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RESEARCH PAPER

Modulation of vascular function by perivascular adipose tissue: the role of endothelium and hydrogen peroxide

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Background and purpose: Perivascular adipose tissue (PVAT) attenuates vascular contraction, but the mechanisms remain largely unknown. The possible involvement of endothelium (E) and hydrogen peroxide (H_2O_2) was investigated.
Experimental approach: Aortic rings from Wistar rats were prepared with both PVAT and E intact (PVAT + E +), with either PVAT or E removed (PVAT-E +, or PVAT + E-), or with both removed (PVAT-E-) for functional studies. Nitric oxide (NO) production was measured.
Key results: Contraction to phenylephrine and 5-HT respectively was highest in PVAT-E-, lowest in PVAT + E +, and intermediate in PVAT + E- or PVAT-E +. In bioassay experiments, transferring bathing solution incubated with a PVAT + ring (donor) to a PVAT- ring (recipient) induced relaxation in the recipient. This relaxation was abolished by E removal, NO synthase inhibition, scavenging of NO, high extracellular K^+, or blockade of calcium-dependent K^+ channels (K_{Ca}). The solution stimulated NO production in isolated endothelial cells and in PVAT-E + rings. In E- rings, the contraction to phenylephrine of PVAT + rings but not PVAT- rings was enhanced by catalase or soluble guanylyl cyclase (sGC) inhibitor, but reduced by superoxide dismutase and tiron. In PVAT-E- rings, H_2O_2 attenuated phenylephrine-induced contraction. This effect was counteracted by sGC inhibition. NO donor and H_2O_2 exhibited additive inhibition of the contraction to phenylephrine in PVAT-E- rings.
Conclusion: PVAT exerts its anti-contractile effects through two distinct mechanisms: (1) by releasing a transferable relaxing factor which induces endothelium-dependent relaxation through NO release and subsequent K_{Ca} channel activation, and (2) by an endothelium-independent mechanism involving H_2O_2 and subsequent activation of sGC.
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Keywords: aorta; endothelium; hydrogen peroxide; nitric oxide; perivascular adipose tissue; potassium channels; rat; reactive oxygen species; relaxation

Abbreviations: 4-AP, 4-aminopyridine; ADRF, adventitium-derived relaxation factor; CCh, carbamylcholine chloride; carboxy-PTIO, carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide; ChTX, charybdotoxin; DAF-2DA, 4,5-diaminofluorescein diacetate; E, endothelium; EC_{50}, the concentration required to induce 50% maximal response; EDHF, endothelium-derived hyperpolarizing factor; K_{ATP}, ATP-dependent K^+ channels; K_{Ca}, calcium-dependent K^+ channels; K_v, voltage-dependent K^+ channels; L-NNA, N^{ω}-nitro-L-arginine; NO, nitric oxide; ODQ, 1*H*-(1,2,4)oxadiazolo (4,3-A)quinazoline-1-one; PVAT, perivascular adipose tissue; PVRF, perivascular adipose tissue-derived relaxation factor; PSS, physiological salt solution; ROS, reactive oxygen species; sGC, soluble guanylyl cyclase; SOD, superoxide dismutase; TEA, tetraethylammonium

Introduction

Perivascular adipose tissue (PVAT) is situated outside the adventitial layer and surrounds most of the systemic blood vessels. Recent studies have shown that PVAT can attenuate vessel contraction to various agonists including phenylephrine, 5-HT, angiotensin II and U 46619, as demonstrated in the aorta and the mesenteric arteries of rats (Lohn *et al*., 2002; Verlohren *et al*., 2004; Gao *et al*., 2005a), and in the internal thoracic arteries of humans (Gao *et al*., 2005b). The mechanisms for the attenuation of contraction by PVAT are not fully understood, but the release of transferable relaxation factor(s) with unknown identity, termed adventitium-derived relaxation factor (ADRF), has been proposed (Lohn *et al*., 2002; Dubrovska *et al*., 2004). However, as this factor is

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released by PVAT and not by adventitia, it is more appropriate to call this factor perivascular adipose tissue-derived relaxation factor (PVRF). Nevertheless, we by no means discount the possible interaction between PVRF and adventitia. Results to date suggest that the putative PVRF induces vessel relaxation through membrane hyperpolarization of smooth muscle cells because (1) rat mesenteric arterial smooth muscle was more hyperpolarized when PVAT was preserved, (2) high K^+ in physiological salt solution, which reduces the K^+ gradient across the cell membrane, abolished the relaxation by PVRF, and (3) K^+ channel blockade counteracted the anticontractile effects of PVAT (Lohn *et al*., 2002; Verlohren *et al*., 2004; Gao *et al*., 2005b; Galvez *et al*., 2006). Thus, PVRF shows some similarities to endothelium-derived hyperpolarizing factor (EDHF) in its action. It is now clear that EDHF may induce hyperpolarization of smooth muscle cells through endothelium-dependent or -independent pathways (Feletou and Vanhoutte, 2006). Therefore, whether PVRF directly induces relaxation of smooth muscles or indirectly mediates relaxation through the endothelium and whether other mechanisms are involved in the anticontractile effects of PVAT, remain to be defined.

