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PPARδ reduces abdominal aortic aneurysm formation in angiotensin II-infused apolipoprotein E-deficient mice by regulating extracellular matrix homeostasis and inflammatory responses ☆

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ABSTRACT

Background: Abdominal aortic aneurysm (AAA) is an inflammatory disorder characterized by a localized degradation of connective tissue and apoptosis of vascular smooth muscle cells. This study examined whether the ligand-activated peroxisome proliferator-activated receptor (PPAR) δ can directly antagonize angiotensin II (Ang II)-induced AAA formation in apoE-deficient mice.

Methods and results: Six-month-old male apoE-deficient mice were infused with Ang II and/or GW501516 (1.44 and 3.3 mg/kg/day, respectively) *via* osmotic mini-pumps. At day 28, aortic size was measured and tissues were collected for analyses. Co-infusion of GW501516, an activator of PPARδ, attenuated both the incidence and the severity of Ang II-induced AAA in apoE-deficient mice. Ligand-activated PPARδ also reduced infiltration of macrophages, resulting in significant decreases in chemotactic proteins such as monocyte chemoattractant protein-1, macrophage inflammatory protein-1β, and inducible nitric oxide synthase. The anti-inflammatory effect of GW501516 was associated with the suppression of apoptotic cell death, along with the inhibition of medial smooth muscle cell loss and focal elastin destruction, which leads to a medial dissection and aortic rupture. These ameliorative effects of GW501516 on Ang II-induced aneurysm were correlated with increased expression of extracellular matrix (ECM) proteins, such as types I and III collagen, fibronectin, and elastin, along with the up-regulation of transforming growth factor-β1. In addition, ligand-activated PPARδ also increased the expression of tissue inhibitor of metalloproteinase (TIMP)-2 and TIMP-3, while it strongly suppressed that of matrix metalloproteinase-2.

Conclusions: PPARδ attenuates Ang II-induced AAA formation by regulating ECM homeostasis and inflammatory responses, suggesting a novel strategy for the treatment of AAA.

1. Introduction

Abdominal aortic aneurysm (AAA) is a multifactorial vascular disorder manifested by a localized structural degeneration of the aortic wall, which leads to permanent segmental dilatation and rupture [1,2]. The pathological features of AAA are characterized by chronic inflammation of the aortic wall, apoptosis of the vascular smooth muscle cells (VSMCs), and destruction of elastic media [3–5]. Apoptosis of medial smooth muscle cells is increased in the aneurysm lesion [6], and depletion of these cells eliminates a cell population capable of synthesizing matrix proteins, which enables tissue repair [7]. A number of molecular mediators and proteins related to extracellular matrix (ECM) homeostasis contribute to the destruction of elastic media [1,2]. Among the matrix proteins–elastin, type I collagen, and type III collagen–are the major structural components of the aortic wall. An accelerated turnover of these matrix proteins was demonstrated in the development of AAA [8,9]. Matrix metalloproteinases (MMPs) contribute to the breakdown of ECM [10], among which MMP-2 and MMP-9 have been intensively studied in the pathology of aneurysm formation. In fact, both MMP-2 and MMP-9 mRNA are significantly elevated in the aneurysm lesion,

