Perivascular adipose tissue promotes vasoconstriction:
The role of superoxide anion
Yu-Jing Gaoca,*, Kumiko Takemoria, Li-Ying Sua, Wen-Sheng Ana, Chao Lua, Arya M. Sharmab, Robert M.K.W. Leea

a Smooth Muscle Research Program and Department of Anaesthesia, McMaster University, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5
b Department of Medicine, McMaster University, Hamilton, Ontario, Canada

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Abstract

Objectives: Recent studies have demonstrated that perivascular adipose tissue (PVAT) releases vascular relaxation factor(s). In this study, we examined if PVAT releases other vasoactive factors in response to perivascular nerve activation by electrical field stimulation (EFS).

Methods and results: In Wistar-Kyoto rats, rings of superior mesenteric artery (MA) with intact PVAT (PVAT (+)) showed a greater contractile response to EFS than rings with PVAT removed (PVAT (−)). Superoxide dismutase (SOD) reduced the contractile response to EFS more in PVAT (+) MA than in PVAT (−) MA. Inhibitors of NAD(P)H oxidase and cyclooxygenase exerted a greater inhibition on EFS-induced contraction in PVAT (+) MA than in PVAT (−) MA. Inhibitors of tyrosine kinase (tyrphostin A25) and MAPK/ERK (U 0126) attenuated EFS-induced contraction in PVAT (+) MA in a concentration-related manner, while inactive forms of these inhibitors (tyrphostin A1 and U 0124) did not inhibit the response. Exogenous superoxide augmented the contractile response to EFS and to phenylephrine in PVAT (−) MA, and this augmentation was blunted by inhibition of tyrosine kinase and MAPK/ERK. EFS increased superoxide generation in isolated PVAT and PVAT (+)/−) MA, which was attenuated by NAD(P)H oxidase inhibition. RT-PCR showed the mRNA expression of p67phox subunit of NAD(P)H oxidase and immunohistochemical staining confirmed its localization in the adipocytes of PVAT.

Conclusion: These results show that PVAT enhances the arterial contractile response to perivascular nerve stimulation through the production of superoxide mediated by NAD(P)H oxidase, and that this enhancement involves activation of tyrosine kinase and MAPK/ERK pathway.

Keywords: Adipose tissue; Contraction; MAPK; Mesenteric artery; Perivascular nerve stimulation; Superoxide anion

1. Introduction

Despite the fact that perivascular adipose tissue (PVAT) surrounds almost all systemic blood vessels, the possible role of PVAT in modulating vascular function had received little attention. In studies with isolated vessels, PVAT is routinely removed. Although Solitiis and Cassis [1] demonstrated that the contractile response of rat aorta to norepinephrine was attenuated in the presence of PVAT, they attributed this attenuation to increased uptake of this monoamine in the presence of PVAT. By virtue of its location and its ability to produce a host of vasoactive substance [2,3], PVAT has the potential to regulate vascular function, but the specific role of PVAT in this regulation remains largely unknown. Recent studies including ours have shown that PVAT releases relaxation factor(s) in rat aorta [4,5], rat mesenteric artery [6], and human internal thoracic artery [7]. We have further shown that the ability of the aortic PVAT to release this relaxation factor was impaired in obese rats induced by prenatal exposure to nicotine [8]. Although the principle of this putative relaxation factor remains to be determined, these findings strongly suggest that PVAT indeed plays an important physiological/pathological role in modulating vascular function.
The modulation of vascular function by PVAT may not be limited to the secretion of a relaxing factor. Most of the regulation in biological system is composed of a balance between acting and counteracting factors. For example, vascular endothelial cells have the potential to produce both relaxing factors (e.g. nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor) and contracting factors (e.g. endothelins and endothelium-derived contracting factor), depending on the type of stimulation. Furthermore, epidemiological studies have shown that accumulation of body fat (obesity) is a significant etiologic factor for hypertension [9,10], which is characterized by an increased peripheral resistance due to enhanced vasoconstriction. The finding that PVAT releases relaxation factor(s) does not provide explanation to this association between obesity and hypertension, although many other factors could be involved. Here we report that PVAT also enhances the contractile response elicited by perivascular nerve activation by electrical field stimulation (EFS), and that this enhancement involves activation of tyrosine kinase and MAPK/ERK pathway by superoxide generated in PVAT.

