Osteoprotegerin Deficiency Limits Angiotensin II–Induced Aortic Dilatation and Rupture in the Apolipoprotein E–Knockout Mouse

Corey S. Moran, Roby J. Jose, Erik Biros, Jonathan Golledge

Objective—Mounting evidence links osteoprotegerin with cardiovascular disease. Elevated serum and aortic tissue osteoprotegerin are associated with the presence and growth of abdominal aortic aneurysm in humans; however, a role for osteoprotegerin in abdominal aortic aneurysm pathogenesis remains to be shown. We examined the functional significance of osteoprotegerin in aortic aneurysm using an Opg-deficient mouse model and in vitro investigations.

Approach and Results—Homozygous deletion of Opg in apolipoprotein E-deficient mice (ApoE−/−Opg−/−) inhibited angiotensin II-induced aortic dilatation. Survival free from aortic rupture was increased from 67% in ApoE−/−Opg+/+ controls to 94% in ApoE−/−Opg−/− mice (P=0.040). Serum concentrations of proinflammatory cytokines/chemokines, and aortic expression for cathepsin S (CTSS), matrix metalloproteinase 2, and matrix metalloproteinase 9 after 7 days (early-phase) of angiotensin II infusion were significantly reduced in ApoE−/−Opg−/− mice compared with ApoE−/−Opg+/+ controls. In addition, aortic expression of markers for an inflammatory phenotype in aortic vascular smooth muscle cells in response to early-phase of angiotensin II infusion was significantly lower in Opg-deficient mice. In vivo, human abdominal aortic aneurysm vascular smooth muscle cells produced more CTSS and exhibited increased CTSS-derived elastolytic activity than healthy aortic vascular smooth muscle cells, whereas recombinant human osteoprotegerin stimulated CTSS-dependent elastase activity in aortic vascular smooth muscle cells.

Conclusions—These findings support a role for osteoprotegerin in aortic aneurysm through upregulation of CTSS, matrix metalloproteinase 2, and matrix metalloproteinase 9 within the aorta, promoting an inflammatory phenotype in aortic vascular smooth muscle cells in response to angiotensin II. (Arterioscler Thromb Vasc Biol. 2014;34:2609-2616.)

Key Words: aneurysm ■ cathepsin S ■ matrix metalloproteinase 2 ■ osteoprotegerin

Abdominal aortic aneurysm (AAA) is the degenerative weakening and dilatation of the abdominal aorta. The main complication of AAA is aortic rupture, which is associated with a mortality of ≈80%.1 There is no currently accepted drug treatment to limit AAA rupture.2

Despite advances in our understanding of its pathophysiology, precise mechanisms involved in AAA are uncertain. It is commonly accepted that an unknown inciting event results in injury of the aortic wall and recruitment of leukocytes to the site. Release of proteolytic enzymes from these cells and resident vascular cells (aortic smooth muscle cells [AoSMC]) in response to local proinflammatory cytokine production contributes to the destruction of the extracellular matrix (ECM). Apoptosis of AoSMC results in the loss of cells primarily responsible for the synthesis of ECM proteins, which limits ECM repair.3

Osteoprotegerin is a secreted glycoprotein member of the tumor necrosis factor receptor superfamily (TNFRSF11B). Since its initial discovery as a key regulator of bone metabolism, osteoprotegerin has attracted interest for its role in vascular disease.4 High concentrations of serum osteoprotegerin have been identified as a marker and risk factor for cardiovascular disease, as well as a predictor for future incident cardiovascular events and mortality.5-8 In line with this, we have reported previously that patients with AAA have elevated aortic and serum osteoprotegerin concentrations that are positively associated with aneurysm diameter and growth9,10 and independently associated with AAA after adjusting for other risk factors such as coronary heart disease.11 In vitro, osteoprotegerin stimulates production of elastolytic enzymes in human monocyte/macrophages and AoSMC9,10 and promotes an aneurysm phenotype in healthy AoSMC through limiting cell proliferation and inducing apoptosis.10 The in vivo role of osteoprotegerin in AAA pathogenesis remains unclear.

We positively correlated human AAA concentrations of osteoprotegerin with aneurysm diameter in a recent study.9 Historically, matrix metalloproteinases (MMPs) have been the main proteolytic enzymes implicated in ECM degradation in AAA.12 More recently, other proteolytic enzymes such as the

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cysteine cathepsin proteases have been implicated in AAA pathogenesis.\textsuperscript{12,13} Cathepsin S (CTSS) is a potent elastolytic/collagenolytic protease for which elevated expression and activity have been demonstrated in human AAA tissue.\textsuperscript{14} Plasma levels of total, pro- and active CTSS have been positively associated with AAA.\textsuperscript{15,16} Experimentally, aortic aneurysm induction in mice is dependent on AoSMC expression of CTSS\textsuperscript{17} and inhibited by CtsS deficiency.\textsuperscript{18}

The aim of this study was to assess the effect of Opg deficiency on aortic aneurysm development in a mouse model. Using an Opg-deficient angiotensin II (AngII)–infused apolipoprotein E–deficient (ApoE\textsuperscript{−/−}) mouse model, we provide in vivo evidence for the involvement of osteoprotegerin in structural (ECM) and cellular (AoSMC) destabilization within the aortic wall. A novel functional association between osteoprotegerin and CTSS is reported.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Opg Deficiency Inhibits AngII-Induced Aortic Dilatation and Rupture in a Mouse Model of Aortic Aneurysm

Aortic and serum osteoprotegerin concentrations are elevated in patients with AAA and positively associated with AAA expansion in humans.\textsuperscript{9,10} The in vivo importance of osteoprotegerin in the development of aortic aneurysm was investigated in Opg-deficient mice infused with AngII. A preliminary study using C57BL/6 (ApoE\textsuperscript{−/−}) mice demonstrated that Opg deficiency limited AngII-induced aortic dilatation in these mice compared with wild-type controls, most significantly within the aortic arch and thoracic aorta (Table I in the online-only Data Supplement). We subsequently investigated the effect of Opg deficiency on AngII-induced aortic aneurysm in the ApoE\textsuperscript{−/−} mouse model. A time-dependent increase in suprarenal aorta diameter in response to AngII infusion during the 28-day experimental period was observed by ultrasound in both ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} control (\(P<0.001\)) and ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} experimental (\(P<0.001\)) mice (Figure 1A).