PVAT, in conjunction with vascular components including adventitia and endothelium, is a rich source of vascular reactive oxygen species (ROS) (Gao *et al*., 2006). It is well documented that ROS has direct vasomotor effects (Ardanaz and Pagano, 2006) and modulates vessel response to other stimulus (Hubel *et al*., 1993; Sasaki and Okabe, 1993). Therefore, PVAT-derived ROS may play an important role in PVAT-mediated modulation of vessel function. Indeed, we have recently found that superoxide generated by PVAT potentiated vessel constriction to perivascular nerve stimulation in rat mesenteric arteries (Gao *et al*., 2006). However, the role of other ROS, especially hydrogen peroxide (H_2O_2), a known activator of vascular K^+ channels (Barlow and White, 1998; Gao *et al*., 2003), which can be produced by membrane-bound NAD(P)H oxidase (Krieger-Brauer and Kather, 1992) and dismutation of superoxide in adipocytes, in PVAT-mediated modulation of vessel function remains to be defined. This study was designed to examine the mechanisms of the anticontractile actions of PVAT in rat aorta, with focus on the involvement of endothelium and the role of H_2O_2. Here, we report that PVAT modulates vessel function through two distinct mechanisms: an endothelium-dependent mechanism through a transferable PVRF and nitric oxide (NO) release from endothelium, and an endothelium-independent mechanism through the generation of H_2O_2.

Methods

Animals

Male Wistar rats (300–350 g) were used for this study (Harlan, Indianapolis, IN, USA). The care and the use of these animals were in accordance with the guidelines of the Canadian Council on Animal Care.

Preparation of aortic rings and contractility experiments

The procedure for the preparation of aortic rings has been described in our previous reports (Gao and Lee, 2001; Gao *et al*., 2005a). Briefly, the rat was anaesthetized by an overdose of sodium pentobarbital (60 mg kg^{-1}, intraperitoneal), and the thoracic aorta was collected in oxygenated physiological salt solution (PSS) with the following composition (in mM): NaCl, 119; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; $CaCl_2$, 1.6; glucose, 11 at 4°C. Paired aortic rings with or without PVAT (PVAT+ and PVAT−, 4 mm long for each) were prepared with either intact endothelium (E+) or with endothelium removed (E−) using the middle segment of the thoracic aorta. To prepare E− rings, endothelium was removed by gently rubbing the internal surface with a fine wooden stick, and successful removal of endothelium was confirmed by the absence of a relaxation response to carbamylcholine (1 μM) in rings precontracted with phenylephrine (1 μM). A computerized myograph system was used to record the isometric tension of the aortic rings. After equilibration for at least 90 min at 3 g of preload, which is the optimal preload defined in our previous experiment (Gao and Lee, 2001), the arterial rings were challenged with 60 mM KCl twice at an interval of 30 min to establish a baseline contractile response. Contractile response to agonists was expressed as a percentage of the KCl contraction and relaxation response was calculated as a percentage of precontraction load. Cumulative concentration–response curves for phenylephrine and 5-HT were constructed. The concentration of phenylephrine required to induce 50% maximal response (EC_{50}) was estimated by fitting each concentration–response curve. Some PVAT+ and PVAT− rings were fixed in 10% formaldehyde for morphological examinations.

Bioassay experiments

To examine the effects of transferable PVRF, bioassay experiments were carried out using PVAT+ aortic rings as donors, and PVAT− rings with or without endothelium as recipients. The transfer of solution incubated with PVAT− rings served as control. The donor and recipient vessels were precontracted with phenylephrine (0.3 μM) or KCl (60 mM), and 3 ml of donor solution was transferred to the recipient chamber when the precontraction reached its plateau (usually within 3–5 min), as described in previous studies (Gao *et al*., 2005a, b). To test the involvement of NO, cyclooxygenase metabolites, cytochrome P450 monooxygenase metabolites and activation of K^+ channels in PVRF-induced relaxation, recipient vessels were incubated with respective enzyme inhibitors or K^+ channel blockers for 25–30 min before transfer of solution was carried out. An equal amount of inhibitor or blocker was added to the donor solution to avoid any dilution of blocking agents in the recipient chamber when donor solution was introduced. Involvement of ROS was tested using specific scavenging enzymes (catalase and superoxide dismutase (SOD)) and the SOD mimetic, tiron. Viability of the vessels was tested with KCl at the end of experiment.

Detection of NO production in endothelial cells and in aortic rings

Under sterile condition, endothelial cells were scraped from the thoracic aorta into cell culture media (Dulbecco's

modified Eagle's medium) (Gao and Lee, 2005) and were identified by positive staining with von Willebrand factor (Magid *et al*., 2003). In another set of experiments, the aortic rings (3 mm long) were carefully inverted so that the endothelium was on the outside to facilitate fluorescence detection in a 96-well microplate (with 200 μl PSS in each well). After 30 min equilibration, the endothelial cells and the aortic rings were loaded with 4,5-diaminofluorescein diacetate (DAF-2DA, 10 μM) for 30 min at 37°C in PSS containing L-arginine (100 μM), and then rinsed with PSS three times to remove DAF-2DA in the media (Mukhopadhyay *et al*., 2007; Zhu *et al*., 2004). DAF-2DA, a membrane permeable NO-sensitive fluorescence dye, reacts with NO to generate a fluorescence compound diaminotriazolofluorescein (Kojima *et al*., 1998; Nakatsubo *et al*., 1998). Images of endothelial cells were obtained using a fluorescence microscope (Carl Zeiss MBX 75, Jena, Germany), and the intensity of fluorescence of aortic rings was measured with a fluorescence microplate reader (Fluoroskan Ascent FL, Thermo Fisher Scientific Inc., Waltham, MA, USA; 485 nm for excitation and 527 nm for emission) before and 10 min after replacement of PSS with solution incubated with PVAT+ aortic rings, with endothelium-denuded aortic rings as background control. Carbamylcholine (1 μM) was used as positive control to induce endogenous NO generation, carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (carboxyl-PTIO) (100 μM) as NO scavenger to verify the specificity of DAF-2DA for NO detection, and solution incubated with PVAT-aortic rings was used as negative control.

