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# Endothelial Cell-Specific Deficiency of Ang II Type 1a Receptors Attenuates Ang II-Induced Ascending Aortic Aneurysms in LDL Receptor<sup>-/-</sup> Mice

Debra L. Rateri, Jessica J. Moorleghen, Anju Balakrishnan, A. Phillip Owens III, Deborah A. Howatt, Venkateswaran Subramanian, Aruna Poduri, Richard Charnigo, Lisa A. Cassis, Alan Daugherty

**Rationale:** Human studies and mouse models have provided evidence for angiotensin II (Ang II)-based mechanisms as an underlying cause of aneurysms localized to the ascending aorta. In agreement with this associative evidence, we have published recently that Ang II infusion induces aneurysmal pathology in the ascending aorta.

**Objective:** The aim of this study was to define the role of angiotensin II type 1a (AT<sub>1a</sub>) receptors and their cellular location in Ang II-induced ascending aortic aneurysms (AAs).

**Methods and Results:** Male LDL receptor<sup>-/-</sup> mice were fed a saturated fat-enriched diet for 1 week before osmotic mini-pump implantation and infused with either saline or Ang II (1000 ng/kg per minute) for 28 days. Intimal surface areas of ascending aortas were measured to quantify ascending AAs. Whole body AT<sub>1a</sub> receptor deficiency ablated Ang II-induced ascending AAs ( $P < 0.001$ ). To determine the role of AT<sub>1a</sub> receptors on leukocytes, LDL receptor<sup>-/-</sup> × AT<sub>1a</sub> receptor<sup>+/+</sup> or AT<sub>1a</sub> receptor<sup>-/-</sup> mice were irradiated and repopulated with bone marrow-derived cells isolated from either AT<sub>1a</sub> receptor<sup>+/+</sup> or AT<sub>1a</sub> receptor<sup>-/-</sup> mice. Deficiency of AT<sub>1a</sub> receptors in bone marrow-derived cells had no effect on Ang II-induced ascending AAs. To determine the role of AT<sub>1a</sub> receptors on vascular wall cells, we developed AT<sub>1a</sub> receptor floxed mice with depletion on either smooth muscle or endothelial cells using Cre driven by either SM22 or Tek, respectively. AT<sub>1a</sub> receptor deletion in smooth muscle cells had no effect on ascending AAs. In contrast, endothelial-specific depletion attenuated this pathology.

**Conclusions:** Ang II infusion promotes aneurysms in the ascending aorta via stimulation of AT<sub>1a</sub> receptors that are expressed on endothelial cells. (*Circ Res.* 2011;108:00-00.)

**Key Words:** ascending aneurysm ■ angiotensin II ■ AT<sub>1a</sub> receptor ■ Tek-cre

Ascending aortic aneurysm (AA) is an asymptomatic expansion of this restricted region in which rupture has catastrophic consequences.<sup>1</sup> It has now become apparent that ascending AAs have a significantly higher incidence than originally thought. Furthermore, studies and communities in which autopsies are routinely performed have demonstrated that the incidence of aortic aneurysms is increasing.<sup>2</sup>

Recent human and experimental studies have inferred a role for angiotensin II (Ang II) in the development of ascending AAs.<sup>3-6</sup> Ascending AAs can be generated in transgenic mice that express a common mutation of fibrillin-1 present in patients with Marfan syndrome. There are also several other genes that have been associated with AAs.<sup>4</sup> The aortic dilation that is localized to the ascending region is prevented by the administration of the AT<sub>1</sub> receptor antagonist, losartan.<sup>5</sup> Retrospective analysis of pharmacological treatments given to individuals with Marfan syndrome demonstrated that

administration of losartan attenuated dilation of the ascending aorta.<sup>3</sup> Prospective evaluations of angiotensin receptor antagonists are currently being performed in populations afflicted with Marfan syndrome.<sup>7</sup>

Ang II has diverse effects that could be implicated in aneurysm formation in the ascending aorta. Ang II exerts its bioactive effects mainly via stimulation of AT<sub>1</sub> receptors in most species, and the AT<sub>1a</sub> receptor subtype in rodents. This receptor subtype is ubiquitously present on the cell types involved in vascular pathology including endothelium, smooth muscle cells (SMCs), and macrophages.

We have demonstrated recently that Ang II infusion into hypercholesterolemic mice promotes pronounced dilation that is localized to the ascending aorta.<sup>3</sup> This region of lumen dilation also has concentric medial thickening that is most pronounced on the adventitia aspect of the aorta. In many mice infused with Ang II, ulceration develops on the anterior

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Non-standard Abbreviations and Acronyms	
<b>5-HT</b>	5-hydroxytryptamine (serotonin)
<b>AA</b>	aortic aneurysm
<b>AAA</b>	abdominal aortic aneurysm
<b>AT<sub>1a</sub></b>	angiotensin II type 1a
<b>Ang II</b>	angiotensin II
<b>LDL</b>	low-density lipoprotein
<b>MCP</b>	monocyte chemoattractant protein
<b>SBP</b>	systolic blood pressure
<b>SMC</b>	smooth muscle cell

aspect of ascending aortas. Using this model as an experimental paradigm of AAs, we determined the role of AT<sub>1a</sub> receptor expression and cellular localization on the development of Ang II-induced ascending AAs.

