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Mechanisms for perivascular adipose tissue-mediated potentiation of vascular contraction to perivascular neuronal stimulation: The role of adipocyte-derived angiotensin II

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ABSTRACT

In rat mesenteric arteries we have recently found that perivascular adipose tissue (PVAT) promoted vasoconstriction to perivascular neuronal activation (by electrical field stimulation, EFS) through generation of superoxide. In this study, we examined the role of adipocyte-generated angiotensin II in PVAT-mediated potentiation of contraction to nerve stimulation. In rat mesenteric PVAT, the presence of angiotesinogen and angiotensin I-converting enzyme (ACE) mRNA was confirmed by RT-PCR. Immunohistochemical staining showed the presence of angiotensin II in mesenteric PVAT. In rat mesenteric arteries, treatment of the vessels with an ACE inhibitor (enalaprilat) or angiotensin II type 1 receptor antagonist (candesartan) reduced PVAT-mediated potentiation of EFS-induced contraction. Exogenously applied angiotensin II enhanced EFS-induced contraction in arteries without PVAT, but not in the arteries with intact PVAT. Chronic treatment with an ACE inhibitor quinapril (14 days) lowered blood pressure and alleviated the potentiation effects of PVAT in EFS-induced contraction. Mesenteric arteries from quinapril-treated group now exhibited the potentiation response to exogenously applied angiotensin II in arteries with intact PVAT to a comparable level as in arteries with PVAT removed. Treatment with hydralazine reduced blood pressure to the same level as quinapril treatment, but did not affect PVAT-associated potentiation of vasoconstriction to EFS and the response to exogenously applied angiotensin II in PVAT-intact arteries. These results showed that adipocyte-derived angiotensin II is critically involved in PVAT-mediated potentiation of EFS-evoked contraction in rat mesenteric arteries.

1. Introduction

Perivascular adipose tissue (PVAT), which surrounds almost every systemic vessel, has emerged as a dual modulator of vascular function due to its two way regulation: attenuating contraction to agonists and promotes contraction to neuronal stimulation (Gao, 2007), similar to endothelium. PVAT attenuates contraction to various agonists (phenylephrine, serotonin, angiotensin II, and U 46619 (Lohn et al., 2002; Verlohren et al., 2004; Gao et al., 2005a, b). The attenuation of contractile responses to agonists by PVAT involves at least two mechanisms in rat aorta: the endothelium-dependent mechanism that involves the release of a transferable relaxation factor(s) (Lohn et al., 2002; Gao et al., 2005a) and the endothelium-independent mechanism that involves the production of hydrogen peroxide (Gao et al., 2007). We have further proven that the modulation of vascular function by PVAT is not limited to reducing contraction to agonists, because in rat mesenteric arteries PVAT potentiates vasoconstriction to perivascular neuronal excitation by electrical field stimulation (EFS), and the mechanisms for the potentiation involve superoxide production and subsequent activation of MAPK/ERK pathway (Gao et al., 2006). PVAT is a rich source of superoxide (Gao et al., 2006), the primary type of reactive oxygen species, which can be converted to hydrogen peroxide through dismutation. Both superoxide and hydrogen peroxide are involved in PVAT-associated regulation of vascular function, because superoxide promotes vasoconstriction to perivascular neuronal stimulation, while hydrogen peroxide attenuates vasoconstriction to agonists (Gao et al., 2007). However, the mediator that stimulates superoxide production in PVAT still remains unknown. Here we studied the role of adipocytes-originated angiotensin II in the potentiation of EFS-induced contraction by PVAT, because adipocytes are known a rich source of angiotensin peptides, and angiotensin II is a potent stimulator of superoxide production.

2. Materials and methods

2.1. Animals

Male Wistar rats (300–350 g) were used in this study (Harlan, Indianapolis, IN, USA). The study conforms with both the Guide for the

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Care and Use of Laboratory Animals published by the US National Institutes of Health and the guidelines of the Canadian Council on Animal Care, and was approved by the Animal Research Ethics Board of McMaster University (06-12-68).