Chemicals

The following chemicals were used: H_2O_2 (BDH Inc., Toronto, Canada); acetylcholine, 4-aminopyridine (4-AP), angiotensin II, apamin, carboxy-PTIO (potassium salt), catalase, carbamylcholine chloride, charybdotoxin (ChTX), DAF-2DA, diclofenac, glipizide, iberiotoxin, mahma NONOate, N^{ω}-nitro-L-arginine (L-NNA), 1*H*-(1,2,4)oxadiazolo(4,3-A)quinazoline-1-one (ODQ), phenylephrine, 5-HT, SOD, tetraethylammonium (TEA) and tiron (Sigma-Aldrich, St Louis, MO, USA); 17-octadecynoic acid (Cayman Chemical, Ann Arbor, MI, USA); H-89, KT-5823 and tyrphostin A25 (Calbiochem, San Diego, CA, USA). Angiotensin II, ChTX and apamin were dissolved in oxygen-free deionized water. H-89, KT 5823, ODQ and tyrphostine A25 were dissolved in dimethyl sulphoxide. 17-octadecynoic acid was dissolved in absolute ethanol and diluted in 50% ethanol. All other agents were dissolved in deionized water and prepared fresh daily.

Statistical analysis

Results were expressed as mean ± standard error of the mean (s.e.m.) where n represents the number of rats. Statistical analysis was performed by two-way repeated measurements or one-way analysis of variance followed by *post hoc* *t*-test to determine any significant difference between the concentration–response curves to phenylephrine and to 5-HT, or by Student's *t*-test, using the SPSS software (SPSS Inc., Chicago, USA) to test treatment effects. The differences were considered significant when $P \leqslant 0.05$.

Results

Morphology of PVAT

The thoracic aorta of Wistar rats is surrounded by a significant amount of PVAT (Figure 1a). Removal of PVAT did not affect the integrity of adventitia and smooth muscles (Figure 1b). The average wet weight of PVAT attached to each aortic ring was 47.5 ± 2.5 mg in PVAT+ E+ rings and 45.5 ± 2.2 mg in PVAT+ E− rings ($n = 7$, $P = 0.55$).

Contraction to phenylephrine and 5-HT in aortic rings with or without PVAT or endothelium

The presence or absence of PVAT or endothelium did not affect the maximal tension induced by 60 mM KCl (Table 1). Phenylephrine induced a concentration-dependent contractile response in all the aortic rings, with the highest response in PVAT− E− rings, the lowest in PVAT+ E+ rings and intermediate responses in PVAT+ E− or PVAT− E+ rings (Figure 2a). The maximal contraction to phenylephrine was significantly higher in PVAT− rings than respective PVAT+ rings either with or without endothelium, and the EC_{50} of

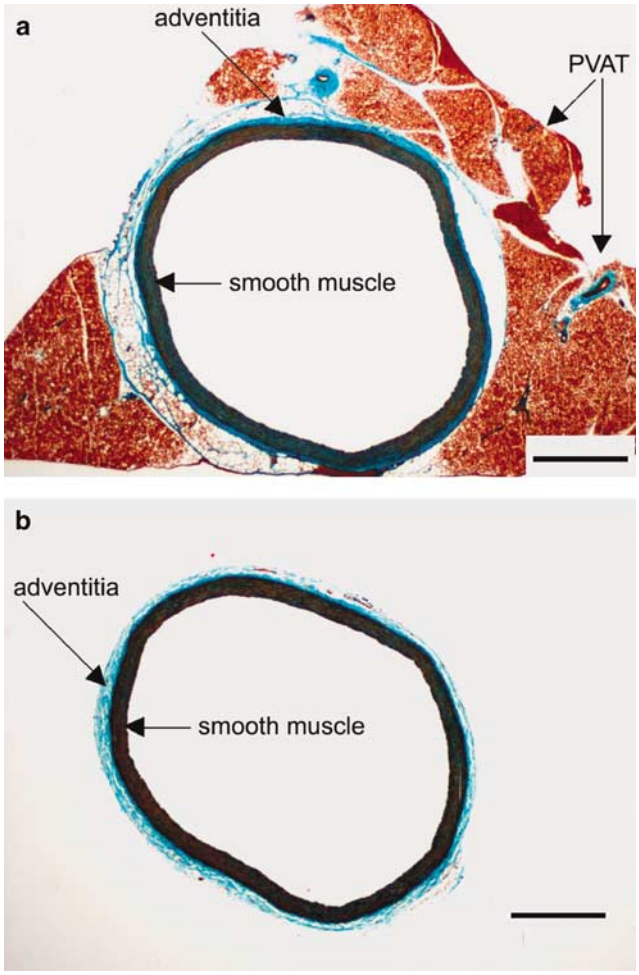

Figure 1 Five-micron cross-sections of aorta before (**a**) and after removal of PVAT (**b**) sections were stained with Gomori's trichrome. PVAT, perivascular adipose tissue. Magnification bar represents 500 μM.

