Interaction between Angiotensin II, Osteoprotegerin, and Peroxisome Proliferator-Activated Receptor- γ in Abdominal Aortic Aneurysm

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Key Words

Abdominal aortic aneurysm • Angiotensin II • AT1 receptor • PPAR- γ • Smooth muscle cells • Osteoprotegerin • Metalloproteinase 9

Abstract

Background and Aims: Osteoprotegerin (OPG) has been associated with abdominal aortic aneurysm (AAA) expansion. Angiotensin II (AngII) receptor blockade has been shown to reduce OPG expression in human AAA tissue. Interaction between vascular AngII and OPG was further examined using cell culture and the AngII-infused ApoE^{-/-} mouse AAA model. The ability of peroxisome proliferator-activated receptor- γ (PPAR γ) activation to target OPG as potential therapy for AAA was also investigated. **Methods and Results:** Human aortic smooth muscle cells (AoSMC) exposed to AngII exhibited dose-dependent increase in the production OPG. A 3-fold increase in suprarenal aortic concentration of OPG was observed in AngII-infused ApoE^{-/-} mice. AngII type 1 receptor expression in human AAA tissue, and AoSMC in vitro, was stimulated up 4-fold in the presence of OPG. This effect in AoSMC was counteracted in the presence of the PPAR γ ligand, pioglitazone. Addition of PPAR γ ligand to cultured human AAA explant reduced OPG secretion by 60% and tissue concentration of OPG and metalloproteinase 9 by 2- and 3-fold, respectively. Administration of pioglitazone to AngII-

infused ApoE^{-/-} mice significantly reduced aortic concentrations of OPG and metalloproteinase 9. **Conclusions:** These data support an interaction between AngII and OPG in aneurysm formation. Activation of PPAR γ may have a role in treatment of AAA.

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Introduction

Inflammation, proteolysis, and vascular smooth muscle cell (VSMC) loss are important mechanisms in progression of abdominal aortic aneurysm (AAA) [1]. A range of cytokines are thought to play a role in promoting aortic wall destruction [2]. We recently demonstrated high concentrations of the glycoprotein osteoprotegerin (OPG) in human AAA biopsies [3]. In vitro OPG induces development of an aneurysm phenotype in normal aortic smooth muscle cells (AoSMC) by inhibiting proliferation, promoting apoptosis and stimulating metalloproteinase (MMP) production [3]. Work by other investigators suggested a pathway by which OPG might be modulated within human aorta in both the pathological and therapeutic situations [4, 5]. Angiotensin II (AngII), thought to play an important role in AAA formation, up-regulates OPG expression in human AoSMC [6]. Furthermore, the expression of OPG by VSMC is inhibited

by activation of the nuclear receptor, peroxisome proliferator-activated receptor- γ (PPAR γ) [5]. PPAR γ has been identified as the target receptor of the thiazolidinedione group of insulin sensitizing drugs now widely used for treating type 2 diabetes [4]. In addition to influencing VSMC expression of OPG, thiazolidinediones have been shown to downregulate expression of other cytokines and proteolytic enzymes, suggesting a potential role of this medication in inhibiting progression of AAA [7].

In the present study, we investigated the link between AngII and OPG in vitro and in vivo, and hypothesized that thiazolidinediones, by downregulating OPG within AoSMC in addition to anti-inflammatory pathways, would reduce pathology associated with aortic wall degeneration and AAA expansion.

Methods

Cell Culture

Human AoSMC (Cambrex) were maintained at 37°C, 5% CO₂, in culture medium (Clonetics) containing 10% FBS. Mouse AoSMC were harvested from 17-week-old male ApoE^{-/-} C57BL/6 mice (Animal Resource Centre, Perth, Australia) using enzymic dispersion. Briefly, mice were euthanized by CO₂ asphyxiation and the aorta harvested from arch to iliac bifurcation, immediately placed into chilled Hanks Balanced Salt Solution (Gibco) and cleaned. After dissection into small pieces, the aortic tissue was incubated in a sterile 1% collagenase solution at 37°C for 2 h with gentle agitation. The suspension was filtered through 63- μ m sterile gauze prior to centrifugation at 200 g, 4°C, for 5 min. After several washes in serum-free DMEM, cells were resuspended in DMEM containing 10% FBS and maintained at 37°C, 5% CO₂. Immunostain with VSMC α -actin confirmed identity. Subconfluent AoSMC were growth arrested for 24 h in serum-free DMEM prior to 48-hour incubation in experimental medium containing 10% FBS in the presence or absence of AngII (0, 0.1, 0.5, 1.0 μ M), recombinant human OPG (0, 0.25, 0.5, 1.0 nM), or pioglitazone (5.0 μ M).