## Methods

An expanded Methods section is available in the Online Data Supplement at <http://circres.ahajournals.org>.

### Mice

LDL receptor<sup>-/-</sup>, AT<sub>1a</sub> receptor<sup>-/-</sup>, SM22-Cre, Tek-Cre, and ROSA26 mice were purchased from The Jackson Laboratory. AT<sub>1a</sub> receptor floxed mice were generated by InGenious Targeting Laboratory. Male mice were fed a saturated fat-enriched diet and implanted subcutaneously with mini-osmotic pumps (Alzet) to infuse saline or Ang II (1000 ng/kg per minute) for 28 days as described previously.<sup>8</sup>

### Genotyping

Genotypes of mice were confirmed by PCR of genomic DNA.

### Detection of AT<sub>1a</sub> Receptor mRNA

Total RNA was isolated from liver, aortic SMCs, and endothelial cells. cDNAs were transcribed using iScript cDNA synthesis kits and primers specific for AT<sub>1a</sub> receptor mRNA were used in real time PCR, with confirmation of amplicons by visualized detected on agarose gels.

### Detection of $\beta$ -Galactosidase Activity in Tissues

Aortas were removed and  $\beta$ -galactosidase activity was detected by X-gal.

### Detection of AT<sub>1a</sub> Receptor Protein

Several commercially available and newly developed antibodies were tested for specificity.

### Systolic Blood Pressure Measurements

Systolic blood pressure (SBP) was measured using the Kent Scientific or Visitech tail cuff machine as described previously.<sup>9</sup>

### Serum Measurements

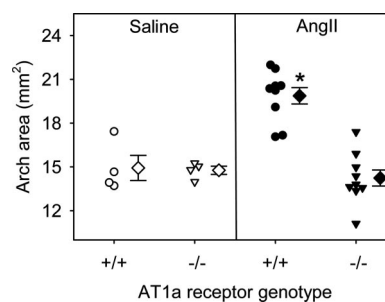
Total serum cholesterol concentrations were determined as described previously.<sup>10</sup> Serum monocyte chemoattractant protein (MCP)-1 concentration was measured by ELISA (R&D Systems).

### Bone Marrow Transplantation

Bone marrow transplantation was performed as described previously.<sup>8,11</sup>

### Aortic Contractility Studies

Aortic segments were isolated and subjected to KCl, serotonin (5-hydroxytryptamine [5-HT]), and Ang II incubations.



**Figure 1. Whole body deficiency of AT<sub>1a</sub> receptors ablated Ang II-induced ascending AAs.** Circles and triangles represent data from individual mice, diamonds are means, and bars are SEMs. \* $P < 0.001$ , when comparing Ang II vs saline infusion within +/+ group and when comparing +/+ vs -/- genotype in Ang II infusion by 2-way ANOVA followed by the Holm-Sidak method.

## Measurements and Pathohistology of Ascending AAs

Ascending aortic aneurysms were measured by intimal area and elongation. Aortas were prepared for en face and elongation measurements as described previously.<sup>12</sup> The incidence of ascending aortic ulceration was also quantified.

To quantify medial thickness and elastin breaks, aortas from each group were chosen based on measurement nearest to the mean of the arch area. Serial sections of ascending aortas were stained with Movat's pentachrome.

### Statistics

Analyses were performed by tests that were appropriate for the number of groups being compared and for the parametric or nonparametric characteristics of the data. Data are represented as means  $\pm$  SEM.  $P < 0.05$  was considered statistically significant.

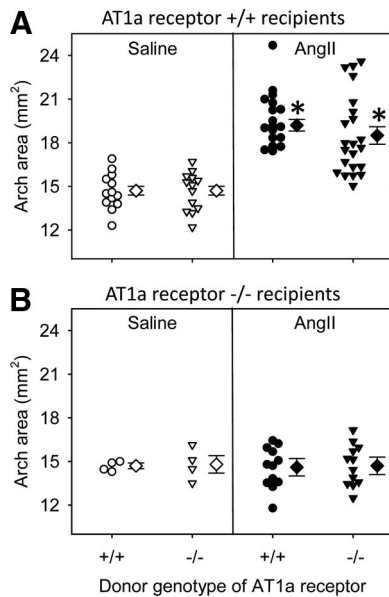
## Results

### Whole Body Deficiency of AT<sub>1a</sub> Receptors Ablated Ascending AAs

We first defined the effects of whole body AT<sub>1a</sub> receptor deficiency on Ang II-induced ascending AA formation in LDL receptor<sup>-/-</sup> mice fed a diet enriched in saturated fat. Ascending aortic intimal area was measured as an index of severity of aneurysm. This area was similar in both AT<sub>1a</sub> receptor<sup>+/+</sup> and AT<sub>1a</sub> receptor<sup>-/-</sup> mice infused with saline. Ang II infusion significantly increased the ascending aortic area in AT<sub>1a</sub> receptor<sup>+/+</sup> mice ( $P < 0.001$ ). In contrast, there was no significant increase in this region of AT<sub>1a</sub> receptor deficient mice infused with Ang II (Figure 1).

