT is observed along with overall changes in adipocyte function (3). Thus, the relationship between the adipocytes within PVAT and blood vessel function is of interest.

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The present study tested 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 mesenteric resistance arteries increases peripheral resistance, a contributing event toward 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 (40).

PVAT of mesenteric resistance arteries most closely resembles white adipose tissue in that it contains adipocytes that have
large unilocular lipid droplets (10). We focused 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 used PVAT from normal male Sprague-Dawley rats for HPLC measures of NE in PVAT and isolated adipocytes. We also measured uptake of NE and used pharmacological inhibitors to transporters to reveal the main transporters that transport NE in PVAT. Confocal microscopy of PVAT was used using the fluorescent NE transport substrate dye methylpyridinium ASP+ (61) in addition to immunohistochemistry, immunocytochemistry, and gene expression analysis of mesenteric and aortic PVAT to reveal the role of organic cation transporter 3 (OCT3).

MATERIALS AND METHODS

Chemicals. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The pharmacological inhibitors nisoxetine (inhibits NET), citalopram [inhibits the 5-hydroxytryptamine (serotonin) transporter (SERT)], corticosterone (inhibits OCT3), and desipramine [inhibits NET and SERT at the concentration used (10 μM)] were purchased from Bio-Techne (Minneapolis, MN). Pargyline (a monoamine oxidase inhibitor), Ro 41-0960 (a catechol o-methyltransferase inhibitor), and NE were purchased from Sigma-Aldrich. ASP+ was synthesized and provided by James N. Wilson (University of Miami, Miami, FL) (73).

Animals. Male Sprague-Dawley rats (225–275 g or ~8–10 wk of age, Charles River, Indianapolis, IN) were used. All protocols were approved by the Institutional Animal Care and Use Committee of Michigan State University and followed the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (8th ed., 2011). Rats were anesthetized with pentobarbital sodium (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 were placed in microcentrifuge tubes containing physiological salt solution (PSS) [containing (in mM) 130 NaCl, 4.7 KCl, 1.8 KH2PO4, 1.7 MgSO4·7H2O, 14.8 NaHCO3, 5.5 dextrose, 0.03 CaNa2EDTA, and 1.6 CaCl2·(pH 7.2)] within 30 min of tissue removal 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 min at 37°C. The concentrations were selected based on their specificity for the transporter in question. NE (10 μM) or vehicle (either H2O or ethanol) was added for another 30 min. 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, samples were thawed and sonicated for 3 s. Samples were centrifuged at 18,000 g for 15 min 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 (catalog no. BCA1, Sigma-Aldrich).

ASP+ uptake. The mesenteric arcade was dissected from Sprague-Dawley rats and stored in PSS without calcium [containing (in mM) 140 NaCl, 5 KCl, 1 MgCl2·7H2O, 10 HEPES, and 10 glucose; pH 7.4] at 4°C until use, for up to 5 h. Immediately before 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 superfused with PSS with calcium (1.8 mM CaCl2·2H2O) 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 superfused 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 superfused 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 ASP+ uptake experiments where nisoxetine or citalopram were used, tissue imaging was performed with a Leica DMLFA confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Yokogawa CSU10 spinning disk confocal head (Yokogawa, Tokyo, Japan) coupled to a XR-Mega10 intensified charge-coupled device (Stanford Photonics, Palo Alto, CA) using a ×40 water-immersion objective. Illumination was provided by an X-cite Exacte Illuminator (Excites Technologies, Waltham, MA). Images were recorded with Piper-Control (Stanford Photonics) and analyzed using ImageJ (NIH). For the corticosterone, citalopram with corticosterone, 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) controlled by µManager (66a) was used for image acquisition. Images were captured as stacks of 50 TIFF (16-bit) images, which were then combined with the average z-projection function in ImageJ. Fluorescence intensity was quantified in relative fluorescent units.