Human AAA Biopsies

Informed consent was obtained for tissue collection in accordance with protocols approved by the relevant ethical bodies. Biopsies collected at surgery from 66 male patients undergoing elective repair of 5–6 cm aneurysms were obtained from the infrarenal aorta opposite the inferior mesenteric artery. Twenty biopsies were used for Western analysis (n = 6), and explant culture (n = 14). Forty-six biopsies from patients receiving statins or AngII inhibitors were excluded due to the influence of these medications on cytokine and protease production [8].

Explant Treatment and Viability

AAA biopsies were dissected into 100-mg full-thickness explants and stabilized in DMEM + 10% FBS for 24 h at 37°C, 5% CO₂, after which they were incubated without treatment (n = 14), with rosiglitazone (5 μ M, n = 8), pioglitazone (5 μ M, n = 8), or

OPG (5 nM, n = 6) over 96 h. Medication doses used in culture were based on safe serum levels measured in patients receiving these medications [9]. Culture supernatant was collected and refreshed at 48-hour intervals and OPG and interleukin-6 (IL-6) assayed by reproducible ELISAs (R&D Systems), following the manufacturer's protocols [3, 10]. Tissue levels of OPG, metalloproteinase activity (MMP-2/MMP-9), and AngII type 1 receptor (AT₁R) were measured in biopsies at completion of the experimental period using Western analysis and zymography. Tissue levels of ATP were compared between the incubated control, medicated explants and a further sample frozen before culture, using a commercially available bioluminescent somatic cell assay (Sigma) as per the manufacturer's instructions.

Pilot Assessment of Effect of Pioglitazone on Aneurysm Formation in AngII-Infused ApoE^{-/-} Mice

Approval for the animal studies was obtained from the local ethics committee. All mouse experiments were carried out in accordance with the institutional and ethical guidelines of James Cook University. Eighteen age- and sex-matched 11-week-old male ApoE^{-/-} C57BL/6 mice (Animal Resource Centre, Perth, Australia) were randomized to three groups: group 1 (control), group 2, and group 3. Six nonmanipulated ApoE^{-/-} mice constituted the experimental control group (group 1). Group 2 (n = 6) received 0.1% carboxymethylcellulose suspension agent (vehicle control) in their drinking water 14 days prior to and during a 28-day infusion of AngII (Sigma). Group 3 (n = 6) received treatment with pioglitazone (20 mg/kg/day) in 0.1% carboxymethylcellulose suspension agent via drinking water 14 days prior to and during a 28-day infusion of AngII. AngII was delivered in sterile 0.9% saline at a rate of 1.0 μ g/kg/min via osmotic minipump (model 2004; Alzet Corporation) placed subcutaneously in the dorsal midline under ketamine (150 mg/kg) and xylazine (10 mg/kg) anesthesia. All mice were maintained on normal diet and sacrificed at 17 weeks of age by CO₂ asphyxiation. Aortas were perfused with PBS via a 20-ml syringe with 20-gauge needle placed in the left ventricle, harvested from arch to iliac bifurcation and photographed with a Nikon Fujix digital camera. Maximum suprarenal aortic diameter was measured by computer aided image analysis (Scion Image, Scion Corporation). Assays for OPG, MMP-9, and PPAR γ were compared between suprarenal aortic tissue of all animals by Western analysis (OPG/PPAR γ) and zymography (MMP-9). Comparative analysis specifically addressed the relationship between AngII, the presence of suprarenal aortic aneurysm and aortic levels of OPG, MMP-9, and PPAR γ , by comparing group 2 with group 1, and, separately, the effect of pioglitazone medication on the same parameters in AngII-infused mice by comparing group 3 with group 2. Results are presented accordingly.