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and the development of AAA is completely abolished in MMP-2- and MMP-9-knockout mice [11]. On the other hand, the activity of MMPs is tightly regulated by tissue inhibitors of metalloproteinases (TIMPs). Accordingly, the mRNA levels of TIMPs were decreased in the aneurysm lesion [12,13], and enhanced formation of AAA was demonstrated in TIMP-1-knockout mice [14]. These findings suggest that deranged homeostasis of ECM proteins is a causing factor in the development of aneurysms.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcriptional factors with multiple functions in energy metabolism and vascular biology [15–17]. These receptors regulate gene expression by dimerizing with the retinoid X receptor and binding to specific recognition sequences, termed as PPAR response elements, which are located in the regulatory regions of target genes [18]. Among isoforms identified in mammals, PPARδ is abundantly expressed in a variety of cell lineages, including VSMCs. It has been postulated that PPARδ exerts anti-atherosclerotic effects by modulating the availability of transcriptional repressors such as BCL-6 [19]. These effects of PPARδ were further supported by the finding that a synthetic PPARδ ligand suppressed the inflammation and proliferation of VSMCs through induction of transforming growth factor (TGF)-β1 [20]. TGF-β1 induced by a synthetic PPARδ ligand increased the deposition of ECM by enhancing the synthesis of collagens, fibronectin, and elastin that affect the stability of the atherosclerotic plaque [20,21]. In addition to the anti-atherogenic effects of PPARδ, accumulating evidence indicates that PPARδ may take part in the regulation of vascular inflammation and fibrosis [20–23]. We therefore hypothesized that the activation of PPARδ may affect the formation of aneurysms through the modulation of inflammation and fibrosis. Here, we demonstrate that the activation of PPARδ reduced angiotensin II (Ang II)-induced AAA formation with transcriptional regulation of ECM-related proteins. Administration of a PPARδ ligand also attenuated inflammatory responses and apoptotic cell death in the abdominal aorta. Compounds that modulate PPARδ may become a potential means to attenuate inflammation and slow the progression of AAA.

2. Methods

2.1. Experimental animals

ApoE$^{-/-}$ mice were obtained from Japan SLC Inc. (Shizuoka, Japan) and housed in a pathogen-free environment. Standard sterilized laboratory diet and water were available *ad libitum* under controlled environmental conditions with a 12 h light/dark cycle (light on 06:00). Six-month-old male apoE$^{-/-}$ mice were randomly assigned to one of three groups: infusion of vehicle (control), infusion of Ang II (1.44 mg/kg/day), or infusion of Ang II plus GW501516 (1.44 and 3.3 mg/kg/day, respectively). The osmotic mini-pumps (model 2004, ALZET, Cupertino, CA) were implanted to deliver indicated reagent(s) subcutaneously for 4 weeks. Blood cholesterol, triglyceride, and free fatty acid levels were profiled by Seoul Clinical Laboratories' services (Seoul, Korea) using standard enzymatic assay kits (Wako Chemical Co., Japan). The abdominal aorta (from the last intercostal artery to the ileal bifurcation) was sectioned and portions of this tissue were either fixed in 4% paraformaldehyde for immunohistochemistry or homogenized for Western blot analyses. All animal studies were approved by the Institutional Animal Care Committee of Gyeongsang National University.

2.2. Systolic blood pressure measurement

Systolic blood pressure (SBP) was measured every week for the duration of the study using a tail–cuff computerized system (BP-2000, Visitech Systems Inc., Apex, NC). Mice were initially accustomed to the instrument for 5 consecutive days prior to the actual measurements.

2.3. Measurement of aortic diameter

After the animals were euthanized, blood was drawn from the right ventricle and the aorta was irrigated with cold phosphate-buffered saline through the left ventricle. Using a dissection stereomicroscope (Olympus SZ-CTV, Tokyo, Japan), the abdominal aorta was exposed and the periadventitial tissue was carefully dissected away from the outer surface. Maximal aortic diameter was determined with the assistance of an operating microscope (Olympus CKX41, Tokyo, Japan) and a calibrated TEM grid (Ted Pella, Inc., Redding, CA). AAA was defined as ≥50% enlargement of maximal abdominal aorta diameter.