2.2. Contractility study

The procedure for the preparation of rat mesenteric arterial rings has been described in our previous report (Gao et al., 2006). Briefly, the rat was euthanized by an overdose of sodium pentobarbital (60 mg/kg, i.p.), and the mesenteric artery was collected in oxygenated physiological salt solution 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 mesenteric arterial rings (4 mm long), one with PVAT intact (PVAT+) and the other with PVAT removed (PVAT−), were prepared from each artery. PVAT removal was carried out with fine scissors under microscope with extra caution not to damage the adventitial layer. Integrity of endothelium was confirmed by the relaxation response to carbamycholine chloride (1 μM) in rings pre-contracted with phenylephrine (1 μM). The integrity of the adventitia layer was not affected by PVAT removal as shown in previous study (Gao et al., 2006). A computerized myograph system was used to study the contraction and relaxation response of the mesenteric arteries. 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 second-train, at 2, 6,12, and 20 Hz at an interval of 3–5 min) as described previously (Gao et al., 2006). Contractile responses to EFS and to phenylephrine were expressed as a percentage of KCl contraction. To test the involvement of angiotensin II, vessel preparation was incubated with an angiotensin I-converting enzyme (ACE) inhibitor enalaprilat (an active form of enalapril) or angiotensin II type I receptor antagonist (candesartan) for 25–30 min before stimulated with EFS. Exogenously applied angiotensin II was introduced 5 min before testing.

2.3. Chronic treatments with an ACE inhibitor quinapril and with a vasodilator hydralazine

To examine the effects of in vivo inhibition of ACE on PVAT-mediated potentiation of contraction to EFS, quinapril (10 mg/kg/day, p.o.) was administered to Wistar rats for 14 days. Blood pressure was monitored with tail–cuff methods. Hydralazine (15 mg/kg/day, p.o.) was given as positive control to induce comparable blood pressure reduction. Rats after treatment with either quinapril or hydralazine were sacrificed with an overdose of sodium pentobarbital (60 mg/kg, i.p.) and the mesenteric arteries were processed as described in contractility study session.

2.4. Immunohistochemical staining for angiotensin II

Mesenteric arteries with intact PVAT were fixed in 10% formaldehyde and embedded in paraffin. All the following staining steps were carried out in humidified chamber and manufacture-recommended procedures (Santa Cruz Biotechnology, USA) were followed. Briefly, sections (5 μm in thickness) were treated with 1% hydrogen peroxide to block endogenous peroxidase activity, followed by incubation with 1.5% blocking serum. Rabbit anti-angiotensin II antibody (Phoenix, USA) was used in a 1:200 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 1.5% 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 min. Slides treated only with the 2nd antibody were used as negative control. Hematoxylin was used as a counter stain.

2.5. Reverse-transcriptase polymerase chain reaction (RT-PCR) measurement of mRNAs for angiotensinogen and ACE

Total RNAs of adipocytes isolated from PVAT were prepared as described by Engeli et al. (2003) with TRIzol reagent. RT-PCR was carried out using the RETRO script, according to the manufacturer's instructions. One μg 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 angiotensinogen (accession number BC087679; forward, 5′-CCTCGCTCTCTGGACTTATC-3′; reverse, 5′-CAGACACTGAGGTGCTGTTGT-3′), and ACE (accession number U03734; forward, 5′-GCAGAACTTCACTGACCAAAAG-3′; reverse, 5′-TCAAAGGAGGGGGACTCATA-3′). The conditions were 35 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 angiotensinogen and ACE PCR products were confirmed by sequencing on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, USA).