The rate of suprarenal aorta expansion in response to AngII was, however, markedly less in ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice (\(P<0.001\); Figure 1A). Aortas from all mice were harvested after fatal-ity or euthanization at study end and regional maximum aortic diameters determined by morphometric analysis (Figure II in the online-only Data Supplement). ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice exhibited significantly smaller median maximum diameter of aortic arch, thoracic aorta, suprarenal aorta, and infrarenal aorta compared with ApoE\textsuperscript{−/−}Opg\textsuperscript{+/+} controls (Figure 1B; Figure III in the online-only Data Supplement). An important complication associated with the infusion of AngII in ApoE\textsuperscript{−/−} mice is fatigue because of aortic rupture. Opg deficiency markedly improved survival from aortic rupture, increasing survival rate from 67% in ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice to 94% in ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice (\(P=0.040\); Figure 1C).

Effect of Opg Deficiency on AngII-Induced Aortic Dilatation Is Blood Pressure Independent and Associated With a Reduced Initial Inflammatory Response to AngII

Infusion of AngII in ApoE\textsuperscript{−/−} mice resulted in a time-dependent increase in blood pressure during the 28-day infusion period (\(P<0.001\); Table 1). The increase in blood pressure induced by AngII was not affected by Opg deficiency. Median systolic, diastolic, and mean arterial blood pressures obtained at baseline (day 0), day 14, and day 28 remained comparable between ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} and ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice (Table 1). The concentration of a range of cytokines and chemokines was measured in the serum of ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} and ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice during a 7-day infusion of AngII (Table 2). Median concentrations of interleukin (IL)-1\(\beta\), IL-1\(\beta\), IL-2, IL-3, IL-6, IL-12, IL-17, monocyte chemotactic protein (MCP)-1, tumor necrosis factor (TNF)-\(\alpha\), macrophage inflammatory protein-1\(\alpha\), granulocyte-macrophage colony-stimulating factor, and regulated on activation, normal T cell expressed and secreted were significantly increased in ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} control mice after 7 days of AngII infusion compared with baseline concentrations (Table 2). In contrast, levels of these cytokines were not markedly elevated in serum from ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice after the same period of AngII infusion (Table 2). Notably, baseline concentrations of IL-6, MCP-1, and TNF\(\alpha\) were significantly lower in ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice compared with ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} controls, and median fold-increase in all markers other than IL-1\(\beta\) and MCP-1 in response to AngII infusion remained significantly lower in ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice (Table 2). The presence of MOMA-2 (macrophage and monocyte antibody)–positive (monocyte/macrophage) cells was demonstrated within the suprarenal aortic adventitia of AngII-infused ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice at 28 days (Figure IV in the online-only Data Supplement).

Reduced Proteolysis in Aortas of AngII-Infused ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} Mice

Based on reported associations between osteoprotegerin and metallo/cysteine proteinases in promoting AAA,\textsuperscript{9,10,19} we examined MMP2, MMP9, and CTSS expression within aortas of ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} (n=6) and ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} (n=6) mice after 7 days of AngII infusion. Median relative expression of Mmp2, Mmp9, and CtsS within the aortas of ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice was reduced by 2-, 3-, and 7-fold, respectively, compared with ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} controls (Table 3). The lower mRNA expression...
translated to reduced aortic levels of these proteases. Median levels of MMP2 and MMP9 \((P=0.001, P=0.028; \text{Figure 2A; Figure V in the online-only Data Supplement})\) and CTSS \((P=0.002; \text{Figure 2B})\) were significantly lower within aortic tissue from \(\text{ApoE}^{-/-}\text{Opg}^{-/-}\) mice compared with \(\text{ApoE}^{-/-}\text{Opg}^{+/+}\) controls after the 7-day AngII infusion period. Interestingly, median activity of CTSS within aortic tissue of \(\text{ApoE}^{-/-}\text{Opg}^{-/-}\) mice \((n=16)\), measured using a substrate-specific assay, was 2-fold lower than in \(\text{ApoE}^{-/-}\text{Opg}^{+/+}\) controls \((n=12)\) after 28 days of AngII infusion \((3.27, 2.17–4.44, \text{versus} 6.89, 5.02–8.99 \text{AU fluorescence unit/min per microgram protein}; P=0.009)\).

**Elevated Production of CTSS by Human AAA Vascular Smooth Muscle Cells and Stimulation of CTSS-Derived Elastolytic Activity in Healthy AoSMC by Osteoprotegerin In Vitro**

Serum concentrations of osteoprotegerin and CTSS are elevated in patients with AAA and positively associated with aneurysm diameter.\(^{10,11,15,16}\) Production of osteoprotegerin and CTSS within AAA tissue may contribute to circulating levels of these proteins in patients with AAA. We have previously demonstrated a positive correlation between tissue concentrations of osteoprotegerin and CTSS within human AAA biopsies, and that osteoprotegerin upregulates CTSS in healthy human AoSMC in vitro.\(^8\) The production of CTSS by smooth muscle cells isolated from aortic aneurysm tissue (AASMCs) compared with AoSMC and the ability of osteoprotegerin to stimulate CTSS-dependent elastolytic activity in AoSMC were assessed in vitro. The concentration of total CTSS in supernatants (secreted) and cell lysates (cellular) from human AASMC \((n=6 \text{ cultures})\) and AoSMC \((n=6 \text{ cultures})\) was determined by ELISA. The median concentration of secreted CTSS from AASMC was 5-fold higher than that of AoSMC \((P=0.025; \text{Figure 3A})\). Similarly, median cellular CTSS was 1.6-fold higher in AASMC than that of AoSMC \((P=0.002; \text{Figure 3A})\). CTSS-derived elastase activity in AoSMC and AASMC was measured using a commercial elastin degradation assay that we initially evaluated using AngII-activated AoSMC \((\text{Figure VI in the online-only Data Supplement})\). Cells were coincubated with the CTSS inhibitor Z-FL-COCHO \((20 \text{ nmol/L})\) to assess the contribution of CTSS to total elastase activity \((\text{Figure VI in the online-only Data Supplement})\). CTSS-derived elastase activity was 3-fold higher in unstimulated AASMC than in unstimulated healthy AoSMC \((P=0.002; \text{Figure 3B})\). Incubation of AASMC in the presence of recombinant human osteoprotegerin \((30 \text{ nmol/L}; n=6)\) for 36 hours did not significantly affect elastase activity in these cells. In contrast, the median level of elastase activity in AoSMC cultured in the presence of recombinant human osteoprotegerin was 4.5-fold higher than that measured in control AoSMC \((P=0.002; \text{Figure 3B})\). In the presence of the CTSS inhibitor, median elastase activity stimulated by recombinant human osteoprotegerin, while remaining elevated compared with control cultures, was reduced in both AASMC and AoSMC by 1.5-fold \((P=0.004)\) and 2-fold \((P=0.002)\) \(\text{(Figure 3B)}\).