Table 1 Contractile response to KCl and to phenylephrine of the aortic rings with (+) or without (−) perivascular adipose tissue (PVAT) or endothelium (E) from male Wistar rats (300–350 g)

	KCl	*Phenylephrine*		
	(60 mM; g)	E_{max} *(% of KCl)*	EC_{50} *(μM)*	n
PVAT + E +	1.8 ± 0.1	101 ± 3[a]	0.28 ± 0.05[a]	7
PVAT-E +	1.7 ± 0.07	124 ± 2[b,a]	0.11 ± 0.01[b,c]	7
PVAT + E-	1.9 ± 0.19	132 ± 2[b,a]	0.14 ± 0.03	6
PVAT-E-	1.5 ± 0.05	161 ± 3[b]	0.06 ± 0.01[b]	6

No statistical difference was found between PVAT + E− and PVAT− E + rings ($P > 0.05$).
[a]$P < 0.01$ versus PVAT− E− rings.
[b]$P < 0.01$ versus PVAT + E + rings.
[c]$P < 0.05$ versus PVAT− E− rings.

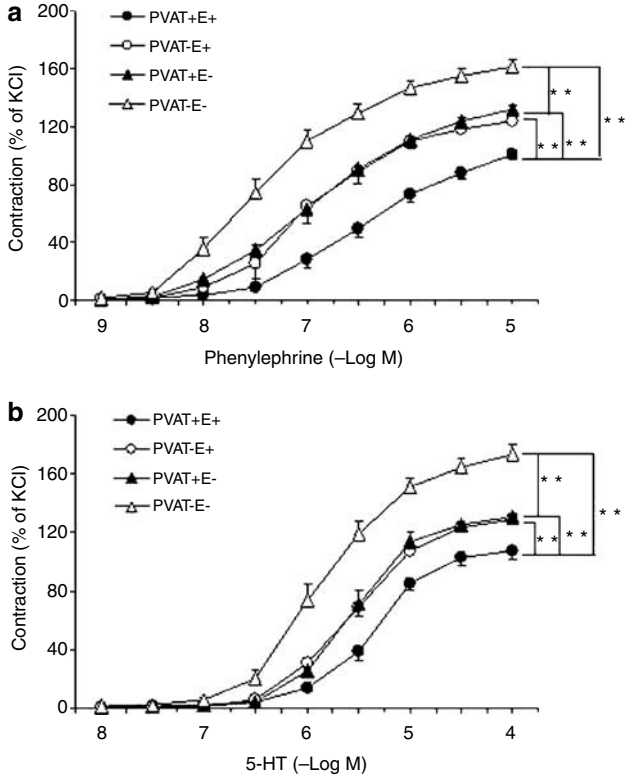

Figure 2 Concentration–response curves to phenylephrine (**a**) and to 5-HT (**b**) in the thoracic aortic rings with (+) or without (−) PVAT or endothelium (E). The presence of PVAT attenuated the contractile response to phenylephrine and to 5-HT in aortic rings with or without E **$P < 0.01$, $n = 5$–7.

phenylephrine was higher in PVAT + E + rings than in PVAT− rings either with or without endothelium (Table 1). Endothelium removal also reduced the EC_{50} of phenylephrine in PVAT− aortic rings. Contraction to 5-HT was similarly affected by PVAT and endothelium (Figure 2b). PVAT also attenuated the contraction to angiotensin II (data not shown).

Response to transferable PVRF

Transferring solution incubated with PVAT + E + aortic rings as a donor induced a marked relaxation response in recipient rings with intact endothelium (PVAT− E +), but not in recipient aorta with endothelium removed (PVAT− E−, Figure 3a and b). Transfer of solution incubated with PVAT + E− aortic rings caused a similar endothelium-dependent relaxation response in recipient rings (PVAT− E +), indicating the origin of this transferable relaxation factor was not from the endothelium (data not shown).

Mechanisms of transferable PVRF-induced relaxation

Incubation of recipient aortic rings (PVAT− E +) with a NO synthase inhibitor (L-NNA) or a NO scavenger (carboxy-PTIO) abolished the relaxation induced by transfer of donor solution from PVAT + E + aorta (Figure 3c). In isolated endothelial cells and aortic rings (PVAT− E +) loaded with NO-sensitive fluorescence dye DAF-2DA, replacement of PSS with the solution incubated with PVAT + rings stimulated a rapid increase in fluorescence density (Figure 4a, b and e), which is similar to the effects of carbamylcholine (Figure 4c and e), and was prevented by NO scavenger carboxy-PTIO (Figure 4d).

The transfer of solution incubated with PVAT + E + rings did not induce relaxation of the recipient rings (PVAT− E +) when the arteries were precontracted with 60 mM KCl (tension in grams; before transfer: 1.59 ± 0.1; after transfer: 1.61 ± 0.2, $n = 5$). Calcium-dependent K^+ channel (K_{Ca}) blocker TEA (1 mM) and a combination of ChTX (0.3 μM) and apamin (0.3 μM) abolished the relaxation caused by the donor solution, whereas blockers of ATP-dependent K^+ channels (K_{ATP}; glipizide, 10 μM) and voltage-dependent K^+ channels (K_v; 4-AP, 1 mM) did not affect the relaxation response (Figure 5). An inhibitor of large conductance K_{Ca} (iberiotoxin, 1 μM) and inhibitors of cyclooxygenase (diclofenac, 10 μM) and cytochrome P450 monooxygenase (17-octadecynoic acid, 3 μM) did not affect the relaxation response in the recipient artery (data not shown).