2.6. Chemicals

The following chemicals were used: angiotensin II, carbamylcholine chloride, 1-[[4-(Dimethylamino)-3-methylphenyl]methyl]-5-(dipheny lacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid (PD123319), phenylephrine, hydralazine, and enalaprilat (Sigma-Aldrich, St Louis, MO, USA); Candesartan was a generous gift from Takeda Pharmaceutical (Osaka, Japan), and quinapril was a gift from Pfizer (USA). Rabbit anti-angiotensin II antibody was purchased from PHOENIX (USA). Trizol reagent was purchased from Invitrogen (Burlington, ON, Canada), and RETROscript was from Ambiuon (Austin, TX, USA). Angiotensin II was dissolved in oxygen-free deionized water. Candesartan was dissolved in 10% Na_2CO_3. All other agents were dissolved in deionized water and prepared fresh daily.

2.7. 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 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 mesenteric arteries 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. Expression of angiotensinogen and ACE mRNA and immunohistochemical staining of angiotensin II in PVAT

Mesenteric arteries (both main trunk and smaller branches) are surrounded by a significant amount of PVAT (Fig. 1A and B). Adipocytes of mesenteric PVAT showed strong expression of both angiotensinogen and ACE mRNA (Fig. 1C). Immunohistochemical staining revealed the presence of angiotensin II in mesenteric PVAT (Fig. 1D and E).

3.2. Effects of ACE inhibition and angiotensin II type I receptor antagonism on EFS-induced contraction

EFS (12 Hz) induced a significant contraction in the mesenteric arteries, which was greater in PVAT+ than in PVAT− vessels, showing the promoting effects of PVAT on the contraction. The contraction to EFS was abolished by tetrodotoxin and by α_1 adrenoceptor antagonist prazosin (data not shown), as previously reported (Gao et al., 2006). Incubation with enalaprilat (10 μM) reduced EFS-induced contraction of PVAT+ arteries, while the contraction to EFS in PVAT− vessels

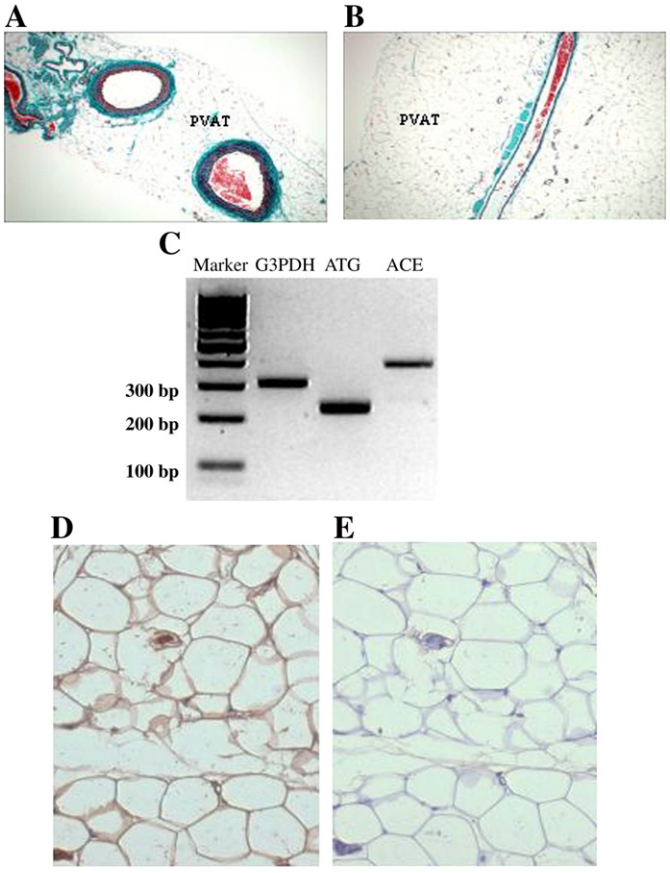

Fig. 1. A and B: presence of perivascular adipose tissue (PVAT) in mesenteric artery and its branch. C: mRNA expression of angiotensinogen (ATG) and angiotensin I-converting enzyme (ACE) from adipocytes of mesenteric PVAT. D: presence of angiotensin II by immunohistochemical staining, and E: negative control for angiotensin II staining, in the PVAT around mesenteric arteries. G3PDH: glyceraldehydes-3-phosphate dehydrogenase.

was not affected by this treatment (Fig. 2A, B, E). Similarly, angiotensin II type I receptor antagonist candesartan (1 μM) attenuated the contraction to EFS in PVAT+ arteries but not in PVAT− arteries (Fig. 2C, D, F). Angiotensin II type II receptor antagonist PD 123319 did not show any inhibitory action on the contraction (data not shown). Treatment with either enalaprilat or candesartan eliminated the differences between PVAT+ and PVAT− preparations in the contractile responses to EFS. These treatments did not affect the contraction to KCl (data not shown).