AT₁R-Probing with FITC-AngII

Detection of AT₁R expression in AoSMC was facilitated by an FITC-labeled AngII method adapted from Bernier and Perry [11]. Briefly, AoSMC grown on 10-mm coverslips in the presence and absence of OPG (1.0 nM) or AngII (100 μ M) were fixed in 4% formaldehyde for 30 s, air-dried, then rehydrated in PBS. Samples were washed in 1.0 mg/ml sodium metabisulfite (3 $\times$ 5 min) to reduce background fluorescence and then rinsed in PBS (3 $\times$ 5 min). Samples were then incubated with FITC-labeled AngII (1.4 μ M) for 2 h at room temperature. As controls, additional cells

Fig. 1. Dose-dependent stimulation of OPG production in human AoSMC by AngII. Data are expressed as mean $\pm$ SEM of seven biological repeat experiments averaged per 24 h; $p < 0.001$.

were either incubated in PBS to determine background fluorescence, preincubated in 950 μ M Ang II solution for 10 min to determine nonspecific binding of FITC-labeled AngII, or pre-treated for 20 min with irbesartan (10 μ M) to block AT₁R. At completion, all coverslips were rinsed in PBS (3 $\times$ 5 mm) and mounted in buffered glycerol solution containing 0.1% *p*-phenylenediamine for fluorescence microscopy.

Western Analysis

Proteins were extracted from human and mouse aortic tissue pulverized under liquid nitrogen, and pelleted AoSMC, using chilled 1 $\times$ PBS buffer containing 1% Triton X-100, 0.1% SDS, and 10 mM cacodylic acid. Homogenates were subjected to several liquid nitrogen freeze/thaw cycles for maximum cell rupture and tissue breakdown, prior to centrifugation at 18,000 *g* for 30 min at 4°C. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories). Extracted proteins from explants (30 μ g), AoSMC (30 μ g), and mouse samples (40 μ g) were separated using SDS gel electrophoresis (12% SDS; Gradient) and Western blotting carried out using either goat primary antisera for OPG (R&D Systems Inc.; 0.2 μ g/ml), or rabbit primary antisera for PPAR γ (sc-7196, Santa Cruz Biotechnology; 0.4 μ g/ml) or AT₁R (sc-7196, Santa Cruz Biotechnology; 0.4 μ g/ml). Bands were visualized using enhanced chemiluminescence (ECL Advance™; Amersham Biosciences) and identified on a ChemiDoc™ imaging system (Bio-Rad Laboratories) supported by QuantityOne™ 1-D Analysis Software (Bio-Rad Laboratories). Quantification of proteins was performed by comparison of band densities and expressed as mean and standard error (SEM) of relative band density units (RDU)/ μ g protein [3].

Gelatin Zymography

Zymography was performed as described previously [3]. Briefly, 10 μ g extracted proteins were separated at 4°C on a 10% acrylamide-SDS gel impregnated with 1 mg/ml gelatin, the gel washed in 2.5% (v/v) Triton X-100, then incubated overnight at 37°C in 50 mM Tris (pH 8) containing 5 mM CaCl₂. Bands were visualized in a 10% ethanol-10% acetic acid solution after staining with 0.125% Coomassie Blue, with enzyme activity quantified with

Table 1. Effect of pioglitazone medication on suprarenal aortic diameter, PPAR γ , OPG, and MMP-9 in the AngII-infused ApoE^{-/-} mouse

	n	SRA _{Max} mm	PPAR γ	OPG	MMP-9
Group 1	6	1.11 $\pm$ 0.03	0.74 $\pm$ 0.07	0.45 $\pm$ 0.09	1.09 $\pm$ 0.10
Group 2	6	1.78 $\pm$ 0.27	0.77 $\pm$ 0.14	1.01 $\pm$ 0.10	3.53 $\pm$ 0.24
Group 3	6	1.33 $\pm$ 0.31	2.63 $\pm$ 0.10	0.51 $\pm$ 0.14	1.26 $\pm$ 0.10
p ¹		0.020	0.937	0.004	0.002
p ²		0.310	0.002	0.026	0.002

Data are expressed as mean $\pm$ SEM; SRA_{Max} = Maximum suprarenal aortic diameter; For PPAR γ , OPG and MMP-9, RDUs per microgram protein; p¹ = group 2 versus group 1; p² = group 3 versus group 2.

densitometric analysis using the ChemiDoc imaging system (Bio-Rad Laboratories) and QuantityOne 1-D analysis software (Bio-Rad Laboratories). Results are expressed as mean and SEM of RDU/ μ g protein.

