Adiponectin and its receptors are involved in hypertensive vascular injury

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Abstract. Adipocyte-derived adiponectin (APN) is involved in the protection against cardiovascular disease, but the endogenous APN and its receptor expression in the perivascular adipocytes and their role in hypertensive vascular injury remain unclear. The present study aimed to detect endogenous APN and its receptor expression and their protective effects against hypertensive vascular injury. APN was mainly expressed in the perivascular adipocytes, while its receptors AdipoR1 and AdipoR2 were ubiquitously expressed in the blood vessels. Angiotensin II (Ang II)-induced hypertension resulted in a significant decrease of APN and AdipoR1 and AdipoR2 in the perivascular adipocytes and vascular cells. The migration assay used demonstrated that APN attenuated Ang II-induced vascular smooth muscle cells migration and p38 phosphorylation. Furthermore, the in vivo study demonstrated that APN receptor agonist AdipoRon attenuated Ang II-induced hypertensive vascular hypertrophy and fibrosis. Taken together, the present study indicated that perivascular adipocytes-derived APN attenuated hypertensive vascular injury possibly via its receptor-mediated inhibition of p38 signaling pathway.

Introduction

Adipose tissue, as a highly active endocrine organ, produces and releases a range of bioactive substances, such as adipokines, inflammatory factors and hormones (1,2). Among these, adiponectin (APN) has been reported as a major anti-inflammatory mediator inhibiting the development of cardiovascular disease (3,4). There is enough evidence indicating that adipocyte-derived APN can exert protective effects in different animal models such as hypertension and obesity (5-7). However, APN attenuates cardiac inflammation and remodeling in the classical angiotensin II (Ang II)-induced hypertension model (8). However, the potential protective role of APN on the Ang II-induced vascular remodeling has not been fully elucidated.

Adiponectin, as a fat-derived hormone, fulfills a critical role as an important messenger between adipocytes and other organs through the adiponectin receptor 1 and/or receptor 2 (AdipoR1/R2). These receptors are ubiquitously expressed in the adipose tissue, skeletal muscle and cardiovascular system (9,10). Previous studies have demonstrated that APN attenuates vascular remodeling through inhibiting vascular smooth muscle cell (VSMC) proliferation and phenotype trans differentiation (11,12). However, little is known about the endogenous APN and its receptor expression and function in the vasculatures of the Ang II-induced hypertensive model. In the present study, APN and AdipoR expression in vascular cells was demonstrated and the effects of APN on VSMCs migration were investigated. The distribution of these molecules in the vasculature of Ang-II induced hypertension were detected in vivo, and whether the APN receptor agonist AdipoRon could attenuate hypertension-associated vascular injury was determined.

Materials and methods

Cell culture. Vascular cells, such as human umbilical vein endothelial cells (HUVECs; American Type Culture Collection, Manassas, VA, USA) and VSMCs (PromoCell GmbH, Heidelberg, Germany), were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) as previously described (13). Cells at 4-6 passages were used in subsequent experiments. 3T3-L1 adipocytes were differentiated from 3T3-L1 fibroblasts (American Type Culture Collection) as previously described (14). Briefly, 3T3-L1 fibroblasts were cultured to reach confluency in a 12-well plate and were differentiated into adipocytes in the specific differentiation medium, consisting of DMEM supplemented with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc.) and 3-isobutyl-1-methylxanthine (5 µM), dexamethasone (0.25 µM) (both from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and insulin (5 µg/ml; Gibco; Thermo Fisher Scientific, Inc.) for 2 days, followed...
by treatment with DMEM supplemented with 10% FBS and insulin (5 µg/ml).

Animal models. Male, 8-12 week-old C57BL/6J background mice were used in the present study. To induce hypertension, mice (n=5) were subcutaneously infused with Ang II (Sigma-Aldrich; Merck KGaA) at a 1,500 ng/kg/min dose for 14 days with ALZET mini osmotic pumps (DURECT Corporation, Cupertino, CA, USA), and in the sham group mice were treated with saline (n=5). Blood pressure was measured non-invasively every day by the tail-cuff method (Softron BP-98A; Softron Co., Ltd., Tokyo, Japan). To determine the role of APN receptor in hypertensive vascular injury, Ang II-infused mice received APN receptor agonist AdipoRon (n=5, 30 mg/kg/day; Selleck Chemicals, Houston, TX, USA) via a gavage tube. The experiments were performed in adherence with the National Institutes of Health guidelines on the Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (Shanghai, China).