3.3. Chronic treatment with an ACE inhibitor (quinapril) or with a vasodilator (hydralazine) on EFS-induced contractile response and on the potentiation effects of exogenously applied angiotensin II

Chronic treatment with quinapril significantly reduced the contractile response to EFS in PVAT+ arteries to a level close to PVAT− vessels, while treatment with hydralazine did not alter the contractile response to EFS (Fig. 3A–D). In mesenteric arteries from control rats, exogenously applied angiotensin II enhanced the contraction to EFS in PVAT− but not in PVAT+ vessels. In quinapril-treated rats, angiotensin II-mediated enhancement was observed in both PVAT+ and PVAT− vessels, and the degree of the enhancement in PVAT+ vessels was comparable to the increase in PVAT− arteries. In hydralazine-treated rats, exogenously applied angiotensin II enhanced the contractile response to EFS only in PVAT− vessels, similar to the finding in control rats (Fig 4A and B). The enhancement by angiotensin II was abolished by treatment with angiotensin II type I receptor antagonist (candesartan, data not shown).

In mesenteric arteries from control rats, enalaprilat attenuated the contractile response to EFS in PVAT+ but not in PVAT− vessels, while in quinapril-treated rats enalaprilat-mediated inhibition of contraction was not observed in either PVAT+ or PVAT− vessels. In hydralazine-treated rats, enalaprilat induced a similar pattern of inhibition as in control rats (Fig. 4C and D).