Data Analysis

The effect of increasing doses of AngII and OPG on AoSMC was assessed using the Kruskal-Wallis test. The concentration of OPG, MMP-9, and PPAR γ within mouse suprarenal aorta, expressed as RDU/ μ g protein, was compared using Mann-Whitney U test between AngII-infused (n = 6) and nonmanipulated control mice (n = 6). The influence of OPG on AT₁R expression in human AAA explant, and the effect of pioglitazone on the response of AoSMC, mice aorta and AAA explants were assessed using Mann-Whitney U test. Samples to be compared were run on the same blot or zymogram so that development conditions were comparable. The concentrations of OPG and MMPs were expressed as mean $\pm$ SEM. Significance was assumed at a *p* value < 0.05 .

Results

AngII Stimulates OPG Production by AoSMC in vitro and Upregulates Aortic Concentrations of OPG in vivo

The effects of AngII on the production of OPG in vitro using human AoSMC and in vivo within an AngII-infused animal model were examined. Incubation of human AoSMC with increasing concentrations of AngII resulted in dose-dependent production of OPG by these cells with a 3-fold increase in OPG secretion over 48 h in the presence of 1.0 μ M AngII (fig. 1). Tissue levels of OPG and MMP-9 in the suprarenal aorta of AngII-infused ApoE^{-/-} mice (group 2) were compared with sex- and age-matched nonmanipulated ApoE^{-/-} mice (group 1). Subcutaneous infusion of AngII over 28 days resulted in focal

Table 2. Effect of PPAR γ activation on cytokine and MMP production in human AAA tissue ex vivo

	n	OPGS	OPGT	MMP-2	MMP-9	IL-6
Control	8	6.79 $\pm$ 0.96	1.02 $\pm$ 0.10	0.90 $\pm$ 0.03	1.20 $\pm$ 0.04	20.2 $\pm$ 0.40
Rosiglitazone	8	2.37 $\pm$ 0.51	0.53 $\pm$ 0.03	0.90 $\pm$ 0.05	0.64 $\pm$ 0.02	17.5 $\pm$ 0.40
Pioglitazone	8	3.08 $\pm$ 0.39	0.51 $\pm$ 0.05	0.90 $\pm$ 0.07	0.42 $\pm$ 0.01	17.8 $\pm$ 0.40
p ¹		0.03	0.01	0.600	0.001	0.01
p ²		0.03	0.01	0.900	0.001	0.01

Data are expressed as mean $\pm$ SEM. OPGS = Supernatant (secreted) levels of OPG; OPGT = tissue levels of OPG; for OPGS and IL-6, picomoles per milligram tissue; for OPGT, MMP-2 and MMP-9, RDUs per microgram protein; p¹ = rosiglitazone versus control; p² = pioglitazone versus control.

Fig. 3. Suppression of OPG-induced AT₁R expression in human AoSMC by PPAR γ activation. Cells incubated for 48 h in the presence (+) or absence (–) of pioglitazone (PIO, 5 μ M) and/or OPG (1.0 nM) directly, or following a 48-hour pre-incubation (>) with either compound. Data are expressed as mean $\pm$ SEM of six culture experiments; * $p = 0.002$; ** $p < 0.001$. Right panel: representative Western blots demonstrating comparative AT₁R expression in treated cells (T; descending, OPG, PIO, OPG + PIO, PIO/OPG, OPG/PIO) versus untreated controls (C).

dilation of the suprarenal aorta in group 2 mice, associated with higher suprarenal aortic concentration of OPG and MMP-9 compared to group 1 mice (table 1).

OPG Upregulates AT₁R Expression in Human AAA Tissue and AoSMC in vitro

The effect of exogenous OPG on AT₁R expression in human AAA tissue was examined using explant culture of AAA biopsies. Human AAA biopsies ($n = 6$) were divided into two explant specimens and each incubated in the presence of OPG or vehicle control over 96 h. Basal secretion of OPG by AAA tissue specimens ex vivo over this time was determined at 6.79 ± 0.96 pM per mg tissue (table 2). Explants were subsequently incubated with an approximate 10-fold higher concentration of exogenous OPG at 0.05 nM per mg tissue. Western analysis at 96 h demonstrated an approximate 2-fold increase in AT₁R protein in AAA explants incubated in the presence of OPG compared to control tissue (3.40 ± 0.55 vs. 1.81 ± 0.20 , $p = 0.015$; fig. 2a). The presence of AT₁R in human AoSMC was visualized after 48 h using FITC-labeled AngII, which, based on qualitative inspection, tended to be greater in cells cultured in the presence of OPG (1.0 nM) compared to resting PBS-treated cells (fig. 2b). Incubation of human AoSMC and ApoE^{–/–} mouse AoSMC with increasing concentrations of OPG resulted in a dose-dependent increase in AT₁R protein assessed by Western blotting after 48 h (fig. 2c).