Sample preparation of the mesenteric PVAT, mesenteric resistance vessels, adipocytes, and stromal vascular fraction. 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). PVAT was either flash frozen for whole PVAT measurements or digested to obtain separate cellular factions by the following protocol. PVAT was added to 1 ml PSS with 1 mg/ml collagenase from Clotstridium histolyticum type IA (catalog no. CS9891, Sigma-Aldrich) and incubated at 37°C with slow rotation until fully digested (~1 h). PVAT was centrifuged at 200 g for 5 min, and the stromal vascular fraction (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 200 g for 10 min. For immunocytochemistry, mesenteric PVAT adipocytes were resuspended in PSS and centrifuged onto CellTak (catalog no. 54240, BD Biosciences, Bedford, MA)-coated slides using a Cytospin 4 cytocentrifuge (700 g for 2 min), and an aliquot was saved to assess purity using a hemacytometer. For Western blots, the mesenteric PVAT, mesenteric resistance vessels, adipocytes, and SVF were added to RIPA buffer solution (catalog no. R3792, Teknova, Hollister, CA) with protease inhibitors (0.5 mM PMSF, 1 mM orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) into a 2-ml bead tube (Ommi, Kennesaw, GA). Tissues were homogenized using the Omni Bead Ruptor Homogenizer (Omni) and centrifuged for 15 min at 18,000 g, and supernatants were saved for Western blot analysis. Supernatants were quantified for protein content using a Bicinchoninic Acid Protein Assay Kit (catalog no. BCA1, Sigma-Aldrich). For mRNA isolation and HPLC analysis, the adipocytes and SVF, were placed into separate tubes with PSS and centrifuged 200 g 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 analysis for NET. Fifty micrograms of protein from the mesenteric PVAT, mesenteric resistance vessels, adipocytes, SVF, AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00308.2015 • www.ajpheart.org

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and vena cava (positive control) were separated on a 10% SDS gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked in 4% (wt/vol) chicken egg ovalbumin in Tris-buffered saline and Tween 2 (TBST) for 3 h at 4°C and then incubated with primary antibody [mouse anti-NE (1:500, NET05-2, Mab Technologies, Stone Mountain, GA) and mouse anti-β-actin (1:2,000, A3854, Sigma)] diluted in blocker overnight at 4°C. The blot was washed with TBST (10 min each, three times) and then incubated with IRDye anti-mouse secondary antibody (1:1,000, no. 926-32210, Li-Cor, Lincoln, NE) diluted in Odyssey Blocking Buffer (no. 927-40000, Li-Cor) for 1 h at 4°C. The blot was washed with TBST (10 min each, three times) and developed on the Li-Cor Odyssey. Densitometric analysis was done using ImageJ.

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. PVAT was then dissected from the aorta on a Sylgard-coated petri dish with the use of a stereomicroscope. 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.

RNA extraction. The Investigative Histopathology Laboratory at Michigan State University prepared the fresh frozen rat aorta slides. RNA was extracted with the Quick RNA MiniPrep kit (catalog no. R1054, Zymo Research, Irving, CA), and purity (260-to-280 nm absorbance ratio) was measured using a Nanodrop spectrophotometer (Thermoscientific, Wilmington, DE). RNA was reverse transcribed with qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). RT-PCR was performed using FAST SYBR Green MasterMix (catalog no. 4385612, Applied Biosystems, Foster City, CA) on the ABI 7500 Fast Real Time PCR system (Life Technologies, Carlsbad, CA) using the following parameters: 95°C for 20 s, 95°C for 1 s, and 60°C for 20 s for 40 cycles. The primer sequences for Slc22a3 (OCT3 gene) amplification were as follows: forward 5'-TATGCAGCGGACAGATACGG-3' and reverse 5'-AAAAATTCGGTGCAAACGCCCA-3' (Integrated DNA Technologies, Coralville, IA). The fluorescence intensity ratio (F/F0) was used to quantify OCT3 expression. Images were analyzed using the “surface plot” function in ImageJ (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 OCT3 expression, the fluorescence intensity ratio (F/F0) was used, where F is the fluorescence intensity after incubation with the inhibitor and ASP and F0 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 NE found in PVAT is in the adipocyte fraction (Fig. 1A). Measures of NE by HPLC in mesenteric PVAT and isolated mesenteric PVAT adipocytes were similar (P > 0.05). Representative images of each sample are shown in Fig. 1A, bottom left and bottom right. The white box in Fig. 1A, bottom left, 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