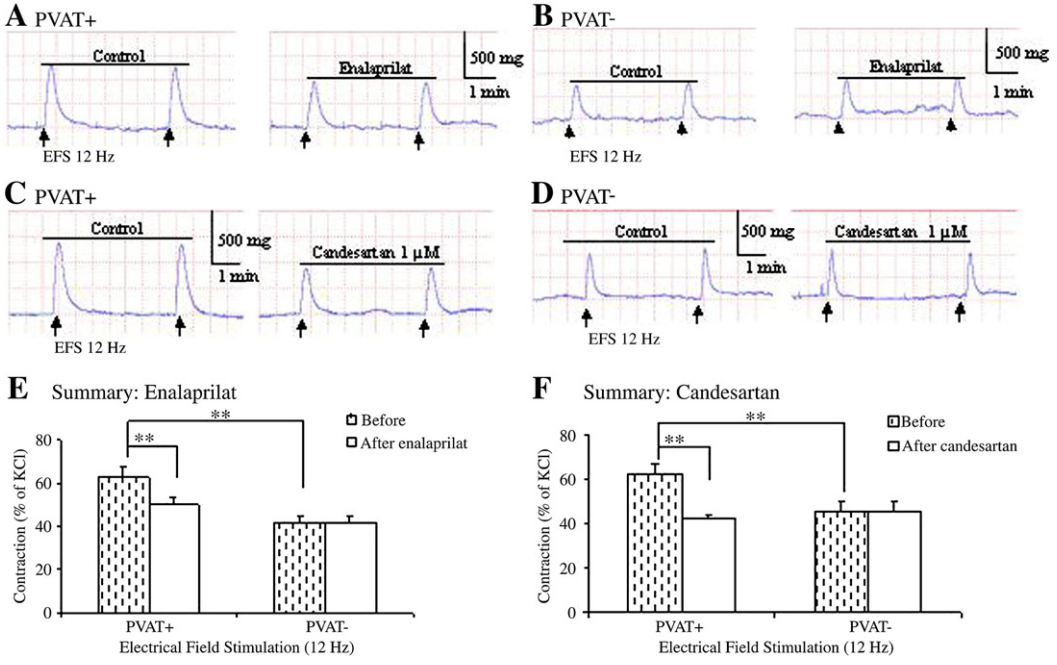

Fig. 2. Effects of ACE inhibitor (enalaprilat, 10 μM) and angiotensin II type I receptor antagonist (candesartan, 1 μM) on the contractile response to electrical field stimulation (EFS, 12 Hz) in mesenteric arteries with or without PVAT (PVAT+, or PVAT−) of Wistar rats. Typical recordings showed enalaprilat- or candesartan-caused attenuation of EFS-induced contraction in PVAT+ arteries (A, C), but not in PVAT− arteries (B, D). Summaries were shown in E and F. ** $P<0.01$. $n=6$ rats for each group.

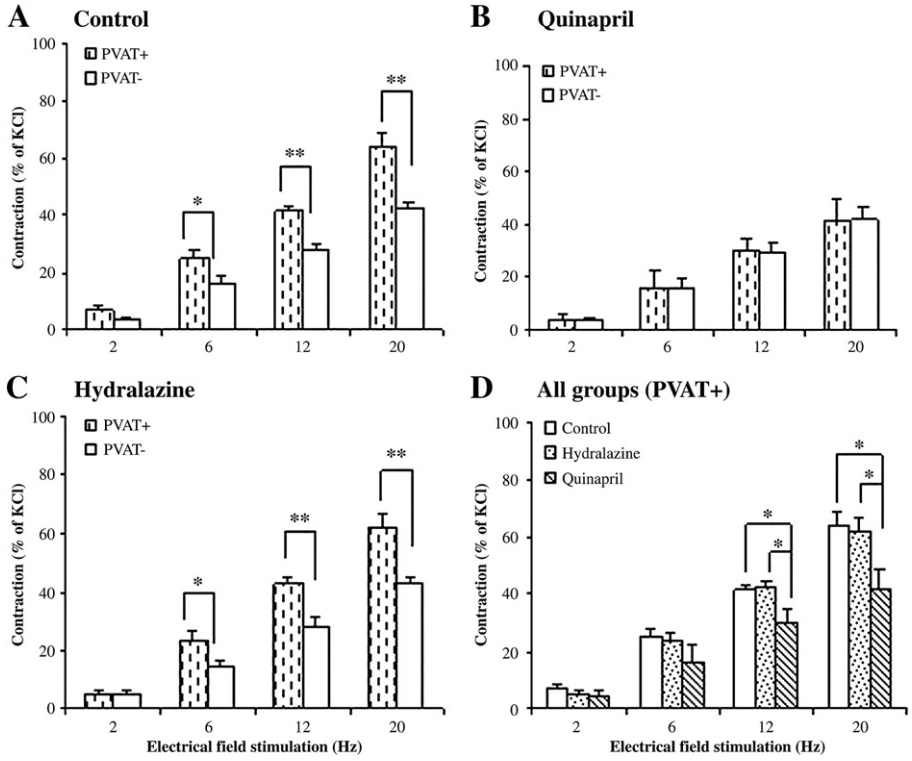

Fig. 3. Effects of chronic treatment (2 weeks) with quinapril (10 mg/kg/day) or hydralazine (15 mg/kg/day) on electrical field stimulation (EFS)-induced contractile responses in arteries with or without PVAT (PVAT+, or PVAT−) of Wistar rats. A: control group, PVAT enhanced the contraction to EFS. B: treatment with quinapril abolished PVAT-associated enhancement of the contraction to EFS. C: treatment with hydralazine did not affect PVAT-associated enhancement of the contraction to EFS, as in control group. D: comparison of contraction to EFS among all groups. Treatment with quinapril but not with hydralazine attenuated EFS-induced contraction. $^{*}P<0.05$, $^{**}P<0.01$. $n=6$ rats for each group.