PPAR γ Activation Suppresses OPG-Induced AT₁R Upregulation in Human AoSMC in vitro

Human AoSMC were cultured in the presence of OPG and/or pioglitazone to examine the effect of PPAR γ activation on the action of OPG to upregulate AT₁R expres-

sion. Treated cells were exposed to either pioglitazone (5 μ M) or OPG (1.0 nM) alone, pioglitazone and OPG concurrently, OPG with addition of pioglitazone at 48 h, or pioglitazone with addition of OPG at 48 h. Figure 3 illustrates the significantly reduced production of AT₁R protein by human AoSMC in the presence of pioglitazone regardless of post, concurrent, or prior incubation with OPG.

PPAR γ -Activation Downregulates OPG, MMP-9 and IL-6 in Human AAA Biopsies

Western analysis demonstrated the expression of PPAR γ within human AAA biopsies (data not shown). The effect of PPAR γ activators on OPG and MMP-9 production in human AAA explants was examined. Human AAA biopsies were divided into three explant specimens and incubated each in the presence of pioglitazone (5 μ M), rosiglitazone (5 μ M), or vehicle control over 96 h. Explant tissue ATP levels at 96 h were comparable with that of control specimens snap frozen before culture, thus confirming specimen viability during culture (control tissue, 0.48 ± 0.11 μ g ATP/mg tissue; untreated explant, 0.57 ± 0.08 μ g ATP/mg tissue; pioglitazone, 0.51 ± 0.07 μ g ATP/mg tissue; rosiglitazone, 0.53 ± 0.12 μ g ATP/mg tissue). Treatment of AAA explant with PPAR γ ligand resulted in the reduction of both OPG and MMP-9. The amount of OPG secreted in a 24-hour period in the presence of PPAR γ ligands was reduced by 60% compared to untreated explant, and corresponded with a 2-fold decrease in tissue OPG demonstrated by Western analysis at 96 h (table 2). The effect of PPAR γ activation on MMP-9 within AAA tissue was most marked in explants incubated with pioglitazone in which MMP-9 was decreased 3-fold compared to untreated explants (table 2). No effect

Group 2

Group 3

Color version available online

Fig. 4. Morphology of supracranial aorta harvested from ApoE^{-/-} mice subcutaneously infused with AngII over 28 days (group 2), compared with corresponding aortic region from AngII-infused mice medicated with pioglitazone 14-days prior to and during AngII infusion (group 3).

of pioglitazone or rosiglitazone on MMP-2 was observed. A modest yet statistically significant decrease in IL-6 secretion of 15% was demonstrated for explants exposed to PPAR γ ligands (table 2).

PPAR γ Activation in vivo Downregulates OPG and MMP-9 within the Supracranial Aorta of AngII-Infused ApoE^{-/-} Mice

Protein level of PPAR γ within the supracranial aorta of nonmedicated AngII-infused group 2 mice determined using Western analysis was not significantly different from levels in group 1 mice (table 1). The administration of pioglitazone to AngII-infused ApoE^{-/-} mice (group 3) was associated with a significant 3-fold increase in supracranial aortic concentration of PPAR γ compared to group 2 animals (table 1). Inspection of the supracranial aorta from group 2 and 3 mice suggested that focal dilatation was more common in group 2 animals (fig. 4). However, maximum supracranial diameter was not significantly different (table 1). Pioglitazone treatment resulted in 2-fold lower mean supracranial aortic OPG concentration than that measured in the nonmedicated group 2 animals (table 1). A similar effect was observed

for MMP-9 (92 kDa) with pioglitazone reducing the MMP activity within the supracranial aorta by approximately 65% (table 1).