2. Methods

2.1. Animals

Male Wistar-Kyoto rats (WKY) at 25–32 weeks of age were obtained from the rat colony maintained at the McMaster University Central Animal Facilities. This study conforms with the guidelines of the Canadian Council on Animal Care and was approved by the Animal Care Committee of McMaster University.

2.2. Contractility study

The procedure for the preparation of superior mesenteric artery (MA) rings has been described in our previous reports [11,12]. Briefly, the rat was euthanized by an overdose of sodium pentobarbital (60 mg/kg, i.p.), and the MA was collected in oxygenated physiological salt solution (PSS) with the following composition (in mM): NaCl, 119; KCl, 4.7; KH2PO4, 1.2; MgSO4, 1.2; NaHCO3, 25; CaCl2, 1.6; glucose, 11, at 4 °C. Paired MA rings (4 mm long), one with PVAT intact (PVAT (+)) and the other with PVAT removed (PVAT (−)), were prepared from each artery. The average weight of PVAT attached to MA was 3.9 ± 0.6 mg (n = 16).

PVAT removal was carried out with fine scissors under a dissection microscope. Caution was taken not to damage the adventitial layer during the dissection. To examine the integrity of the adventitial layer after PVAT removal, some rings were fixed in 10% formaldehyde and embedded in paraffin. Cross sections of MA were stained either with Gomori’s trichrome to measure adventitial layer thickness, or with silver stain to estimate nerve ending density [13]. The dimension of adventitia was analyzed with the software of Image-Pro Plus (Silver Spring, MD, USA), and the density of silver staining in the adventitia was estimated with the software of ImageJ (NIH, MD, USA).

A computerized myograph system was used to study the contraction and relaxation response of the MA. After equilibration for at least 60 min, the arterial rings were challenged with 60 mM KCl twice at an interval of 30 min. Contractile response was elicited with EFS (0.85 ms, 150 V, a 10-s train, at 2, 6, 12 and 20 Hz at an interval of 3–5 min) as described previously [14]. Contractile responses to EFS and phenylephrine were expressed as a percentage of KCl contraction. Relaxation response to sodium nitroprusside was tested in MA rings precontracted with phenylephrine (1 μM for PVAT (−), and 3 μM for PVAT (+), to generate an equivalent pre-contraction level in these arteries), and expressed as a percentage of the precontraction value.

To study the role of superoxide anion, one pair of PVAT (+) and PVAT (−) MA rings were treated with superoxide dismutase (SOD, 200 U/ml, a superoxide scavenger enzyme), and the other pair treated with solvent served as control, for 2 h before exposure to EFS. In another set of experiment, pyrogallol, a superoxide generator, was added to PVAT (−) MA 2–5 min prior to EFS/phenylephrine, to study the direct effects of exogenous superoxide on the contractile response. Arterial preparations were incubated with inhibitors of nitric oxide synthase (Nω-nitro-L-arginine, L-NNA), NAD(P)H oxidase (apocynin and diphenyleneiodonium chloride (DPI)), cyclooxygenase (diclofenac), xanthine oxidase (allopurinol), cytochrome P450 monooxygenase (17-octadecynoic acid), tyrosine kinase (tyrphostin A25), and MAPK/ERK (U 0126) for 25 min before exposure to EFS. The effects of tyrphostin A1 and U 0124, the inactive forms of tyrphostin A25 and U 0126, were also tested to exclude non-specific effects of these inhibitors. Recovery of response to EFS was tested 60 min after washout of SOD or 30 min after washout of other enzyme inhibitors. The inhibitory effect of SOD, apocynin, DPI, and diclofenac was expressed as a percentage of the control. The involvement of α-adrenoceptor in EFS-induced contraction was tested with tetrodotoxin (1 μM), a sodium channel blocker of nerve endings, 15 min prior to EFS.

2.3. Superoxide production by PVAT with lucigenin-enhanced chemiluminescence

Superoxide production by a 4-mm-long segment of MA with and without PVAT and by PVAT alone was measured with lucigenin (5 μM)-enhanced chemiluminescence (recorded every minute for 8 min by a luminometer: T-20/20, USA) [15]. The average weight of isolated PVAT was 2.3 ± 0.2 mg (n = 6). Superoxide level was measured before and immediately after EFS (12 Hz). In some experiments, the effects of NADH (10 μM, 25 min) to stimulate superoxide production and the effects of DPI (100 μM, 25 min) to inhibit
EFS- and NADH-induced superoxide production were examined. Chemiluminescence with the respective background subtracted was expressed as units/min. We had established with our preliminary experiments that EFS with buffer alone did not induce detectable level of superoxide production under this condition (data not shown).