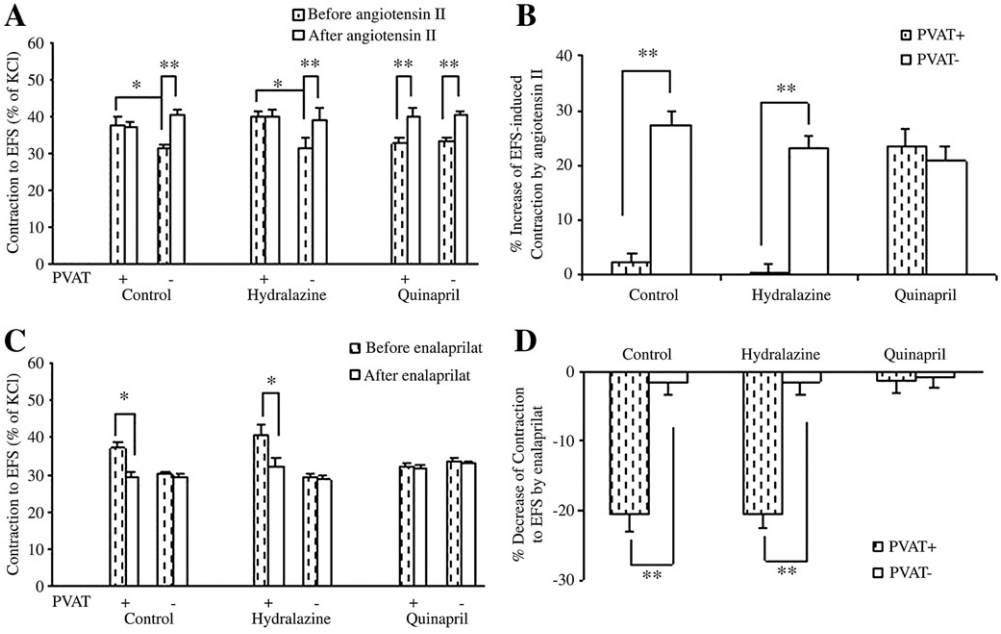

Fig. 4. Effects of chronic treatment with quinapril (10 mg/kg/day) and hydralazine (15 mg/kg/day) for 2 weeks on angiotensin II (10^{-7} M)-induced potentiation (A and B) or enalaprilat (10 μM)-produced inhibition (C and D) of contraction to electrical field stimulation (EFS, 12 Hz) in rat mesenteric arteries with or without PVAT (PVAT+ or PVAT−). A: angiotensin II equally potentiated EFS-induced contraction in both PVAT+ and PVAT− arteries of rats treated with quinapril, but only increased the contraction in PVAT− arteries of rats treated with hydralazine or control. B: percentage of angiotensin II-induced potentiation in these groups. C: treatment with quinapril abolished the inhibition by enalaprilat, but treatment with hydralazine did not affect the inhibition as in the control group. D: percentage of enalaprilat-induced inhibition of EFS-induced contraction in these groups. $^{*}P<0.05$, $^{**}P<0.01$. $n=6$ rats for each group.

3.4. Effects of chronic treatment with an ACE inhibitor (quinapril) and a vasodilator (hydralazine) on blood pressure and contractile response of isolated mesenteric arteries to KCl

Chronic treatment with quinapril or hydralazine produced a comparable blood pressure lowering effect (Fig. 5A) but neither treatment affected the contractile responses to KCl (60 mM) of the arteries (Fig. 5B).

4. Discussion

This study was designed to investigate the role of angiotensin II produced by PVAT in the potentiation associated with PVAT in the contraction to EFS. Here we report that adipocytes-derived angiotensin II mediates PVAT-associated enhancement of contractile response to perivascular neuronal excitation by EFS. To the best of our knowledge, this is the first report to show that adipocytes-originated angiotensin II participates in the neuronal regulation of vascular function in peripheral arteries.