![Fig. 1. Norepinephrine (NE) is present in perivascular adipose tissue (PVAT) adipocytes, and PVAT can take up extracellular NE. A: mesenteric PVAT adipocytes were isolated, and NE content was measured by HPLC. Measures were normalized to tissue weight. Bottom left, representative image of the mesenteric PVAT used. The white box highlights the portion of PVAT used in the experiments in this study. Bottom right, representative image of adipocytes isolated from mesenteric PVAT. B: addition of NE (10 µM) to PVAT in physiological saline solution (PSS) for 30 min increased NE accumulation, as verified by the "Fire" lookup table and constructed surface plots by applying the "surface plot" function in ImageJ (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 fluorescence intensity ratio (F/F0) was used, where F is the fluorescence intensity after incubation with the inhibitor and ASP and F0 is the fluorescence intensity of ASP incubated with vehicle minus the background intensity.](http://ajpheart.physiology.org/)

Statistical analyses were performed using GraphPad Prism 6.0 (La Jolla, CA). When two groups were compared, 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 more than two groups were compared, ANOVA with a Newman-Keuls test was used. With non-normally distributed data, Kruskal-Wallis ANOVA was used followed by a Dunn’s test for multiple comparisons. The tests were unpaired. P values of <0.05 were considered statistically significant. Means ± SE are 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. Pseudocolorization of ASP+ -stained images was performed using the “Fire” lookup table and constructed surface plots by applying the “surface plot” function in ImageJ (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 fluorescence intensity ratio (F/F0) was used, where F is the fluorescence intensity after incubation with the inhibitor and ASP+ minus the background intensity and F0 is the fluorescence intensity of ASP+ incubated with vehicle minus the background intensity.
mediated by transporters. First, we established that NE uptake occurs in PVAT (Fig. 1B). PVAT was then incubated with NE (10 µM) or vehicle in PSS for 30 min, the tissue was washed to remove excess NE, and NE in the tissue was measured by HPLC. As shown in Fig. 1B, mesenteric PVAT NE content was significantly increased after the addition of NE (10 µM) compared with 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 preincubation with inhibitors of NET, SERT, and OCT3: nisoxetine (1 µM), citalopram (100 nM) with corticosterone (100 µM), and desipramine (10 µM) with corticosterone (100 µM). Desipramine, citalopram, or corticosterone alone did not significantly reduce NE uptake compared with vehicle (Fig. 2). These data support transporter-mediated uptake of NE in mesenteric PVAT through NET, SERT, and OCT3.

**ASP**

fluorescently labels monoamine transporters on PVAT adipocytes. The transporter substrate ASP** was used to identify the presence of NET on PVAT adipocytes. ASP** fluoresces upon binding and is transported and accumulates in mitochondria (61). 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 (Fig. 3A). 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** (Fig. 3B). 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 (Fig. 4A, top). The surface plot image of the fluorescence intensity levels with pseudocolorization showed this more clearly (Fig. 4A, bottom). 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 (Fig. 4B, with the top showing an image without color information and the bottom showing the image with pseudocolor; arrows point to perinuclear staining) (19). Perinuclear ASP** fluorescence in PVAT adipocytes suggests transport of ASP** into the adipocyte as opposed to only surface binding.

Preincubation of PVAT with an excess of NE (1 mM) reduced ASP** uptake (Fig. 5). Any residual binding of ASP** in the presence of this saturating concentration of NE can be considered nonspecific 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 (Fig. 5). Representative images are shown of the fluorescence obtained with vehicle only with ASP** (2 µM), background (no ASP**), and each experiment after preincubation with NE or an inhibitor of transport (Fig. 5). 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 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 experiments of NE transport into PVAT (Fig. 2).
**NET is not present in mesenteric PVAT adipocytes.** The presence of NET 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 analysis. We did not observe bands for NET in adipocytes or the SVF but did observe some faint bands for NET in the mesenteric PVAT, resistance vessels, and vena cava, our positive control (Fig. 6), indicating that the 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 (Fig. 5); therefore, we focused on OCT3 for the rest of the experiments.