**Differential Expression of mRNA for Factors Regulating AoSMC Function Within Aortas of AngII-Infused \(\text{ApoE}^{-/-}\text{Opg}^{+/+}\) and \(\text{ApoE}^{-/-}\text{Opg}^{-/-}\) Mice**

The contribution of AoSMC loss to structural degeneration of the aortic media has been suggested through studies in human AAA tissue and animals models of aortic aneurysm.\(^{18,20,21}\) We have shown previously the ability of osteoprotegerin to limit
Table 1. Blood Pressure in ApoE\textsuperscript{−/−} Opg\textsuperscript{−/−} and ApoE\textsuperscript{−/−} Opg\textsuperscript{+/+} Mice During a 28-Day Angiotensin II Infusion Period

<table>
<thead>
<tr>
<th></th>
<th>ApoE\textsuperscript{−/−} Opg\textsuperscript{−/−}</th>
<th>ApoE\textsuperscript{−/−} Opg\textsuperscript{+/+}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Day 14</td>
</tr>
<tr>
<td>SBP</td>
<td>n=19</td>
<td>n=13</td>
</tr>
<tr>
<td>n=19</td>
<td>94 (89–101)</td>
<td>118 (99–125)</td>
</tr>
<tr>
<td>DBP</td>
<td>n=19</td>
<td>n=13</td>
</tr>
<tr>
<td>n=19</td>
<td>70 (62–76)</td>
<td>85 (76–89)</td>
</tr>
<tr>
<td>MAP</td>
<td>n=19</td>
<td>n=13</td>
</tr>
<tr>
<td>n=19</td>
<td>77 (72–84)</td>
<td>96 (90–105)</td>
</tr>
</tbody>
</table>

Data presented as median (interquartile range) pressure presented as mmHg. DBP indicates diastolic blood pressure; MAP, mean arterial pressure; and SBP, systolic blood pressure.

Expression of osteoprotegerin within AAA biopsies is positively correlated with AAA diameter.\textsuperscript{8,9} These association studies implicate osteoprotegerin in AAA; however, previously in vivo evidence for a role of osteoprotegerin in AAA pathogenesis has been absent. Here, we report that Opg deficiency in the ApoE\textsuperscript{−/−} mouse limits AngII-induced aortic dilatation and rupture and present additional in vivo and in vitro data to support a contributing role for osteoprotegerin in aortic wall weakening.

Maximum aortic diameter is commonly presented as the primary outcome measure in experimental AAA studies.\textsuperscript{23–25} Aneurysm diameter is an important clinical indicator of rupture risk for AAA in humans and routinely used in selected patients for surgical intervention.\textsuperscript{3} Ultimately any medical treatment for aortic aneurysm aims to limit the main complication of aneurysm rupture. We used the AngII-infused ApoE\textsuperscript{−/−} mouse model of aortic aneurysm in which aortic dilatation results from breaks in medial elastic lamellae and bleeding into the artery wall, a process that leads to acute aortic rupture in \approx30% of mice.\textsuperscript{26} This model allowed the assessment of both survival free from aortic rupture and maximum aortic diameter. Aortic diameter was assessed by 2 techniques, namely ultrasound

Table 2. Serum Cytokine Concentration in ApoE\textsuperscript{−/−} Opg\textsuperscript{−/−} and ApoE\textsuperscript{−/−} Opg\textsuperscript{+/+} Mice After 7 Days of Angiotensin II Infusion

<table>
<thead>
<tr>
<th></th>
<th>ApoE\textsuperscript{−/−} Opg\textsuperscript{−/−} (n=12)</th>
<th>ApoE\textsuperscript{−/−} Opg\textsuperscript{+/+} (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0 Day 7 MFI</td>
<td>Day 0 Day 7 MFI</td>
</tr>
<tr>
<td>IL-1\textalpha</td>
<td>72.1 (31.2–107.0)</td>
<td>70.4 (54.4–103.0)</td>
</tr>
<tr>
<td>P</td>
<td>2.4 &lt;0.001</td>
<td>1.1 0.492</td>
</tr>
<tr>
<td>IL-1β</td>
<td>146.4 (114.2–191.8)</td>
<td>144.9 (89.1–196.7)</td>
</tr>
<tr>
<td>P</td>
<td>1.7 &lt;0.001</td>
<td>1.2 0.301</td>
</tr>
<tr>
<td>IL-2</td>
<td>105.4 (48.5–174.8)</td>
<td>90.2 (66.2–115.2)</td>
</tr>
<tr>
<td>P</td>
<td>1.6 0.033</td>
<td>1.1 0.661</td>
</tr>
<tr>
<td>IL-3</td>
<td>29.5 (22.7–58.3)</td>
<td>34.6 (18.2–58.6)</td>
</tr>
<tr>
<td>P</td>
<td>1.5 0.038</td>
<td>1.1 0.975</td>
</tr>
<tr>
<td>IL-6</td>
<td>135.5 (100.8–146.2)</td>
<td>105.8 (74.3–118.0)</td>
</tr>
<tr>
<td>P</td>
<td>1.4 &lt;0.001</td>
<td>1.1 0.397</td>
</tr>
<tr>
<td>IL-12</td>
<td>227.4 (174.3–380.1)</td>
<td>259.7 (194.0–312.3)</td>
</tr>
<tr>
<td>P</td>
<td>2.5 &lt;0.001</td>
<td>1.1 0.583</td>
</tr>
<tr>
<td>IL-17</td>
<td>146.4 (106.0–190.8)</td>
<td>117.3 (93.5–170.3)</td>
</tr>
<tr>
<td>P</td>
<td>1.6 0.001</td>
<td>1.1 0.635</td>
</tr>
<tr>
<td>MCP-1</td>
<td>93.6 (90.1–98.3)</td>
<td>74.4 (64.2–79.7)</td>
</tr>
<tr>
<td>P</td>
<td>1.1 &lt;0.001</td>
<td>1.1 0.085</td>
</tr>
<tr>
<td>TNF\alpha</td>
<td>47.9 (41.3–51.9)</td>
<td>30.3 (20.2–40.2)</td>
</tr>
<tr>
<td>P</td>
<td>1.4 &lt;0.001</td>
<td>1.2 0.085</td>
</tr>
<tr>
<td>MIP-1\textalpha</td>
<td>30.8 (26.1–44.6)</td>
<td>29.4 (20.7–36.1)</td>
</tr>
<tr>
<td>P</td>
<td>1.6 &lt;0.001</td>
<td>1.3 0.158</td>
</tr>
<tr>
<td>GMCSF</td>
<td>117.0 (93.0–155.7)</td>
<td>137.8 (86.3–188.5)</td>
</tr>
<tr>
<td>P</td>
<td>1.4 0.007</td>
<td>1.1 0.377</td>
</tr>
<tr>
<td>RANTES</td>
<td>114.4 (86.7–133.8)</td>
<td>103.1 (63.6–116.4)</td>
</tr>
<tr>
<td>P</td>
<td>1.2 0.020</td>
<td>1.0 0.858</td>
</tr>
</tbody>
</table>

Data expressed as median (interquartile range) concentration presented as picograms/mL. MFI indicates median fold-increase.