Mounting evidence has shown that PVAT, a layer of fat tissue that surrounds almost every systemic artery, modulates vascular function through several mechanisms (Gao, 2007). PVAT counteracts vessel constriction to various agonists or induces relaxation of pre-contracted vessels by producing relaxation factors. PVAT also promotes vasoconstriction to perivascular neuronal stimulation through superoxide production and subsequent activation of tyrosine kinase-MAPK/ERK pathway (Gao et al., 2006), but the mechanisms by which superoxide production was stimulated remain unclear. In this study we investigated if angiotensin II played any role in PVAT-associated potentiation, because adipocytes are known as a rich source of angiotensinogen and possess all the enzymes to synthesize angiotensin II (Ahima and Flier, 2000; Engeli et al., 2003; Karlsson et al., 1998; Kershaw and Flier, 2004). We found that rat mesenteric artery was surrounded with a significant amount of PVAT, and that the adipocytes of the PVAT contain a remarkable level of angiotensinogen and ACE mRNA and abundant angiotensin II, which is consistent with previous findings in rat aortic PVAT (Cassis et al., 1988; Cassis et al., 2008; Thatcher et al., 2009) and in rodent or human non-perivascular fat tissues (Campbell et al., 1994; Campbell et al., 1995; Zhang et al., 2006). Therefore, as generally recognized that adipose tissue is a significant source of angiotensinogen and angiotensin peptides other than systemic renin–angiotensin system, PVAT serves as a particular perivascular source of angiotensin peptides locally for blood vessels.

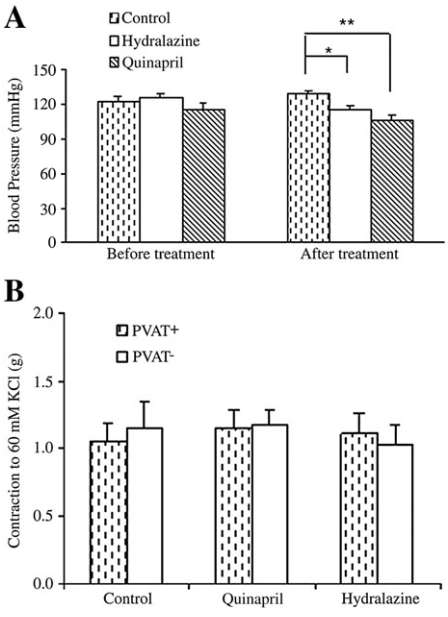

Fig. 5. Effects of chronic treatment (2 weeks) with quinapril (10 mg/kg/day) and hydralazine (15 mg/kg/day) on blood pressure (A) and on the contractile response of mesenteric arteries to KCl (60 mM) (B) in Wistar rats. Quinapril and hydralazine equally reduced systolic arterial blood pressure. Neither treatment affected the contractile response of isolated mesenteric arteries to KCl. $n=6$ rats for each group. * $P<0.05$, ** $P<0.01$.

We have established that angiotensin II produced by PVAT plays an important role in the potentiation of nerve-mediated contraction based on the following in vitro studies using mesenteric arteries from untreated rats. Firstly, inhibition of angiotensin II synthesis with an ACE inhibitor, enalaprilat, attenuated the contraction to EFS in arteries with intact PVAT but not in arteries with PVAT removed. Second, angiotensin II type I receptor antagonist, candesartan, similarly reduced the contraction to EFS in PVAT-intact but not in PVAT-removed vessels. The ineffectiveness of ACE inhibition and angiotensin II type I receptor antagonism in PVAT-removed arteries indicates that PVAT is the main source of angiotensin II, which is consistent with the finding with angiotensinogen mRNA expression in rat aorta (Cassis et al., 1988). Thirdly, exogenously applied angiotensin II mimicked the presence of PVAT by enhancing the contractile response to EFS in the arteries with PVAT removed, but not in the arteries with intact PVAT. This is probably because the local production of angiotensin II by PVAT had already reached a maximum, so that further addition of exogenous angiotensin II had no effect. These results suggested that PVAT-generated angiotensin II was indeed involved in PVAT-associated potentiation of contraction to EFS in rat mesenteric arteries.