2.4. Visualization of superoxide production by adipocytes detected with fluorescent dye dihydroethidium (DHE)

Blocks of PVAT were embedded in Optimal Cutting Temperature compound (OCT from Tissue-Tek®) and flash frozen in liquid nitrogen immediately after EFS stimulation (0 Hz or 12 Hz). Each frozen section (30 μm thick) was covered with 3 μM DHE on top and a cover slip was applied [16]. Slides were incubated in a light-protected humidified chamber at 37 °C for 30 min, and were viewed with a fluorescence microscope (Olympus 1X81, Japan. Excitation: 488 nm; emission: 610 nm). In a separate group of experiments, tissues were incubated with SOD (600 U/ml, 60 min) prior to EFS. Images were obtained using the software of Image-Pro Plus (Silver Spring, MD, USA).

2.5. Immunohistochemistry staining for NAD(P)H oxidase subunit p67phox in the adipocytes of PVAT

PVAT was fixed in 10% formaldehyde and embedded in paraffin. Sections (4–6 μm in thickness) were treated in citrate buffer for antigen retrieval (120 °C for 6 min). All the following staining steps were carried out in humidified chamber and manufacturer-recommended procedures (Santa Cruz Biotechnology, USA) were followed. Briefly, endogenous peroxidase activity was blocked with 1% hydrogen peroxide, followed by incubation with 1.5% blocking serum. Rabbit polyclonal anti-p67phox antibody was used in a 1:50 dilution overnight at 4 °C. The second antibody, biotinylated goat anti-rabbit, was applied for 2 h at room temperature in 1:200 dilutions with PBS containing 0.5% blocking serum. Slides were then exposed to avidin and biotinylated horseradish peroxidase (1:50 dilution, 1 h), followed by staining with a solution containing 3,3’-diaminobenzidine tetrahydrochloride for 5–8 min. Slides treated only with the 2nd antibody were used as negative control. Hematoxylin was used as a counter-stain.

2.6. Detection of p67phox mRNA from adipocytes of PVAT by reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNAs of adipocytes isolated from PVAT were prepared as described by Engeli et al. [17] with TRIzol reagent (Invitrogen). RT-PCR was carried out using the RETRO script (Ambion, USA), according to the manufacturer’s instructions. One microgram of total RNA was reverse-transcribed by MMLV-RT at 44 °C for 1 h, 92 °C for 10 min. Using complementary DNA, PCR amplification was performed with gene-specific primers for p67phox (forward, 5’-CAGTTCAGCTGTGGCTGCT-3’; reverse, 5’-CTCTGGCGCAGCTGACCA-3’) [18]. The conditions were 40 cycles of denaturation at 94 °C (1 min), annealing at 65 °C (1 min), and extension at 72 °C (1 min), followed by a further 7-min extension. The purified p67phox PCR product was confirmed by sequencing on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, USA).

2.7. Chemicals

Allopurinol, apocynin, diclofenac, DPI, lucigenin, L-NNA, polyethylene glycol (PEG), prazosin hydrochloride, pyrogallol, SOD-PEG, sodium nitroprusside, and tetrodotoxin were purchased from Sigma, USA; tyrphostin A 25, tyrphostin A1, U 0126, and U 0124 from Calbiochem, USA, and DHE from Molecular Probes (Eugene, Oregon, USA). DPI, apocynin, tyrphostine A25, tyrphostin A1, U 0126, and U 0124 were dissolved in dimethyl sulfoxide (DMSO). DHE stock solution was made in nitrogen-purged DMSO and diluted in filtered PSS. All other agents were dissolved in deionized water and prepared fresh before use. Polyclonal antibodies for p67phox and ABC staining system were purchased from Santa Cruz Biotechnology (California, USA). TRIzol reagent was purchased from Invitrogen (Burlington, ON, Canada), and RETROscript was from Ambion (Austin, TX, USA).

2.8. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM) where n represents the number of rats. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by post hoc t test for concentration-dependent or frequency-dependent effects, or by Student’s t-test for comparison between MA with and without PVAT, using the software of SigmaStat (SPSS, Inc, Chicago, USA). The differences were considered significant when P ≤ 0.05.