Discussion

Present evidence suggests that smaller AAA measuring 40–55 mm should not undergo surgical intervention [12]. However, approximately 60% of these expand to a diameter requiring invasive treatment if managed conservatively [1]. A medication which can inhibit the pathological changes characteristic of AAA would thus have great potential value both for patients with small AAA and those with large aneurysm not suitable for invasive treatment. We previously identified high concentrations of the glycoprotein OPG in biopsies of human AAA and demonstrated the ability of OPG to stimulate pathological processes integral to AAA progression in vitro at concentrations present within human biopsies [3]. In the present study, we provide evidence to support an interaction between AngII and OPG in AAA development. We also present preliminary data from both human AAA tis-

sue and a mouse model to support the value of PPAR γ activation in regulating aneurysm development.

The ability of AngII to promote inflammation and proteolysis, its effect in stimulating aneurysm formation in vivo, and the high concentration of AngII forming enzymes in human AAA biopsies, support an instrumental role for this peptide in the pathogenesis of AAA [13–15]. Based on our earlier observation of elevated levels of OPG protein present in human AAA tissue [3], we speculated that AngII upregulated aortic OPG in vivo and employed a mouse model of AAA to examine our hypothesis. A number of murine models of AAA have been developed [13, 16–18]. The ApoE^{-/-} mouse develops focal weakening primarily affecting the suprarenal aorta when infused subcutaneously with AngII [13, 19]. A limiting factor in this model is that formation of aortic aneurysm often involves destruction of the internal elastic lamina and focal dissection of the aortic wall rather than the circumferential medial destruction seen in human AAA [20]. However, despite morphological differences, there are significant correlations between this mouse model of AAA and the human condition, including the importance of male gender, inflammation and proteolysis [20, 21]. Furthermore, physical manipulation of the aorta required in other models is not required.

Consistent with previous reports [13, 19], we demonstrated focal aortic dilatation within the suprarenal region in ApoE^{-/-} mice infused with AngII. Concentrations of OPG detected within suprarenal aortic tissue of these animals were significantly higher than in corresponding tissue from ApoE^{-/-} mice not infused with AngII. The ability of AngII to upregulate OPG was confirmed in human AoSMC (fig. 1). We also demonstrated higher activity of MMP-9 in AngII-infused mice compared to controls. We reported previously that OPG acts to upregulate MMP-9 both in VSMC and monocytic cells in vitro [3]. Whether the in vivo upregulation of aortic MMP-9 induced by AngII infusion seen in this study was secondary to OPG upregulation is uncertain. OPG was originally characterized as regulator of bone remodeling by virtue of its ability to act as a decoy receptor for receptor activator of nuclear factor κ B ligand (RANKL) and inhibit osteoclastogenesis [22]. A *direct-effector* function of OPG was later demonstrated by Theoleyre et al. [23], whereby proMMP-9 activity was enhanced in purified osteoclasts via activation of the ras-MAP kinase pathway, involving phosphorylation of p38 and ERK1/2, upon the binding of OPG to a preformed RANK-RANKL duplex at the cell membrane. Both RANK and RANKL are expressed in diseased vasculature by VSMC, endothelial cells, and in-

flammatory cells [24]. Therefore, it is plausible that formation of the RANK-RANKL-OPG tertiary complex on VSMC and macrophages in vivo contributes to the increase in MMP-9 demonstrated here in AngII-infused mouse aorta.

We report here that incubation of human AAA tissue and AoSMC in the presence of exogenous OPG increased expression of the AngII receptor, AT₁R, by up to 2- and 4-fold, respectively (fig. 2). Most cardiovascular effects of AngII are mediated via AT₁R and expression of the receptor in VSMC is upregulated in atherosclerotic disease [25]. Expression of AT₁R is associated with activation of the MAP kinase and NF κ B transduction pathways [26, 27], and, as mentioned above, OPG is able to stimulate MAP kinase signaling upon formation of a tertiary complex with cell membrane-bound RANK-RANKL [23]. Thus, our observation of increased AT₁R levels in AAA explant can be explained in terms of stimulated expression resulting from exogenous OPG binding to RANK-RANKL duplexes already formed on inflammatory, endothelial, and remaining smooth muscle cells present within the diseased tissue. However, the mechanism by which OPG stimulates AT₁R expression in AoSMC in vitro remains unclear. Attributing this novel finding to signaling via the tertiary complex RANK-RANKL-OPG is improbable, as RANK and RANKL are frequently undetectable in normal vessels [24] and thus unlikely to be expressed in AoSMC in vitro. We have reported direct regulatory action of OPG on growth and survival of normal VSMC in vitro previously [3]. This, together with the current finding with respect to AT₁R expression, suggests that OPG possesses the ability to exert direct biological action in VSMC independent of the RANK-RANKL system. For example, it may activate signaling at the cell membrane via an as yet undefined receptor pathway, or, as a soluble molecule, may function intracellularly to influence processes associated with AT₁R upregulation at a transcription/translation level. This remains the subject of ongoing investigation in our laboratory.