Involvement of K^+ channel activation in acetylcholine-induced relaxation

In PVAT− E + rings precontracted with phenylephrine (1 μM), acetylcholine (1 μM) induced a marked relaxation response (% of precontraction: 63.3 ± 4.8, $n = 5$) that was absent in PVAT− E− rings. The relaxation to acetylcholine was prevented when the vessels were precontracted by 60 mM KCl (% of precontraction: 1.4 ± 0.2, $n = 5$), or treated with K_{Ca} blocker (TEA, 1 mM; % of precontraction: 2.2 ± 0.3, $n = 5$).

Involvement of H_2O_2 and sGC in endothelium-independent PVAT modulation of vascular contraction

In vessels denuded of endothelium, catalase enhanced, whereas SOD and tiron reduced, the contraction to phenylephrine (0.3 μM) in PVAT + rings but not in PVAT− rings (Figure 6a). Resting tension was not affected by either catalase or SOD or tiron. Although exogenous H_2O_2 caused an immediate transient contractile response in PVAT− E− aorta at resting tension that returned to baseline within 5–8 min (14.5 ± 5.7% for 30 μM, 34.4 ± 7.2% for 100 μM and 57.5 ± 2.9% for 300 μM) but not in PVAT + E− aorta

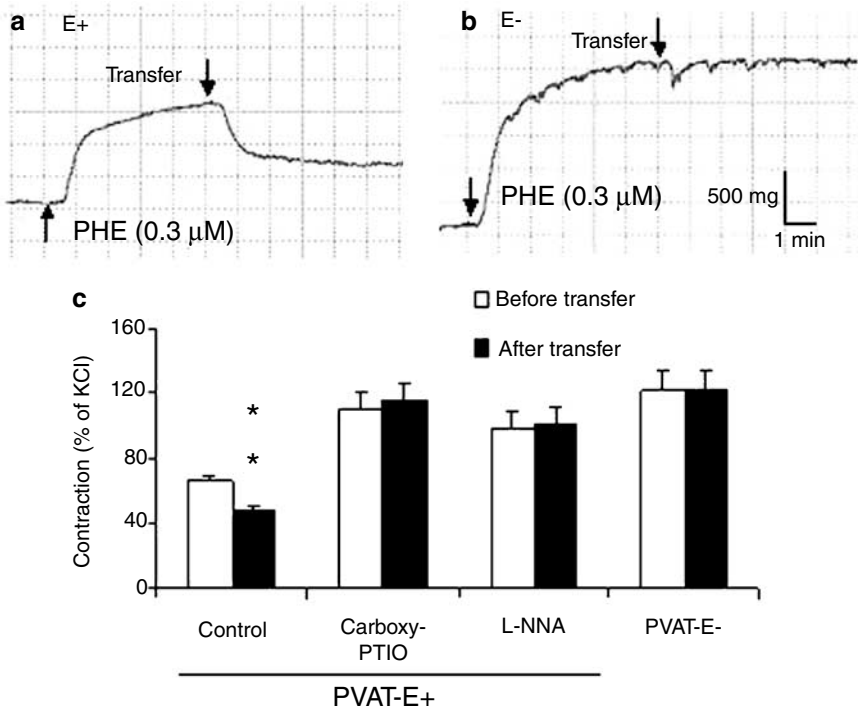

Figure 3 Typical recording showing that transferring solution incubated with PVAT−intact aortic rings to PVAT-denuded aortic rings induced a relaxation response in the recipient artery with intact endothelium (E+) (**a**) but not in endothelium-denuded rings (E−) (**b**). Relaxation induced by this solution transfer was abolished by NO synthase inhibitor (L-NNA, 100 μM) and NO scavenger (carboxy-PTIO, 100 μM) (**c**). Vessels were precontracted with phenylephrine (0.3 μM). $^{**}P<0.01$ versus before transfer (paired Student *t*-test, $n=5$–7).

(0.54±0.54% for 300 μM), H_2O_2 concentration-dependently attenuated the contraction of PVAT−E− rings to phenylephrine after 15 min incubation. The sGC inhibitor (ODQ), which caused a transient increase of the basal tone (PVAT+E− rings: 13.2±4.3%; PVAT− E− rings: 0.6±0.4%; $P<0.01$, $n=8$ for each), enhanced the contractile response to phenylephrine in PVAT+E− rings but not in PVAT− E− rings and counteracted the inhibitory effects of H_2O_2 (300 μM) on phenylephrine-induced contraction in PVAT− E− rings (Figure 6a). Inhibitors of tyrosine kinase (tyrphostin A25, 50 μM), of protein kinase A (H-89, 3 μM) and protein kinase G (KT-5823, 1 μM) did not antagonize the anticontractile effects of PVAT (data not shown).

Combined effects of exogenous NO and H_2O_2

In PVAT− E− aortic rings, a combination of NO donor (MAHMA NONOate, 100 μM) and H_2O_2 (300 μM) additively inhibited the contraction to phenylephrine (0.3 μM) (Figure 6b). In phenylephrine (0.3 μM)-precontracted PVAT− aortic rings, the relaxation to mahma NONOate was similarly inhibited by ODQ (10 μM), either in the presence or absence of L-NNA (100 μM), and neither L-NNA nor ODQ affected the basal tension and the contraction to KCl (data not shown).