\*P value, 2-sided P value for comparison between concentrations at day 0 and concentrations day 7 within each group by paired \textit{t}-test.

†P value, 2-sided P value for MFI in ApoE\textsuperscript{−/−} Opg\textsuperscript{−/−} mice vs MFI in ApoE\textsuperscript{−/−} Opg\textsuperscript{−/−} mice by Mann–Whitney U test.

‡P<0.05 compared with ApoE\textsuperscript{−/−} Opg\textsuperscript{−/−} baseline by Mann–Whitney U test.
performed in live mice and morphometry of harvested aortas. Opg deficiency consistently limited AngII-induced aortic dilation and rupture as assessed by all these outcome measures. The 5-fold lower incidence of fatal rupture because of aortic rupture in Opg-deficient ApoE−/− mice was particularly noteworthy. These findings suggest the importance of osteoprotegerin in promoting aortic wall degeneration in this model.

Consistent with previous reports, we observed a time-dependent increase in blood pressure in ApoE−/− Opg−/+ mice.23,27 Higher levels of serum and aortic osteoprotegerin are measured in AngII-infused ApoE−/− Opg−/+ mice that develop more severe aortic dilatations,19,28 indicating a potential link between elevated blood pressure and increased osteoprotegerin contributing to aneurysm formation in this model. Previous studies however suggest that AngII induces aortic aneurysm by mechanisms independent of blood pressure elevation.23,29 Opg deficiency did not affect the hypertensive response to AngII infusion suggesting that Opg promotes aortic aneurysm by blood pressure–independent mechanisms.

Leukocyte infiltration of the aortic wall is a prominent early response to AngII infusion that has been implicated strongly in aneurysm formation in this model.6,13,33 Human and animal studies suggest that cytokines and chemokines, such as TNFα, IL-6, and MCP-1, play a key role in aortic leukocyte recruitment and activation.6,13,31 In the current study, a lower incidence of AngII-induced aortic dilation in Opg-deficient ApoE−/− mice corresponded with decreased levels of a range of circulating proinflammatory cytokine and chemokines. The most notable of these were TNFα, IL-6, and MCP-1. Baseline concentrations of these proteins were significantly lower in ApoE−/− Opg−/+ mice compared with controls. Importantly, the concentrations of these proteins did not significantly increase in ApoE−/− Opg−/+ mice after AngII infusion unlike the situation in ApoE−/− Opg−/+ controls in which a significant increase in the concentrations of all 3 proteins was identified. These findings suggest that Opg deficiency acted to suppress the inflammatory response to AngII infusion.

Infusion of AngII results in the overexpression of osteoprotegerin, CTSS, MMP2, and MMP9 in the aorta of ApoE−/− mice.19,28 In the present study, although detection of MOMA-2 in suprarenal aorta of ApoE−/− Opg−/+ mice indicated the presence of monocyte/macrophages within the aortic wall, reduced aortic dilation in these mice was associated with downregulation of aortic CTSS, MMP2, and MMP9. A recent study reported that reduced aortic aneurysm in Cts null AngII-infused ApoE−/− mice was associated with decreased aortic activity of MMP2 and MMP9, implicating CTSS in the regulation of these proteases.18 These findings suggest a functional association between osteoprotegerin, CTSS, MMP2, and MMP9 in aortic aneurysm. Indeed, aortic concentrations of osteoprotegerin correlate with those of CTSS, MMP2, and MMP9, and increasing infrarenal aorta diameter in patients with AAA.9 Vascular smooth muscle cells have been identified as a major source of osteoprotegerin within human AAA tissue,7 and MMP production in healthy AoSMC is stimulated by osteoprotegerin in vitro.10 Here, we show AASMCs also produce large quantities of CTSS in vitro. Interestingly, incubation of AASMCs in the presence of osteoprotegerin had no significant effect to increase CTSS in these cells. Cellular and

### Table 3. Relative Expression of Selected Genes Within the Aorta of ApoE−/− Opg−/+ and ApoE−/− Opg−/− Mice After 7 Days of Angiotensin II Infusion

<table>
<thead>
<tr>
<th>Gene</th>
<th>ApoE−/− Opg−/+</th>
<th>ApoE−/− Opg−/−</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctn1</td>
<td>1.50 (0.65–1.83)</td>
<td>15.29 (13.14–22.29)</td>
<td>0.002</td>
</tr>
<tr>
<td>Bax/Bcl2*</td>
<td>0.69 (0.58–0.75)</td>
<td>0.37 (0.34–0.51)</td>
<td>0.032</td>
</tr>
<tr>
<td>Mapk1/3</td>
<td>1.66 (0.98–8.45)</td>
<td>12.12 (8.55–16.58)</td>
<td>0.008</td>
</tr>
<tr>
<td>Mapk14 (p38 Mapk)</td>
<td>3.02 (1.82–4.39)</td>
<td>6.25 (5.00–7.46)</td>
<td>0.041</td>
</tr>
<tr>
<td>Pypag</td>
<td>4.90 (2.62–8.03)</td>
<td>16.05 (8.87–32.00)</td>
<td>0.026</td>
</tr>
<tr>
<td>Nfkb</td>
<td>1.80 (0.52–2.63)</td>
<td>5.98 (4.30–8.74)</td>
<td>0.004</td>
</tr>
<tr>
<td>Mmp2</td>
<td>0.58 (0.48–0.73)</td>
<td>0.26 (0.03–0.33)</td>
<td>0.002</td>
</tr>
<tr>
<td>Mmp9</td>
<td>0.39 (0.36–0.41)</td>
<td>0.13 (0.11–0.16)</td>
<td>0.002</td>
</tr>
<tr>
<td>Cts</td>
<td>0.67 (0.52–0.94)</td>
<td>0.09 (0.06–0.17)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Data expressed as median (interquartile range) mRNA expression relative to house-keeping gene Gapdh. P value, 2-sided P value for comparison by Mann–Whitney U test.

*Ratio of Bax mRNA expression relative to Gapdh to Bcl2 mRNA expression relative to Gapdh.