This notion was further supported by the findings with arteries from animals chronically treated with an ACE inhibitor. After 2-week treatment with quinapril, PVAT-associated enhancement of EFS-induced contraction was blunted, and the potentiation by exogenously applied angiotensin II in response to EFS emerged in arteries with intact PVAT, which was not seen in untreated animals, with a comparable level of potentiation as in the arteries with PVAT removed. The treatment with quinapril also rendered the in vitro ACE inhibitor (enalaprilat) ineffective, because ACE had already been blocked by the chronic treatment.

Quinapril lowered blood pressure in these animals. In order to rule out the possibility that the treatment effect of quinapril on EFS-induced contraction was due to its blood pressure lowering effect, we used rats treated with hydralazine with a similar decrease in blood pressure as controls. Hydralazine does not inhibit ACE. We found that mesenteric arteries from hydralazine-treated rats exhibited the same pattern in reactions as in control rats: the potentiation of contraction to perivascular nerve stimulation by PVAT, and an inhibition of the potentiation by in vitro ACE inhibition. These results provided further support for the critical role that angiotensin II played in PVAT-associated potentiation of vessel contraction to EFS. Vascular contractility was not changed after either quinapril or hydralazine treatment, as contraction to membrane depolarization by KCl was comparable among these groups.

We have found in previous study that PVAT promoted vasoconstriction to EFS through superoxide production (Gao et al., 2006). In this study we established that PVAT-produced angiotensin II is critically involved in the potentiation. As well recognized, angiotensin II is a potent stimulator for NAD(P)H oxidase to produce superoxide (Rahman et al., 2004; Touyz and Schiffrin, 1999), either by enhancing the activity upon short term exposure (in minutes)(Cruzado et al., 2005), or by inducing overexpression of this enzyme upon long-term incubation (hours or days)(Lassegue et al., 2001; Weber et al., 2005). We have also demonstrated the presence of NAD(P)H oxidase, in rat mesenteric PVAT (Gao et al., 2006), and the ability of angiotensin II in stimulating the enzyme to produce superoxide, which mediated angiotensin II-associated enhancement of EFS-induced contraction, in rat mesenteric arteries (Lu et al., 2008). Furthermore, a common signaling pathway, activation of tyrosine kinase-MAPK/ERK1/2, was involved in the potentiation of EFS-induced contraction by superoxide and by angiotensin II (Gao et al., 2006; Lu et al., 2008). Taken together,

these findings suggest that PVAT-derived angiotensin II is a stimulator for NAD(P)H oxidase to produce superoxide, which, in turn, mediates PVAT-associated enhancement of EFS-induced contraction. The detailed mechanisms involved in angiotensin II release from PVAT upon perivascular neuronal stimulation warrant further investigation.

There is substantial evidence indicating that angiotensin peptides formed locally play an important role in paracrine regulation of local tissue function, which may especially be true in vascular tissue because almost every systemic artery is surrounded by a layer of adipose tissue that produces angiotensin peptides (Fleming et al., 2006; Engeli et al., 2003). In rat aorta, we have recently found that angiotensin (1–7) is one of the transferable relaxation factors from PVAT (Lee et al., 2009), and in this study we found that PVAT-originated angiotensin II is critically involved in the potentiation of contraction to sympathetic stimulation. Because vascular tone is constantly under the control of sympathetic activity (Brooks and Osborn, 1995; Luff, 1996), the potentiation of vasoconstriction to EFS by PVAT-originated angiotensin II may represent an important regulation of vascular function.

In summary, we have demonstrated that PVAT-derived angiotensin II is critically involved in PVAT-mediated potentiation of EFS-evoked contraction in rat mesenteric arteries. Together with the finding that angiotensin (1–7) has been identified as one of the PVAT-derived relaxation factors, PVAT-derived angiotensin peptides seem to play a significant role in regulation of vascular function.

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