Discussion

The novel finding of this study is that PVAT attenuates vessel constriction to agonist through two mechanisms: an endothelium-dependent mechanism initiated by transferable PVRF and an endothelium-independent mechanism that is not transferable. The transferable PVRF causes endothelial NO release and subsequent activation of K^+ channels leading to relaxation and the nontransferable anticontractile mechanism involves generation of H_2O_2 by PVAT and subsequent activation of smooth muscle sGC. These findings add new insights to our current understanding about the mechanisms by which PVAT modulates vessel function.

Endothelium-dependent relaxation by PVAT

One of our novel findings is that PVRF exerts its anticontractile effect partly through the endothelium. This is in contrast with the results from Lohn *et al*. (2002), who concluded that PVRF acted through an endothelium-independent mechanism. Their conclusion was based on: (1) in endothelium-denuded preparations, PVAT+ aortic rings still contracted less to 5-HT than PVAT− rings, similar to the situation in E+ rings and (2) in E+ aortic rings treated with NO synthase inhibitor, PVAT+ rings still contracted less to 5-HT than PVAT− rings. However, they did not carry out bioassay experiments to examine if the action of this transferable relaxation was dependent on the presence of endothelium. Our results here clearly showed that the action of this transferable relaxation factor is dependent on the presence of endothelium because removal of endothelium abolished the relaxation response induced by this transferable factor. Our results also showed that this transferable PVRF is not directly causing relaxation of smooth muscle by

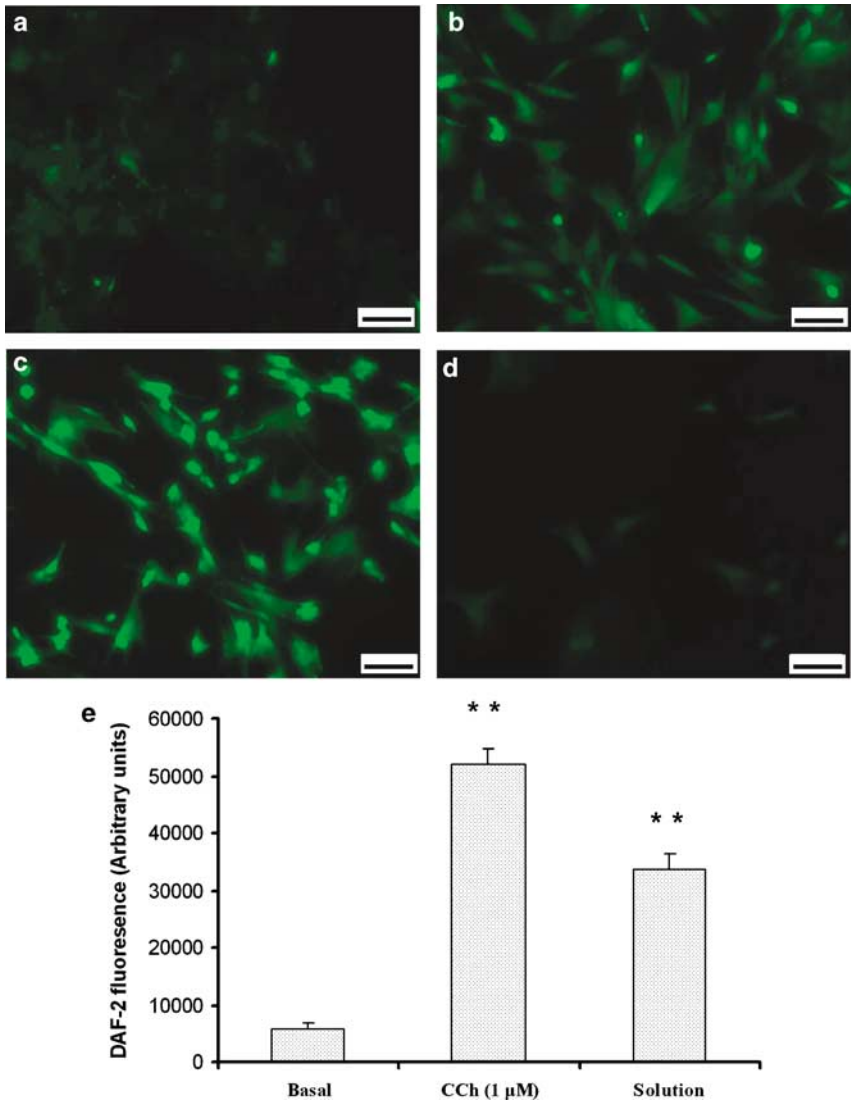

Figure 4 NO production in isolated aortic endothelial cells induced by solution incubated with PVAT+ aortic rings detected with DAF-2DA (**a**–**d**). Images were obtained using fluorescence microscopy before (**a**), 10 min after exposure to solution incubated with PVAT+ aortic ring (**b**) and to carbamylcholine (CCh, 1 μM; **c**) and to the solution in the presence of carboxy-PTIO (100 μM, **d**). Magnification bar represents 25 μm. CCh and incubation solution from PVAT+ aortic rings induced the production of NO in PVAT− E+ aortic rings (**e**). $^{**}P<0.01$ versus basal value. ($n=4$). Solution incubated with PVAT− ring did not change the fluoresence intensity (data not shown).

itself. We found that the release of NO by endothelium is involved in the relaxation to this transferable PVRF because (1) the relaxation was abolished by NO synthase inhibitor and by NO scavenger and (2) solution containing PVRF induced NO production from aortic endothelial cells in culture and in aortic rings. This relaxation factor is not derived from endothelium because solution incubated with PVAT-E+ rings did not induce any relaxation in recipient rings without endothelium.