**Figure 2.** Downregulation of matrix metalloproteinase 2 (MMP2), MMP9, and cathepsin S (CTSS) in the aortas of ApoE−/− Opg−/+ mice assessed after 7 days angiotensin II (AngII) infusion. A. Decreased aortic MMP2 and MMP9 in ApoE−/− Opg−/+ mice (white; n=6) compared with ApoE−/− Opg−/+ controls (gray; n=6) determined by zymographic analysis. Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for relative density units (RDU) per microgram protein (P values calculated by Mann–Whitney U test). B. Reduced CTSS in aortas from ApoE−/− Opg−/+ mice (n=8) compared with ApoE−/− Opg−/+ controls (n=8) determined by TaqMan Protein Assay. Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for expression relative to aortic β-actin protein; *P=0.002 (P values calculated by Mann–Whitney U test).
inflammation and matrix degradation.\textsuperscript{18,20,25,32,33} Exposure of AoSMC to osteoprotegerin in vitro results in inhibition of cell growth and induction of apoptosis.\textsuperscript{10} Dedifferentiation (phenotype modulation) of vascular smooth muscle cells to an inflammatory phenotype in response to vascular injury involves downregulation of genes involved in the regulation and modulation of smooth muscle contraction.\textsuperscript{24} Phenotypic modulation is associated with reduction in mitogen-activated protein kinase (MAPK)1/3 activation resulting in decreased myosin light chain phosphorylation and loss of contractile properties.\textsuperscript{15} Calponin 1 facilitates signal transduction by MAPK1/3,\textsuperscript{36} and Cnn1 deletion in differentiated vascular smooth muscle cells results in impaired MAPK1/3 activity and cell contractility.\textsuperscript{37} Evidence also suggests that MAPK1/3 possesses actin-binding properties and that an intact actin cytoskeleton, degraded during apoptosis, is required for MAPK1/3 signaling.\textsuperscript{38} Our comparison of selected genes in the aortas of ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} and ApoE\textsuperscript{−/−}Opg\textsuperscript{+/−} mice after 7 days of AngII infusion showed a 10- and 7-fold greater expression of Cnn1 and Mapk1/3, respectively, within the aortas of ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice. These findings suggest that Opg deficiency promotes a more stable AoSMC phenotype within the aortic wall, a theory supported by the 2-fold lower expression ratio of the apoptosis markers Bax and Bcl2 also observed within aortas of ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice. Thus, it is postulated that the inhibition of aortic dilatation and rupture in ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice is contributed to by an AoSMC phenotype that is less responsive to functional change after AngII infusion.

Activation of PPARγ downregulates AngII type 1 receptor and osteoprotegerin expression in vascular smooth muscle cells,\textsuperscript{39,40} whereas its own expression is negatively regulated by AngII via activation of AngII type 1 receptor.\textsuperscript{41,42} We have previously shown that PPARγ activation blocks osteoprotegerin-induced upregulation of AngII type 1 receptor in human AAA explant and AoSMC.\textsuperscript{19} A protective role for PPARγ in aortic aneurysm has been demonstrated recently in 2 independent mouse models of AAA incorporating vascular smooth muscle cell–selective Pparγ deletion.\textsuperscript{17,43} Importantly, the absence of vascular smooth muscle cell PPARγ rendered mice more susceptible to CTSS-associated degeneration of medial elastin.\textsuperscript{17} PPARγ binds to a peroxisome proliferator–activated receptor response element upstream of the CTSS gene in AoSMC and knockdown and overexpression of PPARγ results in the increase and decrease, respectively, of CTSS mRNA and activity.\textsuperscript{17} Here, we report increased expression of Pparγ and decreased expression of Ctsl in aortas of ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice associated with reduced incidence of AngII-induced aortic aneurysm. The upregulation of PPARγ in Opg-deficient mice may act to negatively regulate AngII type 1 receptor and CTSS in AoSMC and contribute to the relative resistance of these mice to AngII-induced aortic aneurysm. AngII-induced aortic aneurysm in ApoE\textsuperscript{−/−} mice involves the activation of Nfkb with increases in both p52 and p65 Nfkb subunits within the aortic wall.\textsuperscript{41} The 3-fold increase in Nfkb mRNA observed within aortas of ApoE\textsuperscript{−/−}Opg\textsuperscript{−/−} mice seems inconsistent with the inhibition of AngII-induced aortic dilatation in these mice. An explanation might be in the concurrent increases of aortic Mapk1/3 and Pparγ reported above. Phosphorylation of PPARγ via active MAPK1/3 leads to the physical association

Figure 3. Elevated cathepsin S (CTSS) and CTSS-derived elastase activity in human aortic aneurysm smooth muscle cell (AASMC) and stimulation of CTSS-derived elastase activity in human aortic vascular smooth muscle cell (AoSMC) by recombinant human osteoprotegerin (rhOPG). A. Concentration of CTSS measured in the supernatant (secreted) and cell lysate (cellular) of healthy human AoSMC (gray) and aortic smooth muscle cells derived from human abdominal aortic aneurysm biopsies (AASMC; white) after 96 hours in culture. Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for change in fluorescence (AUU) per minute per micromgram protein from 6 repeat cultures (P values calculated by Mann–Whitney U test). B. CTSS-derived elastase activity measured in AASMC (white) and AoSMC (gray) cultured for 36 hours in control media, in the presence of rhOPG (50 nmol/L), or in the presence of rhOPG+CTSS inhibitor (20 nmol/L). Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for change in fluorescence units (ΔFU) per minute per micromgram protein from 6 repeat cultures (P values calculated by Mann–Whitney U test; *P=0.002 vs AoSMC control; †P=0.002 vs AoSMC control; ‡P=0.004 vs AASMC in OPG alone; #P=0.002 vs AoSMC in OPG alone.

secreted levels of osteoprotegerin\textsuperscript{10} and CTSS are markedly elevated in AASMC in vitro compared with healthy human AoSMC. Thus, it is plausible that exogenous osteoprotegerin had little effect on already maximally stimulated cells from advanced-stage AAA. Nonetheless, our observation that osteoprotegerin stimulated CTSS, and CTSS-derived elastase activity in healthy human AoSMC in vitro provides evidence of an association between osteoprotegerin and upregulation of protease activity within the aneurysmal aortic wall.

Previous human and animal studies suggest that AASMCs exhibit a phenotype that favors aortic ECM degeneration.\textsuperscript{18,20,25,32,33} In particular, they have reduced ability to proliferate, increased apoptosis tendency, and promoted
Disclosure

None.