3. Results

3.1. Effects of dissection on adventitia and nerves

Dissection did not affect the integrity of adventitia (thickness in μm, 35.6 ± 5.6 for PVAT (+) rings, and 32.1 ± 4.4 for PVAT (−) rings, n = 6 for each; P = 0.84) and the density of the nerves (in arbitrary units, 71.3 ± 7.5 for PVAT (+) rings, 77.6 ± 2.9 for PVAT (−) rings; n = 7 for each; P = 0.45).

3.2. PVAT on force generation by the arteries

Maximum force generated in response to 60 mM KCl was not different between PVAT (+) and PVAT (−) MA rings (in grams: 0.75 ± 0.08, 0.67 ± 0.08 for PVAT (+) and
PVAT (+) MA, respectively, \( n = 14 \) for each, \( P = 0.45 \)), although the onset of contraction was slower in PVAT (+) MA than in PVAT (–) MA (Fig. 1A, B). Relaxation response to sodium nitroprusside (10\(^{-6}\)M) in phenylephrine-precontracted arteries was similar in these rings (in % of pre-contraction: 90 ± 3.3 in PVAT (+) MA and 91 ± 3.8 in PVAT (–) MA, \( n = 5 \) for each, \( P = 0.9 \)).

### 3.3. Contraction to perivascular nerve stimulation

EFS elicited a frequency-dependent contractile response in both PVAT (+) and PVAT (–) MA, but the amplitude of contraction was higher in PVAT (+) MA than in PVAT (–) MA (Fig. 1C, D, E). The contraction to EFS was abolished by tetrodotoxin (1\(^{-6}\)M) and by prazosin (1\(^{-6}\)M) (data not shown).

### 3.4. Effects of SOD on the contraction to perivascular nerve stimulation

Incubation with SOD-PEG (200 units/ml) markedly reduced the contraction to EFS in PVAT (+) MA, and removal of SOD partially restored the response (Fig. 2A–D). In PVAT (–) MA, SOD also reduced the contraction response to EFS, but the reduction was less prominent as compared with that of PVAT (+) MA (Fig. 2D and E). Furthermore, contractile response became similar between PVAT (+) and PVAT (–) MA after incubation with SOD (\( P > 0.05 \)). The contraction of the artery to KCl was not affected by SOD in both PVAT (+) and PVAT (–) MA (in grams, for before and after SOD treatment, respectively; PVAT (+) MA: 0.79 ± 0.08 and 0.75 ± 0.09, \( P = 0.51 \); PVAT (–) MA: 0.74 ± 0.11 and 0.7 ± 0.08, \( P = 0.49 \); \( n = 6 \) for each). PEG did not affect the contraction to EFS (data not shown).

### 3.5. Effects of inhibition of superoxide-producing enzymes and of tyrosine kinase-MAPK/ERK pathway on the contraction to perivascular nerve stimulation

Treatment with NAD(P)H oxidase inhibitor (apocynin (100 \(\mu\)M) and DPI (10 \(\mu\)M)) and cyclooxygenase inhibitor (diclofenac, 10 \(\mu\)M) attenuated the contraction to EFS (12 Hz) more in the PVAT (+) MA than in PVAT (–) MA (Fig. 3A and C). Tyrosine kinase inhibitor tyrphostin A25 and MAPK/ERK inhibitor U 0126, but not the inactive forms of these enzymes (tyrphostin A1 and U 0124), concentration-depen-

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**Fig. 1.** (A and B) Typical recording of contractile response to KCl (60 mM) in MA with (PVAT (+)) and without PVAT (PVAT (–)). Maximal force developed was similar in both preparations. (C and D) Typical contractile response of PVAT (+) and PVAT (–) arteries to electrical field stimulation (EFS), and the results are summarized in (E). *\( P < 0.05 \) versus PVAT (–) arteries (\( n = 9 \) rats, 30–32 weeks).