Activation of the nuclear transcription factor PPAR γ has previously been shown to downregulate AT₁R expression in VSMC [7, 28, 29], and, OPG gene expression in human AoSMC [5]. In line with these reports, we observed that pioglitazone inhibited the induction of AT₁R by OPG in human AoSMC (fig. 3), an effect most likely mediated via the PPAR γ activator's action on AT₁R or OPG expression, or both. These findings prompted us to examine the effect of PPAR γ activation on human AAA explants and in the AngII-infused ApoE^{-/-} mouse model of AAA.

We documented the effect of AngII blockade on human AAA explant production of OPG in an earlier report [3]. Treatment of explants with the AT₁R antagonist, irbesartan, resulted in the time-dependent inhibition of OPG secretion highlighting the potential for AT₁R blockade in slowing aneurysm expansion. Interestingly, AT₁R antagonists have recently been shown to induce PPAR γ activity [30]. In the present study, we detected PPAR γ protein within human AAA tissue (data not shown). Incubation of human AAA biopsies with PPAR γ ligands resulted in a 2.5-fold decrease in OPG production, a 3-fold decrease in MMP-9, together with a small reduction in IL-6 production (table 2). Whether thiazolidinedione-induced down-regulation of OPG synthesis in the AAA biopsies resulted from direct action of PPAR γ activation upon OPG gene expression [5], or indirectly via negative regulation of AT₁R and the AngII pathway [28], is not clear from the present data. Even so, the data do support the potential benefit of PPAR γ activation in regulating aneurysm progression in vivo, which we examined using the AngII-infused mouse model.

Examination of the suprarenal aorta in the pioglitazone-treated group 3 mice demonstrated varying degrees of focal dilatation (fig. 4), and whether the gross pattern differed significantly when compared to the non-medicated group 2 mice was subjective. Morphometric analysis did determine a lower mean maximum suprarenal aortic diameter in group 3 mice compared to group 2 (table 1), although statistical significance could not be demonstrated. Within the suprarenal aortic tissue however, OPG concentration (and MMP-9) was significantly decreased in mice receiving pioglitazone treatment (table 1). Nonetheless, the small number of mice per experimental group (n = 6) prevented definitive conclusions being drawn with respect to the effect of PPAR γ activation on aneurysm development within this model, and further study incorporating a larger experimental cohort is presently underway in our laboratory to address this issue.

Low concentrations of PPAR γ were detected in control and AngII-infused ApoE^{-/-} mice in line with previous studies [31]. Administration of pioglitazone via drinking water 14 days prior to the start of AngII infusion and continuing for the duration of the infusion period resulted in upregulation of aortic PPAR γ expression. Although an increase in protein does not necessarily equate to an increase in levels of the active transcription factor, the fact that associated with this change we found significant downregulation of aortic OPG and MMP-9 suggests that AT₁R and/or OPG gene regulation by PPAR γ is present.

The ability of AT₁R antagonists to inhibit OPG production, inflammation and proteolysis suggests these medications may be useful in slowing aneurysm progression. Many patients with AAA are already prescribed AT₁R blockers or AngII converting enzyme inhibitors for the management of hypertension, making these medications difficult to investigate in a randomized controlled clinical trial. The present study describes a potential positive feedback mechanism by which OPG, via AngII and AT₁R, sustains its own production within the aortic wall, which could favor cellular changes associated with aneurysm progression. The ability of PPAR γ activation to block this positive feedback loop suggests the potential for its activation within the aortic wall in limiting AAA progression. Synthetic PPAR γ agonists are available for clinical use but not presently indicated in patients with AAA, and further studies examining the effect of this medication in aneurysmal disease are warranted.

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