References


3. Natafmadja M, West M, Wijesekara K, Pande K, Amlani S, Oparil S, Schairer C, Bolognesi D. Osteoprotegerin and CTSS in advanced-stage human AAA is not limited to vascular cells, but present also in leukocytes, lymphocytes, and plasma cells.9,10,47 The effect of Opg deficiency on these cells requires further study. In summary, the current study suggests a role for osteoprotegerin in aortic aneurysm formation and rupture. In vitro, osteoprotegerin stimulated CTSS and CTSS-derived elastase activity in AoSMC. In vivo, Opg deficiency was associated with downregulation of CTSS, MMP2, and MMP9 within aortas of AngII-infused ApoE−/− mice and an aortic gene expression profile suggesting AoSMC differentiation and viability. It is postulated that Opg deficiency reduced aortic proteolytic activity and stimulation of an AoSMC inflammatory phenotype in response to AngII, thus reducing aortic dilatation and rupture.

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References


**Significance**

Abdominal aortic aneurysm is an important cause of mortality and requirement for surgical intervention in older adults. There is a need to identify effective medical therapies that can slow or halt abdominal aortic aneurysm growth. The work presented in this report suggests that osteoprotegerin is important in the development and rupture of abdominal aortic aneurysm. The authors demonstrate that Opg deficiency inhibits aortic expression of the proteolytic enzymes cathepsin S, matrix metalloproteinase 2, and matrix metalloproteinase 9, limiting vessel dilatation and rupture within a mouse model. These molecules are considered to be among the key proteases responsible for negative remodeling of the aortic wall associated with abdominal aortic aneurysm pathogenesis in humans.
Osteoprotegerin Deficiency Limits Angiotensin II–Induced Aortic Dilatation and Rupture in the Apolipoprotein E–Knockout Mouse
Corey S. Moran, Roby J. Jose, Erik Biros and Jonathan Golledge

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**SUPPLEMENTAL MATERIAL**

Detailed Materials and Methods

**Generation of double knockout (ApoE<sup>-/-</sup>Opg<sup>-/-</sup>) mice**

Osteoclastogenesis inhibitory factor (osteoprotegerin, Opg) gene-knockout mice on a C57 black 6/J background (ApoE<sup>+/+</sup>Opg<sup>-/-</sup>) were obtained from CLEA Japan Inc [1]. Opg-deficient apolipoprotein E-deficient (ApoE<sup>-/-</sup>Opg<sup>-/-</sup>) mice were generated by mating the respective null homozygotes with ApoE<sup>-/-</sup>Opg<sup>+/+</sup> mice (B6.129P2-Apoe tm1Unc/Arc (N10)) sourced from the Animal Resources Centre, Canning Vale, Australia. Resultant double-heterozygous progenies were then inter-crossed and subsequent generations typed to identify and establish homozygous double deficient mouse lines. DNA extracted from ear clippings was amplified on an Applied Biosystems Veriti thermal cycler using forward 5' - GGT CCT CCT TGA TTT TTC TAT GCC-3' and reverse 5'- TGC CCT GAC CAC TCT TAT ACG GAC-3' primers to identify the wild type allele and forward 5’ GCT GCA TAC GCT TGA TCC GGC-3’ and reverse 5’- TAA AGC ACG AGG AAG CGG TCA-3’ primers to detect the Opg-null allele (both primer pairs amplifying at 94°C 30 sec, 62°C 30 sec and 72°C 30 sec). ApoE-null allele was identified using the forward 5’-CGA AGC CAG CTT GAG TTA CAG AA-3’ and reverse 5’-AGA GCC GGA GGT GAC AGA TCA G-3’ primer set (amplifying at 96°C 60 sec, 60°C 60 sec and 72°C 3 min). 5µl of the PCR products were loaded on a 1% agarose gel pre-stained with Gel-Red (Biotium, CA) and electrophoresis performed for 45min at 100V. PCR products were visualised by ultraviolet transillumination (Supplementary Figure I).

**Mouse model of AAA and in vivo studies**

Approval for animal studies was obtained from the local ethics committee and experimental work performed in accordance with the institutional and ethical guidelines of James Cook University, Australia, and conforming to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA). Mice were housed in an individually-ventilated, temperature/humidity-controlled cage system (Aero IVC Green Line; Tecniplast) on a 12-hour light/dark cycle, and maintained on normal laboratory chow and water ad libitum. The angiotensin II (Ang2)-infusion model was implemented as previously described [2]. Briefly, an osmotic micro-pump (ALZET Model 1004, Durect Corporation, USA) containing Ang2 (Sigma-Aldrich) dissolved in sterile water was inserted into the subcutaneous space left of the dorsal midline under anaesthesia (4% isoflurane inhalation) to administer Ang2 or H2O vehicle at a rate of 1.0 µg/kg/min over the experimental period. Three separate animal studies were performed:

1) **Ang2-induced aortic dilatation in Opg-deficient C57BL/6 (ApoE<sup>-/-</sup>Opg<sup>-/-</sup>) mice.** Thirteen week old male ApoE<sup>+/+</sup>Opg<sup>+/+</sup> and ApoE<sup>+/+</sup>Opg<sup>-/-</sup> mice (n=30 per group), were infused with Ang2 over 28 days, after which regional aortic diameters (arch, thoracic aorta (TA), suprarenal aorta (SRA), infrarenal aorta (IRA)) were determined by morphometric analysis following dissection.

2) **Effect of Opg deficiency on aortic dilatation in Ang2-infused ApoE<sup>-/-</sup> mice.** Six month old male ApoE<sup>-/-</sup>Opg<sup>+/+</sup> (n=18) and ApoE<sup>-/-</sup>Opg<sup>-/-</sup> mice (n=17) were infused with Ang2 for 28 days. In vivo dilatation of the SRA was monitored by ultrasound at 14-day intervals, while maximum aortic arch, TA, SRA, and IRA diameters were determined at harvest or by morphometric analysis.

3) **Effect of Opg deficiency on early event mechanisms within the aorta of Ang2-infused ApoE<sup>-/-</sup> mice.** Six month old male ApoE<sup>-/-</sup>Opg<sup>+/+</sup> and ApoE<sup>-/-</sup>Opg<sup>-/-</sup> mice (n=6 per group) were infused with Ang2 for seven days, after which whole aortas were processed for mRNA and protein analysis.
Measurement of mouse aortic diameters
Maximum diameters of aortic arch, TA, SRA, and IRA (Supplementary Figure II) were determined by morphometric analysis (Study 1 & 2) while in vivo dilatation of the SRA was monitored at 14 day intervals by ultrasound (Study 2) as previously described [2]. Necropsy was performed within 24 hours of sudden mouse fatality to confirm aortic rupture as cause of death. Phosphate buffered saline-perfused aortas were harvested from mice completing the study protocol or that died during the study and were placed on a graduated template and digitally photographed (Coolpix 4500, Nikon). Maximum diameter of the arch, TA, SRA, and IRA were determined from the images using computer-aided analysis (Adobe® Photoshop® CS5 Extended version 12, Adobe Systems Incorporated). Ultrasound measurements of the SRA were obtained prior to Ang2 infusion (base-line) and at days 14 and 28 post pump insertion. Scans were performed in sedated mice (i.p., 40 mg/kg ketamine, 4 mg/kg xylazine) using a MyLabTM 70 VETXV platform (Esaote, Italy) with a 40 mm linear transducer at an operating frequency of 10 MHz (LA435; Esaote, Italy) to provide a sagittal image of the SRA. Maximum SRA diameter was measured at peak systole using the calliper measurement feature. Good inter-observer reproducibility of morphometric and ultrasound analysis has been demonstrated previously in our laboratory [3,4]