Studies on PVRF to date have consistently showed that hyperpolarization of smooth muscles is involved in the relaxation to the transferable PVRF, because raising extracellular K^+ abolished the action of the transferable PVRF, and PVAT caused a hyperpolarized membrane potential of the underlined smooth muscle cells (Lohn *et al.*, 2002; Verlohren *et al.*, 2004; Gao *et al.*, 2005b). In this study, we have also found that high K^+ abolished the relaxation response to this transferable PVRF. However, results to date regarding the specific types of K^+ channels involved varied depending on the tissue and animal species. K_{ATP} channels were reported to be responsible in the aorta (Lohn *et al.*, 2002), K_v channels in the mesenteric arteries of Sprague–Dawley rats (Verlohren *et al.*, 2004) and K_{Ca} channels in the internal thoracic arteries of humans (Gao *et al.*, 2005b). In this study, we found that K_{Ca} channels are responsible for the relaxation activated by the transferable PVRF in the aorta of Wistar rats, because the relaxation response induced by donor solution from PVAT+ vessels was inhibited by blockade of K_{Ca} channels but not by blockers of K_v and K_{ATP} channels. The subtypes of K_{Ca} channels involved appear to be intermediate- and small-conductance K_{Ca} because ChTX and apamin inhibited the relaxation to the transferable

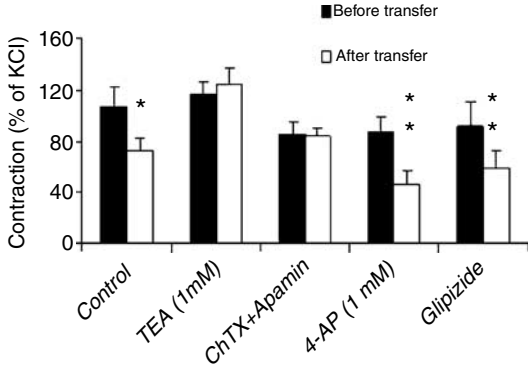

Figure 5 Relaxation of PVAT-denuded aortic rings induced by transferring solution incubated with PVAT-intact aortic rings was inhibited by K_{Ca} channel blockers (tetraethylammonium, TEA, 1 mM), and by a combination of charybdotoxin (ChTX, 0.3 μM) and apamin (0.3 μM), but not by 4-aminopyridine (4-AP, 1 mM) or glipizide (10 μM). Vessels were precontracted with phenylephrine (0.3 μM). $^{**}P<0.01$ versus before transfer (paired Student *t*-test, $n=5$–7).

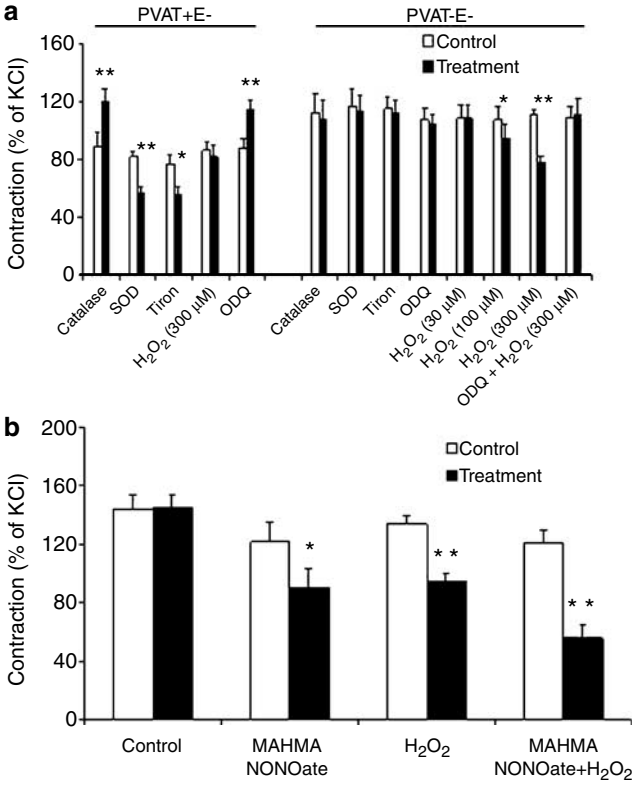

Figure 6 (**a**) Effects of catalase (1000 μ ml^{-1}), superoxide dismutase (SOD, 150 u ml^{-1}), tiron (1 mM), H_2O_2 (30, 100, 300 μM) and ODQ (10 μM) on the contraction of endothelium-denuded aortic rings with/without PVAT (PVAT+ E−, PVAT− E−) to phenylephrine (0.3 μM). Catalase and ODQ enhanced, whereas SOD and tiron reduced the contraction to phenylephrine in PVAT+ E− rings. H_2O_2 had no effect. In PVAT− E− vessels, H_2O_2 concentration-dependently attenuated the contraction to phenylephrine, and this attenuation was blocked by ODQ. (**b**) Treatment with NO donor (MAHMA NONOate, 100 μM) or H_2O_2 (300 μM), or a combined treatment of these two agents attenuated the contraction to phenylephrine (3×10^{-7} M) in PVAT− E−rings. $^{*}P<0.05$, $^{**}P<0.01$ versus respective control (paired Student *t*-test; $n=4$–6).