Histological analysis. Thoracic aortas of Ang II-induced hypertensive mice were obtained as previously described (15), and then were fixed in 4% paraformaldehyde at 4°C for 24 h, embedded in paraffin, and 5 µm sections were prepared for hematoxylin (15 min at room temperature) and eosin (5 min at room temperature) (H&E) and picrosirius red staining (30 min at room temperature) to assess the vascular hypertrophy and fibrosis according to the manufacturer's (Wuhan Servicebio Technology Co., Ltd., Wuhan, China). Morphometric analysis was performed using Image-Pro Plus 6.0 software to assess vascular hypertrophy by measuring the medium and lumen area. Fibrotic staining was expressed as a percentage of stained areas (red) to the total areas examined. For immunofluorescence staining, paraffin sections were first deparaffinized and rehydrated in xylene (30 min), washed with TBS with Tween-20 and stained with hematoxylin (15 min at room temperature) and eosin (5 min at room temperature) and eosin (5 min at room temperature) and incubated at 37°C. Following 2 h incubation, Ang II (100 nM) was added. After 6 h, the cells remaining on the upper surface of the chamber were removed with a cotton swap, migrated cells on the lower surface of the chamber were counted, and stained with hematoxylin (15 min at room temperature) and eosin (5 min at room temperature) and three random microscopic fields (magnification, x200) per well were quantified by a fluorescence microscope (DM3000; Leica, Wetzlar, Germany).

Migration assay. VSMCs migration was measured using a Transwell chamber with a polycarbonate membrane (6.5 mm diameter and 8.0 µm pore size; Corning Incorporated, Corning, NY, USA). Briefly, serum-starved VSMCs were trypsinized and resuspended in DMEM with 0.5% bovine serum albumin (Sigma Sigma-Aldrich; Merch KGaA). Cell suspension (100 µl, 1.0x10^5 cells/ml) was loaded in the upper compartment of the chamber. DMEM with APN (10 µg/ml) were added to lower compartment and incubated at 37°C. Following 2 h incubation, Ang II (100 nM) was added. After 6 h, the cells remaining on the upper surface of the chamber were removed with a cotton swap, migrated cells on the lower surface of the chamber were counted, and stained with hematoxylin (15 min at room temperature) and eosin (5 min at room temperature) and three random microscopic fields (magnification, x200) per well were quantified by a fluorescence microscope (DM3000; Leica, Wetzlar, Germany).

Statistical analysis. Statistical analysis was performed using SPSS 19.0 (SPSS; IBM Corp., Armonk, NY, USA). Data are expressed as the mean ± standard deviation. Comparisons of experimental groups were analyzed by Student's t-test (between two groups) or one-way analysis of variance, followed by the post-hoc Dunnett's test for data with more than two groups (Levene's tests for equal variance and Welch's and Brown-Forsythe's test for unequal variances). P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of APN and AdipoRs in vascular cells. APN and Adipor expression were detected in adipocytes, VSMCs and endothelial cells (ECs) by western blot analysis. As demonstrated in Fig. 1, APN expression was highest in the adipocytes, with the expression of APN in the VSMCs and ECs less than...
in the adipocytes. Additionally, AdipoR1 and AdipoR2 were expressed in the VSMCs, ECs and adipocytes.

**Expression of APN and AdipoR in the vasculature.** To detect APN and AdipoR expression in the blood vessels, immunostaining of cross-sections of thoracic aorta in Sham and Ang II-induced hypertensive mice was performed. Ang II infusion resulted in a dramatic increased blood pressure including systolic and diastolic blood pressure (Fig. 2A). In accordance with the *in vitro* results, APN was mainly distributed in the perivascular adipocytes (Fig. 2B and C, perilipin-positive cells), while AdipoR1 and AdipoR2 were expressed in both VSMCs (α smooth muscle actin-positive cells) and adipocytes (Fig. 2D and E). Ang II-induced hypertension resulted in decreased APN and AdipoR expression in the vasculature.

**Effects of Ang II on APN and AdipoR expression.** To detect the direct effects of Ang II on APN, APN levels in the 3T3-L1 adipocytes pretreated with Ang II were measured. The result demonstrated that APN expression was decreased in a time-dependent manner following Ang II treatment (Fig. 3A). In addition, it was also illustrated that AdipoR1 and AdipoR2 expression was decreased in a time-dependent manner following Ang II treatment in the VSMCs (Fig. 3B).