**Fig. 2.** (A and B) Typical recording showing the inhibition of contractile response to electrical field stimulation (EFS) in PVAT (+) MA by superoxide dismutase (SOD, 200 U/ml). (C) Recovery of the response to EFS after washout of SOD (1 h). (D) A summary of results of SOD treatment on PVAT (+) and PVAT (–) arteries. (E) A summary of inhibition (%) by SOD in PVAT (+) and PVAT (–) MA. *\( P < 0.05 \), **\( P < 0.01 \) versus control arteries (\( n = 6 \) rats, 30–32 weeks).
dently attenuated the contraction in PVAT (+) MA, but showed no effect in PVAT (−) MA (Fig. 3B). Inhibition of xanthine oxidase with allopurinol, and cytochrome P450 monooxygenase with 17-octadecynoic acid did not affect the contraction in either PVAT (+) or PVAT (−) MA, and these compounds did not inhibit the contraction to KCl (data not shown).

3.6. Effects of nitric oxide synthase inhibitor (L-NNA) on the contraction to perivascular nerve stimulation

Incubation of endothelium-intact MA with L-NNA (100 μM, 30 min) did not affect the basal tension, but enhanced the contractile response to EFS (12 Hz) in both PVAT (+) and PVAT (−) MA to similar extents. The contraction to EFS was greater in PVAT (+) MA than in PVAT (−) either in the presence or absence of L-NNA (Fig. 3D).

3.7. Effects of exogenous superoxide on the contraction to perivascular nerve stimulation (12 Hz) and to phenylephrine (0.3 μM) in PVAT (−) MA

Treatment of the arteries with pyrogallol (a superoxide generator; 10 and 30 μM), which did not contract the arteries, enhanced the contraction to EFS and to phenylephrine (Fig. 4A–C). Inhibitors of tyrosine kinase (tyrphos-
3.8. Immunohistochemical staining for p67phox and detection of p67phox mRNA by RT-PCR

The cytoplasm and cell membrane of the adipocytes in the PVAT were positively stained by anti-p67phox antibody (Fig. 5A), showing the presence of this subunit of NAD(P)H oxidase. Negative controls did not show any non-specific staining (Fig. 5B). Expression of p67phox mRNA in PVAT was also detected by RT-PCR (Fig. 5C) and the specificity of PCR product was confirmed by nucleotide sequencing (DNA Data Bank of Japan, accession number: M32011).

3.9. EFS-enhanced superoxide production by PVAT

Basal production of superoxide was greater in PVAT (+) MA than in PVAT (−) MA. EFS (12 Hz) increased the production of superoxide in PVAT (−) MA slightly, but a significant increase was found in MA with intact PVAT (Fig. 6A). In isolated PVAT, NADH (a stimulator of NAD(P)H oxidase) and EFS (12 Hz) enhanced superoxide production, and this enhancement was attenuated by DPI (a NAD(P)H oxidase inhibitor) (Fig. 6B). The production of superoxide was reduced by chemical scavenger tiron (10 mM) and by SOD (200 units/ml; data not shown). EFS-induced superoxide production by adipocytes of PVAT was confirmed in frozen sections of PVAT stained with DHE (Fig. 7A, dark red fluorescent staining of nuclei of adipocytes), while adipocytes in control tissue (0 Hz) only showed a weak fluorescence (Fig. 7B). The increase in fluorescence intensity by EFS was greatly reduced by pretreatment with SOD-PEG (1000 units/ml, 60 min, 37 °C) prior to EFS stimulation (data not shown).

4. Discussion

The novel finding of the present study is that PVAT (+) enhances vasoconstriction in response to perivascular nerve activation by EFS, and this enhancement may involve the release of superoxide by PVAT and the activation of tyrosine kinase and MAPK/ERK pathway. To the best of our knowledge, this is the first report to show that the presence of PVAT enhances vasoconstriction, which, therefore, adds new insights to the current understanding about the role that PVAT may play in the regulation of vascular function.

We have established that the presence of PVAT did not affect the ability of the arteries to contract or to relax, because maximal contraction in response to KCl and relaxation response to sodium nitroprusside were similar between PVAT (+) and PVAT (−) MA. The slower onset of KCl-induced contraction in PVAT (+) than in PVAT (−) MA was probably due to the concomitant release of PVAT-generated relaxing factor, which suppressed the development of contraction to KCl through opening potassium channels [4,6,7], but eventually its effect was rendered ineffective by high concentration of extracellular KCl, as shown in previous studies [19,20]. Adventitia, which is located between PVAT and medial smooth muscle, plays a crucial role in mediating vascular response to perivascular nerve stimulation [21]. However, the difference between PVAT (+) and PVAT (−) MA in their responses to EFS was not due to potential damage of the adventitial tissue during the removal of PVAT, because the dimensions of adventitia and the density of the nerve staining were comparable between PVAT (+) and PVAT (−) MA. We have also established that the contractile response of the MA to EFS was through the activation of perivascular sympathetic nerves and was mediated by \(\alpha_1\)-adrenoceptors.
because tetrodotoxin and prazosin abolished this response, which is consistent with previous report [22]. Since the presence of PVAT attenuated contractile response of blood vessels to several exogenously applied agonists [4,6–8], the enhancement of the contractile response to EFS by PVAT appears to be a specific phenomenon associated with perivascular nerve stimulation.