**OCT3 is present in PVAT adipocytes.** PVAT expression of Slc22a3 (the gene for OCT3) was compared with its relative expression in the heart, a positive control (15) (Fig. 7A). Mesenteric and aortic PVATs expressed Slc22a3 at higher relative expression than in the heart (Fig. 7, A and B). Mesenteric PVAT was separated into adipocytes and the SVF to allow for the assay of Slc22a3 expression in each fraction. Relative expression of Slc22a3 was higher in adipocytes than in the SVF (Fig. 7C). We used the reference gene β₂-microglobulin because it gave us the most similar expression among all of the sample types compared with other housekeepers we assayed (data not shown). The cycles at which each sample reached threshold for β₂-microglobulin were similar between the heart and mesenteric PVAT (C_T: 20.0 and 19.4, respectively) as well as the heart and aortic PVAT (C_T: 17.8 and 17.5, respectively) but were dissimilar for the heart, adipocytes, and 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 in the heart, 23.0 in adipocytes, and 30.7 in the SVF). Immunostaining revealed OCT3 protein in aortic PVAT (Fig. 8A) and mesenteric PVAT adipocytes (Fig. 8B) using the aorta as a positive control for OCT3 (70). Aortic staining for OCT3 was located to the tunica media (labeled M in Fig. 8A) but not the tunica adventitia (labeled V in Fig. 8A). Immunostaining for OCT3 was present on the periphery of the adipocytes in both aortic and mesenteric PVATs (Fig. 8, A and B). Immunostaining was not present when the primary antibody was excluded (Fig. 8, C and D). Inhibition of OCT3 with corticosterone (100 µM) reduced NE uptake in aortic PVAT by 47.0 ± 11.0% (Fig. 8E). These data support the presence of OCT3 on adipocytes and the potential for OCT3 to transport NE in PVAT.

**DISCUSSION**

PVAT modulates blood vessel function (5, 9, 14, 67, 68), and, in the current study, 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 vascular smooth muscle to induce contraction and vasoconstriction. This could be a physiologically relevant mechanism by which PVAT modulates vascular tone. In the present 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 (12). NE released from the nerves that innervate PVAT and the vascular smooth muscle cell could be taken up by PVAT, thus reducing vascular contraction. 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, as evidenced by the discovery that mesenteric adipose tissue adipocytes synthesize NE and serotonin (66, 69). We previously demonstrated that PVATs possess measurable

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Fig. 4. ASP⁺⁺ is transported into mesenteric PVAT adipocytes. ASP⁺⁺ (2 µM) was added to mesenteric PVAT for 10 min in PSS and imaged by confocal microscopy. A: pseudocolored representation of the fluorescence intensity of ASP⁺⁺ (2 µM) binding revealing that ASP⁺⁺ binds along on the periphery of the adipocyte in a punctate pattern (top). The surface plot of the same image shows this more clearly (bottom). Refer to the RFU 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 a PVAT adipocyte imaged at the level of the nucleus. Arrows point to areas of perinuclear staining. Pseudocolorization of the images (bottom) shows that intense staining was present in the perinuclear region. Images are representative of six animals. Scale bars = 50 µm. Samples were imaged with a ×40 objective.
catecholamines (5). The source of these catecholamines may be from the adipocyte fraction (Fig. 1A). Other sources of NE could be found in the SVF of PVAT, such as macrophages (11), lymphocytes (58), and neurons. The catecholamines present in PVAT are releasable by tyramine and support contraction in the rat superior mesenteric artery independent of sympathetic innervation (5). PVAT-dependent contraction to tyramine was reduced by the NET inhibitor nisoxetine (5), directing us further into the investigation of NE transport in PVAT. In the present study, we used the transporter inhibitors desipramine [10 μM, inhibitory constant ($k_i$) for NET: 7.36 nM (55) and for SERT: 129 nM (54)], nisoxetine [1 μM, $k_i$ for NET: 0.46 nM and for SERT: 158 nM (16)], and corticosterone [100 μM, OCT3 IC$_{50}$: 120–290 nM (39)]. Desipramine, a NET inhibitor, at higher concentrations can also inhibit SERT [$k_i$: 129.00 nM (54)] and OCT3 [IC$_{50}$ of 700 nM (75)]. Since our experimental samples were intact tissues, not isolated transporters or membranes, each inhibitor was used at a concentration that was 50–100 times above the $k_i$ values to assure the inhibitors reached their target, considering 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) was even lower than with one. These possibilities include the inhibitory effects of two drugs on only one transporter, or the inhibitory effect of one drug on the other. However, this was not investigated further in the present study.

Fig. 5. ASP$^+$ uptake is reduced by transporter inhibitors. Mesenteric PVAT was incubated with inhibitors of transport or vehicle for 10 min followed by the addition of ASP$^+$ (2 μM). Data are reported as percent uptake from vehicle. Numbers above the bars indicate numbers of animals (N) used in each experiment. Values are means ± SE. *P < 0.05, **P < 0.005, and ***P < 0.001 vs. vehicle; #P < 0.05 vs. corticosterone. Representative images from each experiment are located beneath the corresponding bar.