Cytokine/chemokine assay of mouse serum
Serum samples collected after seven days of Ang2 infusion (Study 3) were assessed using a multiplex quantitative ELISA-based assay (Q-Plex™, Quansys Biosciences). Q-Plex™ technology is based on placement of immobilized capture antibody in 350–500 μm spots at the bottom of polypropylene 96-well plates to capture target proteins (for more details see http://www.quansysbio.com/assay-development/). Each spot is printed with a different analyte capture antibody, in this case against IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12, IL-17, MCP-1, TNFα, MIP-1α, GMCSF, and RANTES. Two internal control assays are designed within the assay to ensure integrity of results from each well. The Q-Plex™ multiplex array system has been previously validated as an alternative to individual immunoassays, with acceptable inter assay variability and very sensitive levels of detection [5,6,7]. Analyte concentration was quantified against known standards by densitometry using the ChemiDoc™ imaging system (Bio-Rad Laboratories) supported by QuantityOne™ 1-D Analysis Software (Bio-Rad Laboratories), and expressed as pg/ml.

Preparation of mouse aortic samples
Simultaneous isolation of nucleic acids and protein from whole aortas was performed using TRIzol® Reagent (Sigma-Aldrich) as per the manufacturer’s instructions. Total RNA and protein samples were prepared for real time PCR and protein analysis (TaqMan® Protein Assay and zymography), respectively.

Real time PCR
QuantiTect® Primer Assays were used to determine gene expression for Mmp2 (QT00116116), Mmp9 (QT00108815), Ctss (QT00102116), Pparg (QT00100296), Nfkb (QT00154091), Bax (QT00102536), Bcl2 (QT00156282), Cnn1 (QT00105420), Mapk1 (QT00133840), Mapk3 (QT00103355), and Mapk14 (QT00161945) in mouse aortas using quantitative real time (qPCR) as previously described [8]. The relative expression of these genes in aortas of experimental and control animals was calculated by using the concentration-Ct-standard curve method and normalized using the average expression of glyceraldehyde-3-phosphate dehydrogenase (Gapdh; QT01658692) for each sample using the Rotor-Gene Q operating software version 2.0.24. Gapdh was chosen as the ‘housekeeping’ gene since analyses showed its expression to be similar in categories assessed. The QuantiTect SYBR® Green one-step RT-PCR Kit (Qiagen) was used according to the
manufacturer’s instructions with 40ng of total RNA as template. All reactions were independently repeated in duplicate to ensure the reproducibility of the results.

**TaqMan® Protein Assay**

CTSS protein in mouse aortic tissue was quantified using the TaqMan® Protein Assay (TPA, Applied Biosystems) [9]. Polyclonal antibodies to cathepsin S (ab18822, Abcam) and β-Actin (4967, Cell Signaling Technology) were biotinylated using the Lightning-Link™ Biotin Conjugation Kit (Innova Biosciences) prior to oligonucleotide labelling with the ‘TaqMan® Protein Assay Open Kit’ (Applied Biosystems) as per manufacturer’s instructions. The protein assay was performed using the ‘TaqMan® Protein Assay Core Reagents Kit’ with ‘Master Mix’ (Applied Biosystems) according to manufacturer’s instructions. Real-time quantitative PCR was performed using the Rotor-Gene Q operating software version 2.0.24. Relative protein expression between control and experimental samples was calculated by using the concentration-Ct standard curve method and normalized using the average expression of β-actin for each sample. Each assay included a no-protein control (NPC) to calculate ΔCt values (Ct value (sample) - Ct value (NPC)), a linear range generated for each sample, and a ΔCt threshold was designated. The fold change between samples was calculated between the crossover points of each linear trend line at the ΔCt threshold.

**Zymography**

Measurement of MMP2 and MMP9 in mouse aortic tissue using gelatin zymography was performed as previously described [10]. Extracted protein from mouse aortas was separated at room temperature on a 10% acrylamide-SDS gel containing 0.5% gelatin. Following several washes in 2.5% (vol/vol) Triton X-100, gels was incubated for 12 hours at 37°C in 50 mM MTris (pH 8) containing 5 mM CaCl2. Bands were visualized in a 10% ethanol/10% acetic acid solution after staining with 0.5% Coomassie blue (R-250), with enzyme activity semi-quantified with densitometric analysis using the ChemiDoc™ imaging system (Bio-Rad Laboratories) and QuantityOne™ 1-D Analysis Software (Bio-Rad Laboratories).

**Cathepsin S activity assay**

Measurement of CTSS activity within mouse aorta was performed using a commercial assay (K144-100; Biovision) as per manufacturer’s instructions. The fluorescence-based activity assay utilized the preferred CTSS substrate sequence Val-Val-Arg (VVR) labelled with amino-4-trifluoromethyl coumarin (AFC). Free AFC cleaved from the synthetic Z-VVR-AFC substrate was detected using a POLARStar Omega™ fluorescence plate reader (BMG Labtech) at 400 nm excitation and 505 nm emission and used as a quantitative measure of CTSS activity expressed as change in fluorescence units (ΔFU) per minute per μg protein.