It is well known that endothelium, smooth muscles and adventitia are local sources of superoxide [23–25], and NAD(P)H oxidase is the main superoxide-producing enzyme [26]. However, it is not known whether PVAT, which surrounds most of the systemic vessels, also contributes to vascular superoxide production. Our study showed that PVAT is another source of vascular superoxide in addition to vessel wall components, because (1) PVAT (+) MA generated more superoxide than PVAT (−) MA, (2) isolated PVAT also produced superoxide, (3) EFS enhanced superoxide production by PVAT and the origin of superoxide from adipocytes in PVAT was confirmed by fluorescent labeling with DHE. We further found that the main enzyme responsible for superoxide production by PVAT was probably NAD(P)H oxidase, because superoxide production by PVAT was inhibited by the inhibitor of this enzyme (DPI) and enhanced by the stimulator of this enzyme (NADH).

Fig. 5. Immunohistochemical staining of NAD(P)H oxidase subunit p67phox and its mRNA expression in the PVAT of mesenteric artery. Cytoplasm and cell membrane of the adipocytes were positively stained as indicated with arrowheads (A). No positive staining was found in the negative control by omitting the primary antibody in the staining process (B). Expression of P67phox mRNA in PVAT (C). Data are representative of four rats (30–32 weeks).
Expression of p67phox mRNA was also detected from adipocytes isolated from PVAT, and the PCR product was confirmed by sequencing. We had selected p67phox subunit among all the subunits of NAD(P)H oxidase because the expression of this subunit in mouse adipose tissue was the most abundant [27]. Expression of gp91phox mRNA was also detected in the mesenteric PVAT (Gao et al., unpublished data). This is the first report, to the best of our knowledge, showing that NAD(P)H oxidase subunit is present in the adipocytes of PVAT and contributes to vascular superoxide production.

We further showed that superoxide generated by PVAT is involved in the enhanced contraction to EFS of PVAT (+) MA, based on the following findings. Firstly, enzyme scavenger for superoxide (SOD-PEG) exerted greater attenuation of EFS-induced contraction in PVAT (+) MA than in PVAT (−) MA. PEG, a hydrophilic polymer associated with SOD, did not reduce EFS-induced contraction, although it attenuated oxidative stress in an animal model of spinal injury through stabilization of disrupted membrane [28]. Secondly, NAD(P)H oxidase inhibitors (apocynin and DPI) reduced EFS-induced contraction more in PVAT(+) MA than in PVAT (−) MA. Thirdly, pyrogallol, a superoxide-generating compound, potentiated the contraction of PVAT (−) MA in response to perivascular nerve stimulation, mimicking the presence of PVAT. Contractile response to phenylephrine was also enhanced by pyrogallol in PVAT (−) MA. Xanthine oxidase and cytochrome P450 monoxygenase, which have also been shown to play a role in vascular superoxide production [29,30], did not seem to be involved in superoxide production in PVAT because inhibitors of these enzymes did not show any effects on EFS-induced contraction. Cyclooxygenase inhibition reduced the contraction to EFS more in PVAT (+) MA than in PVAT (−) MA, indicating its involvement in superoxide generation [31] in PVAT, and/or its involvement in signal transduction pathway of superoxide-induced enhancement of contraction, as shown in vascular tissue [32,33], although we cannot rule out the possibility that suppression of vasoconstrictive prostanoid could be involved.