2.4. Western blot analyses

Aortic tissue was homogenized using a FastPrep-24 instrument with ceramic spheres (MP Biomedicals, OH), and an aliquot of the protein subjected to SDS-polyacrylamide gel electrophoresis was transferred onto a Hybond-P^{+} polyvinylidene difluoride membrane (Amersham BioSciences UK Ltd., UK). Membranes blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 overnight at 4 °C were reacted with the indicated specific antibodies in TBS containing 1% BSA and 0.05% Tween-20 overnight at 4 °C, and incubated with a peroxidase-conjugated goat antibody diluted to 1:3000 for 2 h at room temperature. After extensive washing in TBS containing 0.1% BSA and 0.1% Tween-20, immuno-reactive bands were detected using West-ZOL Plus (iNtRON Biotechnology, Seoul, Korea). Polyclonal antibodies specific for type I and III collagens, elastin, fibronectin, monocyte chemoattractant protein (MCP)-1, MMP-2, MMP-9, TIMP-1, TIMP-2, TIMP-3, and TGF-β1, as well as horseradish peroxidase-conjugated IgG, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal goat anti-mouse macrophage inflammatory protein (MIP)-1β and rabbit anti-actin antibodies were purchased from R&D Systems (Minneapolis, MN) and Sigma-Aldrich Co. (St. Louis, MO), respectively. Polyclonal rabbit anti-mouse inducible nitric oxide synthase (iNOS) and anti-alpha smooth muscle actin (α-SMA) antibodies were obtained from Abcam plc. (Cambridge, UK).

2.5. Histological analyses

Following fixation with 4% paraformaldehyde, tissues were embedded in paraffin. The paraffin blocks were sectioned into 5-μm slices and mounted on double-gelatin-coated glass slides. Before histological process, sections were deparaffinized and hydrated in H_2O. Aortic sections were routinely stained with hematoxylin–eosin and Masson's trichrome. To assess elastic fiber integrity, sections were also stained with Weigert's Resorcin Fuchsin kit (Cat # 26370, EMS, PA, USA) as per the manufacturer's instructions. For immunohistochemistry, tissues were boiled in 10 mmol/L sodium citrate buffer (pH 6.0) for 10 min to retrieve antigens. Normal serum (1%) from the same species as that of the secondary antibodies was used in the blocking buffer and antibody diluent. Each section was incubated with primary antibody overnight at room temperature or 4 °C. To detect MCP-1, iNOS, α-SMA, and macrophages, Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (Invitrogen) were used. Macrophages were immunostained with a rabbit anti-serum against murine macrophages (AIA31240, Accurate Chemical and Scientific Corporation). Biotinylated secondary antibodies and streptavidin-conjugated Alexa Flour 594 were used for types I and III collagen, elastin, fibronectin, MMP-2, MMP-9, TIMP-1, TIMP-2, TIMP-3, and TGF-β1. Apoptotic cells in the abdominal aorta were visualized with a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using an *in situ* cell death detection kit (Roche Applied Science, Indianapolis, IN). Sections were mounted with a ProLong® Gold antifade reagent with DAPI (Invitrogen) and photographed using a fluorescence microscope (Olympus, Tokyo, Japan).

2.6. Statistical analyses

Data are expressed as the means ± SEM. Statistical significance was determined by ANOVA with post-hoc Bonferroni test or Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Ligand activation of PPARδ suppresses Ang II-induced formation of abdominal aortic aneurysm

To elucidate the effects of ligand-activated PPARδ in Ang II-induced formation of AAA, we first examined the effect of GW501516, a specific ligand of PPARδ, on aneurysm formation induced by Ang II infusion. At four weeks after the infusion of Ang II to apolipoprotein E-deficient (apoE$^{-/-}$) mice, the majority of mice developed AAA, whereas the co-infusion of GW501516 with Ang II dramatically attenuated the development of AAA (Fig. 1A & B). The maximal diameter of the abdominal aorta in apoE$^{-/-}$ mice treated with Ang II was significantly greater than that in vehicle-treated mice. Concurrent infusion of GW501516 blunted the Ang II-induced increase in the aortic diameter (Fig. 1C). Moreover, infusion of GW501516 with Ang II improved the survival of apoE$^{-/-}$ mice compared with mice treated with Ang II alone (Fig. 1D).

Because increased blood pressure induced by Ang II is a well-known risk factor for AAA formation, we monitored SBP in conscious apoE$^{-/-}$ mice using a noninvasive tail–cuff system. A time-dependent increase in SBP was demonstrated in Ang II-infused mice, and no difference in SBP was observed between mice treated with or without GW501516 throughout the study (Fig. 1E). These findings indicate that the effects of GW501516 on aneurysm formation are not mediated by a reduction