Fig. 6. NE transporter (NET) is not located in mesenteric PVAT (mPVAT) adipocytes. Whole PVAT was assayed for NET by Western blot along with the mesenteric resistance vessels (MRV), PVAT adipocytes (adip), and PVAT stromal vascular fraction (SVF). The arrow points to the band of interest. β-Actin was used as the loading control, and the vena cava was used as a positive control. N = 4.
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, dopamine (DA), and serotonin uptake by specific inhibitors (30). 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 (18, 32, 37). 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 OCT isoforms, is required for the transporter to be placed on the plasma membrane (38). While oligomerization would be interesting to study, it 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 PVATs.

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, as previously validated in NE uptake assays using radiolabeled

Fig. 7. Slc22a3 mRNA is expressed in mesenteric and aortic PVAT. A: relative mesenteric (mes) PVAT expression of Slc22a3 mRNA was measured and compared with the heart as a positive control. B: whole aortic PVAT relative expression of Slc22a3 was measured and compared with the heart as a positive control. C: PVAT adipocyte relative expression of Slc22a3 mRNA was higher than that in the SVF (**P < 0.005) compared with the heart as a positive control. Measures were normalized to B2M. Values are means ± SE for the number of animals (N) shown. **P < 0.005.

Fig. 8. Immunohistochemical and immunocytochemical staining revealing the presence of organic cation transporter 3 (OCT3) in aortic PVAT and mesenteric PVAT adipocytes and that aortic PVAT NE uptake is reduced by inhibition of OCT3. A: the aorta was used as the positive control for OCT3. Aortic staining for OCT3 protein was evident in the media (M) but not in the adventitia (V). PVAT (P) around the aorta also stained for OCT3. L, lumen. B: mesenteric PVAT 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: the aorta with PVAT was stained without the inclusion of the primary antibody to serve as a negative control. D: mesenteric PVAT adipocytes were stained without the inclusion of the primary antibody as a negative control. Adipocyte images are representative of four animals. Samples were imaged with a ×40 objective. Scale bar = 50 µm. E: NE uptake of aortic PVAT was assayed after incubation with vehicle or corticosterone (100 µM). Data are reported as percentages from vehicle, and measures are expressed as NE concentration to protein content. Values are means ± SE for the number of animals (N) shown. *P < 0.05.
Although in vitro NE transport influences the contractility of blood vessels to NE (5, 64), there is no confirmation that this occurs in vivo. The mesentery and omentum are considered “visceral fat” (33, 52), and the adipose tissue around mesenteric resistance arteries is most specifically referred to as mesenteric PVAT. We showed an image of mesenteric PVAT in Fig. 1 to clarify this point. Mesenteric PVAT is a common PVAT depot that is studied for its function NE transport molecules on adipocytes when NE enters the cell. The finding that ASP was capable of binding and being transported 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 punctuate pattern of ASP fluorescence in the adipocyte is strikingly similar to that observed when adipocyte mitochondria were stained using rhodamine 123 (19). 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 (8), additionally supporting our observation that ASP was able to bind and be transported into the cell. The finding that ASP was calopram sensitive is in line with the finding that adipocytes express functional SERT (66). Moreover, a study by Pizzinat et al. (57) found that [3H]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. 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 nonspecific for NET. Although convincing evidence that NET is present on mesenteric PVAT adipocytes was not found (Fig. 6), it is possible that ASP is a more sensitive tool for the detection of NET. We did not investigate the presence of DA transporters (DAT) further due to the finding that GBR-12935 [100 nM, a DAT inhibitor, k: 3.7 nM (59)] 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 plays a role in NE uptake in PVAT.