The mechanisms for superoxide-induced enhancement of contraction to EFS were investigated. A recent report had found that superoxide potentiated vasoconstriction in rat mesenteric arteries to α1-adrenoceptor agonist, but the...
mechanisms for this enhancement were not addressed [34]. We investigated the involvement of tyrosine kinase and MAPK/ERK in PVAT-enhanced contraction to EFS because activation of MAPK/ERK pathway by superoxide was involved in stretch-induced enhancement of contraction of bovine coronary artery [35]. We found that inhibitors of tyrosine kinase (tyrophostin A25) and MAPK/ERK (U 0126) concentration dependently attenuated the contraction to EFS in PVAT (+) MA, but not in PVAT (−) MA, suggesting their involvement in the enhanced contractile response to EFS in PVAT (+) MA. The inhibition seemed to be specific because the inactive forms of these inhibitors (tyrophostin A1 and U 0124) did not affect the contractile response. In PVAT (−) MA, these inhibitors of tyrosine kinase and MAPK/ERK (but not their inactive forms) also attenuated the enhancement of EFS-induced contraction by superoxide generator pyrogallol, further suggesting that potentiation of the contraction to EFS by PVAT-derived superoxide was mediated through activation of tyrosine kinase and MAPK/ERK pathway. It should be noted that some tyrosine kinase inhibitor such as genistein and its inactive form daidzein also possess antioxidant property [36–38]. In our preliminary experiments we did find that daidzein attenuated contractile response to EFS. We also found that SB 202474, the inactive form of SB 203580 (a MAPK/P38 kinase inhibitor) attenuated the response to EFS.

It is known that superoxide may function as messenger molecules in signal transduction cascades in some physiological processes [39,40], but the mechanisms for activation of tyrosine kinase and MAPK/ERK pathway to promote vasoconstriction to EFS as shown in this study are not clear. Superoxide facilitates synaptic neurotransmission in central nervous system [41], but whether this also happens in peripheral synaptic or neuromuscular transmission in perivascular nerves remains to be studied. Although superoxide may also induce vasoconstriction directly at certain concentration [33], it does not seem to be the case because pyrogallol, a superoxide generating compound, enhanced the contraction to EFS and to phenylephrine at the concentration which did not show any contraction by itself. Another possibility for superoxide to enhance vasoconstriction is the inactivation of endothelial nitric oxide (NO) [32]. Inhibition of NO synthase by L-NNA, which did not affect basal tension, greatly increased the contractile response to EFS in endothelium-intact MA either with or without PVAT, suggesting that endothelial NO indeed plays a role in antagonizing EFS-induced contraction. The fact that PVAT (+) MA still showed a higher response to EFS than PVAT (−) MA in the presence of L-NNA suggests that inactivation of NO by superoxide is not responsible for the enhanced response to EFS in PVAT (+) MA. NO may serve as a neurotransmitter in rat mesenteric arteries [42,43], but further studies had already shown that neuronal NO did not participate in EFS-stimulated vasomotor response of WKY MA [44,45]. Therefore, inactivation of either endothelial or neuronal NO by PVAT-generated superoxide did not appear to be responsible for the enhanced contractile response to EFS in PVAT (+) MA.

Our finding that PVAT-derived superoxide enhances EFS-induced contraction may have physiological significance, because PVAT is ubiquitous in almost all systemic blood vessel, and vascular tone is mainly under the control of sympathetic innervations. An alteration in PVAT property such as the amount of superoxide produced may affect local sympathetic control of vascular tone. This finding may also have clinical relevance because obesity is a well-known risk factor for the development of hypertension [46–48], and both the quantity and the quality of PVAT were altered in obese animals [8]. A recent report on human and mice have shown that subcutaneous adipocytes from obese individual produced more reactive oxygen species than that from the normal and thus lead to systemic oxidative stress in obesity [27]. Therefore, although enhanced superoxide production by PVAT in obesity has not been reported, systemic and/or perivascular oxidative stress in obesity may enhance vasoconstriction to activation of sympathetic innervations.

Taken together, our results suggest that in rat MA, EFS induces vasoconstriction through the release of norepinephrine from perivascular nerves and simultaneously stimulates superoxide generation from PVAT and arteries, and that the superoxide generated from PVAT potentiates vasoconstriction to norepinephrine, resulting in a greater contraction to EFS in PVAT (+) MA than in PVAT (−) MA. Our finding that PVAT enhances vasoconstriction to perivascular nerve activation, in conjunction with the finding that PVAT releases a relaxing factor, suggests that PVAT may play a dual regulatory role in the modulation of vascular function, and this enhancement may be part of the mechanisms for obesity-associated hypertension.

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