PVRF. The reason behind this diversity in K^+ channel subtypes activated by PVRF is not clear, but it may be related to differences in the distribution of these K^+ channels among different vessels or different species.

Our results showed that relaxation caused by NO in response to stimulation by this transferable PVRF was mediated through K_{Ca} channel activation, resulting in the hyperpolarization of smooth muscle. NO can hyperpolarize vascular smooth muscle cells by activating K^+ channels in either a cyclic-GMP-dependent or -independent manner (Yuan *et al*., 1996; Chauhan *et al*., 2003; Feletou and Vanhoutte, 2006). In this case, activation of K_{Ca} channels by PVRF-induced NO does not seem to be mediated through a cGMP-protein kinase G-dependent pathway, because an inhibitor of protein kinase G did not affect the relaxation. In PVAT− E+ aortic rings, endothelium-dependent relaxation to acetylcholine, which was inhibited by L-NNA, was also blocked by high-extracellular KCl and by K_{Ca} channel blockade with TEA, supporting the involvement of membrane hyperpolarization in NO-induced relaxation.

Endothelium-independent relaxation by PVAT

Endothelium-independent anticontractile effects of PVRF had been previously reported in Sprague–Dawley rat aorta (Lohn *et al*., 2002). It was established that the reduced contractile response of PVAT+ aortic rings to phenylephrine was not owing to physical restriction of PVAT on vessel contractility because the maximal contractile response to KCl and the relaxation response to sodium nitroprusside were not altered by the presence of PVAT, as shown in this study and previous reports (Lohn *et al*., 2002; Gao *et al*., 2005a). Our results here suggested that PVAT-derived H_2O_2 mediates the endothelium-independent anticontractile property of PVAT, because incubation with catalase, a scavenger of H_2O_2, enhanced the contractile response to phenylephrine in PVAT+ E− aortic rings, but not in PVAT− E− aortic rings. H_2O_2 is mainly generated by dismutation of superoxide by SOD, although adipocytes can also produce H_2O_2 directly through membrane-bound NAD(P)H oxidase (Krieger-Brauer and Kather, 1992; Krieger-Brauer *et al*., 2000). The involvement of H_2O_2 is further supported by the findings that SOD and tiron (a membrane permeable SOD mimetic) reduced the contractile response to phenylephrine in PVAT+ rings but not in PVAT− rings, and that exogenously applied H_2O_2 attenuated the contraction of PVAT−E− arteries to phenylephrine in a concentration-dependent manner. It also suggested that superoxide produced by PVAT serves as the substrate for SOD to generate H_2O_2. The ability of PVAT to produce superoxide had been shown in rat mesenteric arteries (Gao *et al*., 2006) and adipose tissue contains a significant amount of SOD (Nakao *et al*., 2000). In rat aortic PVAT, the presence of SOD has been detected (Gao *et al*., unpublished data). As H_2O_2 is membrane permeable, PVAT-derived H_2O_2 can easily diffuse to underlying smooth muscles. This may also explain why exogenously applied H_2O_2 did not attenuate vessel constriction to phenylephrine in PVAT+ E− rings, as PVAT-derived H_2O_2 had already suppressed the contraction. Although adventitia can also produce superoxide (Wang *et al*., 1998),

this factor does not seem to be involved as removal of PVAT did not affect adventitial integrity, as shown in Figure 1 and in a previous study (Gao *et al*., 2006).

H_2O_2 has both contractile and relaxation actions on blood vessels depending on its concentrations, vessel type, contractile status and animal species through a variety of mechanisms (Ardanaz and Pagano, 2006). In this study, H_2O_2 produced a transient contraction in PVAT− E− rings at resting tension, which is consistent with previous reports (Yang *et al*., 1998; Gao and Lee, 2001). The direct relaxation effects of H_2O_2 were not shown in the present experiments because the vessels were not contracted before application of H_2O_2. Instead, an attenuation of the following contractile response to phenylephrine was observed in the presence of H_2O_2.

The mechanisms by which PVAT-derived H_2O_2 modulates vessel constriction were investigated in this study. We found that activation of sGC was involved because sGC inhibition counteracted the anticontractile action of PVAT in PVAT+ E− arteries, and eliminated the inhibitory effect of exogenously applied H_2O_2 on phenylephrine-induced contraction in PVAT− E− arteries. These findings are consistent with previous reports that H_2O_2 acts as a non-NO activator of sGC in bovine and porcine coronary arteries (Hayabuchi *et al*., 1998; Iesaki *et al*., 1999). Inhibition of sGC also induced a small but significant contraction of the PVAT+ E− but not PVAT− E− arteries, suggesting that sGC was consistently activated in the aortic rings with intact PVAT.

In summary, we found in this study that PVAT exerts its relaxation effects through two distinct mechanisms: (1) through a transferable PVRF, which induces an endothelium-dependent relaxation through the release of NO and the activation of K^+ channels and (2) through an endothelium-independent mechanism, which involves the production of H_2O_2 by PVAT and its subsequent activation of sGC. The effects of these two mechanisms are additive. The pathways for the interaction between transferable PVRF and endothelium warrant further investigation.

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Conflict of interest

The authors state no conflict of interest.

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