**APN attenuates VSMCs migration.** Vascular active substance-induced VSMCs migration serves a pivotal role in hypertension-associated vascular remodeling (16). Whether APN affects Ang II-induced VSMCs migration was examined by utilizing the Transwell chamber assay. As depicted in Fig. 4A, APN (10 µg/ml) significantly attenuated Ang II-induced VSMCs migration. The p38 signaling pathway is known to be involved in the regulation of VSMC migration (17). In the present study, the phosphorylation of p38 mitogen-activated protein kinase (MAPK) was reduced following APN treatment in Ang II-stimulated VSMCs (Fig. 4B).
AdipoRon attenuates hypertensive vascular remodeling. AdipoRon is an orally active APN receptor activator, which potentially exerts protective effects against cardiac remodeling (18,19). In order to detect the effects of AdipoRon in hypertension-associated vascular remodeling, the Ang II-infused mice were orally treated with AdipoRon (30 mg/kg/day). As demonstrated in Fig. 5A, AdipoRon treatment resulted in decreased p38 phosphorylation. In addition, AdipoRon attenuated Ang II-induced perivascular collagen deposition and vascular hypertrophy as detected by Sirius Red and H&E stainings (Fig. 5B and C, respectively).

Discussion
In the present study, the distribution of APN and AdipoR in the vasculature was detected and it was demonstrated that APN...
was predominantly expressed in the perivascular adipocytes, while AdipoR1 and R2 were ubiquitously expressed in the blood vessels. APN attenuated Ang II-induced VSMCs migration and p38 phosphorylation. Furthermore, APN receptor agonist, AdipoRon, appeared to attenuate Ang II-induced hypertensive vascular hypertrophy and fibrosis.

Perivascular adipocytes are an important source of adipokines and their role in regulating vascular function is well established (20-22). APN, an abundant adipocyte-derived factor with a variety of biological functions, was first described in 1995 (23). Over the past two decades, numerous studies have elucidated its physiological functions, which may improve insulin sensitivity in insulin target tissues, modulate inflammatory responses, and serve a crucial role in the regulation of energy metabolism (24,25). In the cardiovascular system, APN ameliorates Ang II-induced cardiac remodeling by attenuating inflammation, it inhibits connective tissue growth factor-induced VSMCs proliferation, and promotes endothelial nitric oxide synthase expression and nitric oxide release from endothelial cells (24,26). Previous studies have demonstrated that hypertensive mice express decreased APN plasma levels compared with the controls (27). To the best of our knowledge, the present study was the first to demonstrate that hypertension induced APN downregulation in local perivascular adipocytes. VSMC dysfunction, including migration, proliferation and phenotype transdifferentiation, has an important role in hypertension-induced vascular remodeling. VSMC migration is associated with vascular remodeling when exposed to high stress, such as hypertension. It has been demonstrated that Ang II significantly increased the VSMC migration (28). APN receptors, AdipoR1 and AdipoR2, were originally identified by screening a skeletal muscle expression library for cDNAs that encoded proteins that to the globular domain of APN (29). Both receptors contain seven transmembrane domains and belong to the progestin and adipoQ receptor family, which has the opposite transmembrane topology to the G-protein coupled receptors, and have the N-terminus in the cytoplasm and the C-terminus facing the extracellular space. Although ubiquitously expressed, AdipoR1 is most abundant in skeletal muscle whereas AdipoR2 is mainly restricted to the liver (30,31).

In the present study, ubiquitous expression of AdipoR1 and AdipoR2 in the vasculature was demonstrated, including ECs, VSMCs and perivascular adipocytes. In an obesity and diabetic mouse model, AdipoR1 expression was reduced in the target tissue (32,33). However, in a rat model of chronic kidney disease, AdipoR1 and AdipoR2 expression was significantly increased in renal cells (34). In the present study, AdipoR1 and AdipoR2 expression was decreased in the cultured VSMCs treated with Ang II. In addition, Ang II-induced hypertension resulted in a significant decrease of AdipoR1 and AdipoR2 expression in the perivascular adipocytes and VSMCs in vivo. AdipoR1 or AdipoR2 mediate the effects of APN by regulating multiple signaling pathways, including the peroxisome proliferator-activated receptor-α, the AMP-activated protein kinase, and the p38 MAPK (12,35,36). Previous studies have demonstrated that APN could attenuate lipid deposition and apoptosis through activating p38 MAPK pathway (37,38). In the present study, it was demonstrated that APN alone could induce...
p38 phosphorylation, which is in accordance with previous studies (39,40). Furthermore, Ang II promoted p38 phosphorylation. It could be speculated that the APN/p38 axis may have an inhibitory feedback effect on p38 phosphorylation, with p38 phosphorylation level decreased following Ang II treatment. APN receptor agonist, AdipoRon, inhibited p38 phosphorylation in the vasculature of Ang II-induced hypertensive mice.

In conclusion, the present study demonstrated that Ang II-induced hypertensive mice had decreased expression of APN and AdipoR in the peripheral adipocytes and VSMCs. APN attenuated Ang II-induced VSMCs migration and p38 phosphorylation. APN receptor agonist AdipoRon attenuated Ang II-induced hypertensive vascular remodeling. These results suggest that APN and APN receptors may be potential therapeutic targets for inhibiting hypertensive vascular injury.

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