The “anticontractile” effect of PVAT to NE in the rat thoracic aorta is attenuated by desipramine plus deoxycorticosterone (64), and this observation was the impetus to study PVAT NE transport. Since the anticontractile effect of PVAT due to NE transport at least in the rat aorta has already been shown (64), we did not pursue these experiments in the present study. 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 experiments. Interestingly, in contrast to our NE uptake experiment, which required both desipramine and corticosterone or calopram and corticosterone to reduce uptake, ASP fluorescence was reduced by preincubation with corticosterone or calopram alone. This could be due to a difference in transporter affinity for ASP versus NE. ASP has been used as a surrogate for [3H]NE, due to the similarity in their pharmacological profiles, but there are differences in their affinity to certain transporters (28). This was also evidenced by the observation that adding a high concentration of NE to saturate NET failed to abolish ASP staining, indicating the presence of ASP fluorescence nonspecific to NE transport mechanisms. Schwartz et al. (61) observed nonspecific fluorescence of ASP in experiments using human embryonic kidney cells. Therefore, care needs to be taken when interpreting findings from ASP-binding studies to mechanisms of specific NE transport. Both ASP and NE uptake experiments pointed to OCT3 as being important, and, thus, the was the focus in our final experiments.

**OCT3 is highly expressed in mesenteric and aortic PVATs.** OCT3 is a low-affinity, high-capacity uptake transporter, formally known as extraneuronal monoamine transporter and also termed as the uptake-2 system, and is broadly expressed in non-neuronal cells (51). OCT3 expression on PVAT adipocytes has not yet been investigated. We were surprised to find a higher expression of OCT3 in mesenteric and aortic PVATs 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 (57). This would support the idea that the function of NE transporters is to deliver NE into the cell to be metabolized.

We observed high expression of OCT3 in aortic PVAT and inhibition of OCT3 reduced NE uptake. The presence of OCT3 in the aorta, a conduit artery, would lead one to question the role of OCT3 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 (57), but there may be other physiological roles for OCT3 that may not involve blood pressure regulation, such as polyamine transport (60) and clearance of toxins (35). Therefore, OCT3 may be more necessary in the aortic PVAT due to its other roles versus mesenteric resistance PVAT. This would have to be investigated further.

**Limitations.** Although in vitro NE transport influences the contractility of blood vessels to NE (5, 64), there is no confirmation that this occurs in vivo. The mesentery and omentum are considered “visceral fat” (33, 52), and the adipose tissue around mesenteric resistance arteries is most specifically referred to as mesenteric PVAT. We showed an image of mesenteric PVAT in Fig. 1 to clarify this point. Mesenteric PVAT is a common PVAT depot that is studied for its relevance to alterations in vascular response and blood pressure (5, 23, 43, 49, 71, 72), whereas PVAT around skeletal muscle arteries is more associated with mechanisms of insulin resistance (7, 27, 47, 48, 74). Therefore, mesenteric PVAT was the most relevant PVAT for us to study.

Aortic PVAT was included in the immunohistochemistry, NE uptake, and RT-PCR analyses as this is the most discrete and widely studied PVAT depot and was therefore useful when we compared our findings with those of other studies. Nonetheless, it should be noted that mesenteric PVAT mechanisms of NE uptake are more physiologically relevant with regard 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 analysis for NET lacked strong reactivity to the positive control (Fig. 6), 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 using knockout animals and/or small interfering RNA toward differ-
ent 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 (17). This has been observed in the brains of NET knockout mice (63), which exhibit increased SERT and DAT expression. In addition, mice with reduced SERT expression overexpress OCT3 and exhibit increased serotonin clearance through OCT3 (6). These points have been discussed in a previous report as it pertains to SERT knockout rats (44). This 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 knockout rodent in this study.

The anticontractive effect of PVAT due to NE transport at least in the rat aorta was discovered by Soltis and Cassis in 1991 (64), 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 anticontractile effect of PVAT. We fully recognize that other mechanisms exist that reduce NE-induced contraction, such as the release of adiponectin from PVAT adipocytes (26). 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. In addition, 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 euthanized the animal before 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. (31) reported inhibition of NE (noradrenaline) and DA release after exposure of rat brain striatal slices to barbiturates, including pentobarbital. Other groups have similarly reported an inhibition of NE release (34, 50, 62) or no effect (41, 56) by pentobarbital in their studies. Our laboratory has previously euthanized 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 was euthanized 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 was 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⁺.

Conclusions, novelty, and significance. The present 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 study 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 (1). 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 rat superior mesenteric artery was reduced by tetrabenazine (a vesicular monoamine transporter inhibitor). This finding, published by our group, supports that local stores of NE in PVAT could contribute to vascular contraction (5). 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. The role of PVAT 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 (nondisease model) rats to test whether uptake is an important physiological mechanism in PVAT. Understanding the normal functional characteristics of NE uptake in the nondiseased 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.

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AUTHOR CONTRIBUTIONS

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