**Cell culture**

Healthy human aortic vascular smooth muscle cells (AoSMC; Clonetics® human aortic SMC, Lonza) and AASMC isolated from human AAA biopsies (n=6) by combined collagenase and elastase digestion [11] were seeded at 1x10⁵ cells/ml into separate cultures and maintained in DMEM+10% FBS at 37°C, 5% CO₂. Culture supernatants and cell lysates were harvested at confluency or after a maximum of 96 hours and assayed by ELISA for secreted and cellular CTSS, respectively. In a separate study, AASMC and AoSMC were seeded separately at 1x10⁵ cells/ml and maintained in DMEM+10% FBS at 37°C, 5% CO₂ to ~80% confluency. Following 24 hours in low-serum (1%) medium, cells were refreshed with phenol red-free DMEM+10% FBS and incubated at 37°C, 5% CO₂ in the presence of recombinant human (rh)Opg (Enzo Life Sciences; 50 nM, n=6), rhOpg + Ctss inhibitor (Z-FL-COCHO, EMD Millipore; 20 nM), or media alone (control). Cells were harvested after 36 hours and assayed for elastase activity.
Assessment of elastase activity
Elastase activity in cell lysate was assessed using a commercial assay (EnzChek® Elastase Assay; Molecular Probes) as per manufacturer's instructions. The fluorescence-based activity assay utilized soluble bovine neck ligament (DQ™) elastin labelled with BODIPY® FL dye. Fluorescent fragments released upon elastolytic digestion were detected using a POLARStar Omega™ fluorescence plate reader (BMG Labtech) at 505 nm excitation and 515 nm emission and used as a quantitative measure of elastase activity expressed as fluorescence units per minute per μg protein.

ELISA for CTSS
Cell culture samples were assayed using the Total CTSS DuoSet ELISA systems (DY1183; R&D Systems) as per manufacturer's instructions. Use of these assays previously in our laboratory have demonstrated excellent recovery and intra- and inter-assay reproducibility [12,13]. Sample optical density was measured using a Sunrise™ absorbance plate reader (Tecan) at 450 nm. Assay detection limits for CTSS was 16-1000 pg/ml. Concentrations were expressed as pg/mg protein.

Statistics
Data were analysed using GraphPad Prism (version 6) and TIBCO Spotfire S+ (version 8.2). Parametric or non-parametric tests were applied appropriate to distribution of data. Comparison of cell culture end-point data was performed using Mann-Whitney U test. For mouse studies, aortic end-point data for maximum diameter, MMP2/9/CTSS protein, and mRNA expressions, were compared between ApoE−/−Opg−/− and ApoE−/−Opg+/+ mice by Mann-Whitney U test. Comparison between baseline and end-point serum cytokine concentrations was performed within each mouse group using paired-t test, while median fold-increase in concentrations of individual cytokines was compared between ApoE−/−Opg−/− and ApoE−/−Opg+/+ mice by Mann-Whitney U test. Mouse data obtained as a function of time, i.e. blood pressure and ultrasound of SRA diameter, was compared within each group by repeat measures one-way ANOVA, and between ApoE−/−Opg−/− and ApoE−/−Opg+/+ mice by mixed-effects linear regression. Kaplan-Meier survival curves were analysed using log-rank (Mantel-Cox) test. In all cases P values less than 0.05 were considered significant.

References


Figure I: Homozygous null mutations for osteoprotegerin (Opg<sup>−/−</sup>) generated in apolipoprotein E-deficient C57Bl/6 (ApoE<sup>−/−</sup>) mice. Representative agarose gel electrophoretic profiles of PCR-amplified products of Opg (A) and ApoE (B) PCR. PC, positive control; NTC, no template control; WT, wild-type; KO, knock-out; MW, molecular weight ladder (100bp).
**Figure II: Aortic region boundaries.** *Aortic arch*: from heart to left subclavian artery; *thoracic aorta* (TA): from left subclavian artery to the aortic hiatus in the diaphragm; *suprarenal aorta* (SRA): from the aortic hiatus to the left renal artery; *infrarenal aorta* (IRA): from left renal artery to the aortic bifurcation at the left and right common iliac arteries.

**Figure III: Effect of Opg deficiency on AngII-induced aortic dilatation in ApoE+/− mice.** Gross morphology of aortas harvested from *ApoE−/− Opg+/+* (control) and *ApoE−/− OPG−/−* mice subcutaneously infused with AngII (1.0 µg/kg/min) for 28 days.
Figure IV: Immuno-detection of monocyte/macrophages within ApoE<sup>−/−</sup>Opg<sup>−/−</sup> aorta. Immunohistochemical localisation of MOMA-2 (brown stain) identifying monocyte/macrophages (white arrows) within the adventitia of 5μm frozen-sectioned SRA from an Opg-deficient ApoE<sup>−/−</sup> mouse infused with AngII over 28 days. A, adventitia; L, lumen; T, intramural thrombus; black arrows indicating elastic lamellae (media); Scale bar = 0.1 mm.
Figure V: Effect of Opg deficiency on aortic levels of MMP2 and MMP9 in AngII-infused ApoE−/− mice. Zymographic detection of MMP2 and MMP9 in aortic tissue from ApoE−/− OPG+/+ (n=6; Gel 1) and ApoE−/− Opg−/− (n=6; Gel 2) mice following infusion of AngII for seven days. Boxed numerals 1 and 2 are duplicate samples from Gel 1 included on Gel 2 for normalisation of densitometry between gels; CL, cell lysate and conditioned media (CM) from cultured vascular smooth muscle cells as positive control (marker) for MMP2 and 9.
Figure VI: AngII-induced CTSS-derived elastase activity in AoSMC *in vitro*. Elastase activity in healthy human AoSMC cultured in the presence and absence of AngII (100 nM) over 36 hours measured using an elastin degradation assay. Addition of a specific CTSS inhibitor Z-FL-COCHO (CTSSi, 20 nM) to AngII-activated AoSMC confirmed CTSS-derived elastase activity induced by AngII. AngII, angiotensin II; CTSS, cathepsin S; AoSMC, aortic smooth muscle cells; Data expressed at each time point (n=6 cultures) as mean and standard deviation CTSS activity (fluorescence/µg protein); *P<0.001 and #P=0.042 calculated by mixed-effects linear regression for difference between vehicle and AngII and AngII+CTSSi, and between AngII and AngII+CTSSi, respectively.
Table I: Regional aortic diameters in AngII-infused wild-type \((ApoE^{++}/Opg^{++})\) and \(Opg^{-/-}\) null \((ApoE^{++}/Opg^{-/-})\) mice

<table>
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<tr>
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<th>ApoE^{++}/Opg^{++}</th>
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<td>1.23 (1.12-1.33)</td>
<td>0.014</td>
</tr>
<tr>
<td>SRA</td>
<td>1.37 (1.26-1.69)</td>
<td>1.27 (1.17-1.48)</td>
<td>0.065</td>
</tr>
<tr>
<td>IRA</td>
<td>0.79 (0.67-0.84)</td>
<td>0.77 (0.68-0.88)</td>
<td>0.562</td>
</tr>
</tbody>
</table>

\(Opg\), osteoprotegerin; \(n\), number; TA, thoracic aorta; SRA, suprarenal aorta; IRA, infra-renal aorta; data presented as median (interquartile range) maximum diameter \((\text{mm})\); \(P\), 2-sided \(P\)-value for comparison between groups by Mann-Whitney \(U\) test.