### AT<sub>1a</sub> Receptor Deficiency in Bone Marrow-Derived Cells Did Not Influence Development of Ascending AAs

Following the demonstration that whole body deficiency of AT<sub>1a</sub> receptors completely attenuated development of ascending AAs, we sought to determine the cell types expressing this receptor that were responsible for this pathology. To examine the role of hematopoietic cells, LDL receptor<sup>-/-</sup> mice that were either AT<sub>1a</sub> receptor<sup>+/+</sup> or AT<sub>1a</sub> receptor<sup>-/-</sup> were irradiated and repopulated with bone marrow-derived cells harvested from AT<sub>1a</sub> receptor<sup>+/+</sup> or AT<sub>1a</sub> receptor<sup>-/-</sup> donors. The development of ascending AAs was determined by the AT<sub>1a</sub> receptor genotype of recipients. Ang II infused into AT<sub>1a</sub> receptor<sup>+/+</sup> recipients significantly increased arch



**Figure 2. Deficiency of AT<sub>1a</sub> receptors in bone marrow-derived cells did not affect Ang II-induced ascending AAs.** AT<sub>1a</sub> receptor<sup>+/+</sup> (A) or AT<sub>1a</sub> receptor<sup>-/-</sup> (B) recipients were irradiated and repopulated with bone marrow-derived cells harvested from either AT<sub>1a</sub> receptor<sup>+/+</sup> or AT<sub>1a</sub> receptor<sup>-/-</sup> mice. After repopulation, chimeric mice were infused with either saline or Ang II. **Circles and triangles** represent individual mice, **diamonds** are means, and **bars** are SEMs. Statistical analyses were performed using 2-way ANOVA. There was no significant effect of genotype of the donor cells on aortic arch intimal areas.

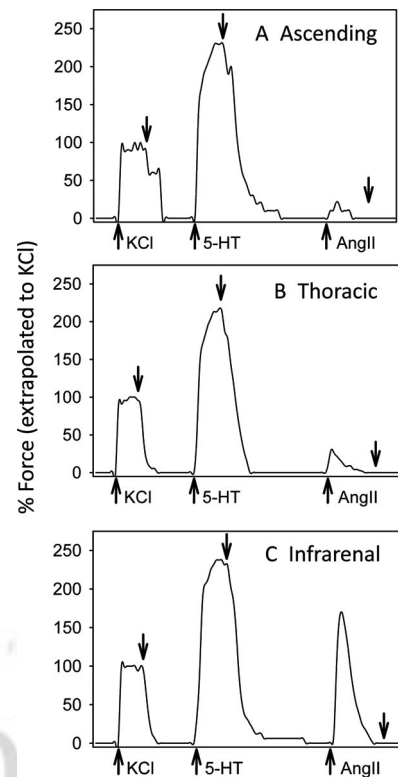
area in both donor genotypes (Figure 2A), whereas AT<sub>1a</sub> receptor<sup>-/-</sup> recipients did not develop ascending AAs (Figure 2B). No significant effects were observed for donor genotype, irrespective of recipient AT<sub>1a</sub> receptor genotype.

### Ang II Failed to Contract SMCs in the Ascending Aorta

Because we failed to detect an effect of leukocytic AT<sub>1a</sub> receptors in forming ascending AAs, we next focused on mechanisms within the aortic wall. Ang II infusion leads to changes localized to ascending aortic media layers.<sup>3</sup> Therefore, we determined whether Ang II exerted direct effects on SMC contractile responses that were differed in ascending aortas compared to other aortic regions. To accomplish this, we performed contractile studies using aortic rings from selected regions, as described previously.<sup>13</sup> KCl and 5-HT contracted all aortic regions (ascending, descending thoracic, and infrarenal). However, Ang II produced minimal discernible contraction of ascending aortas (Figure 3). Consistent with a previous report, only infrarenal aortic regions contracted in response to Ang II stimulation.<sup>14</sup> These studies failed to provide any evidence of differential contractile responses to Ang II in SMCs of ascending and descending aortas.

### AT<sub>1a</sub> Receptor Deficiency on SMCs Had No Effect on Development of Ascending AAs

To determine whether Ang II interacted directly with AT<sub>1a</sub> receptors on SMCs within ascending aortas to promote aneurysms, we generated mice with loxP sites on either side of exon 3 (Online Figure I). Mice were bred to transgenic

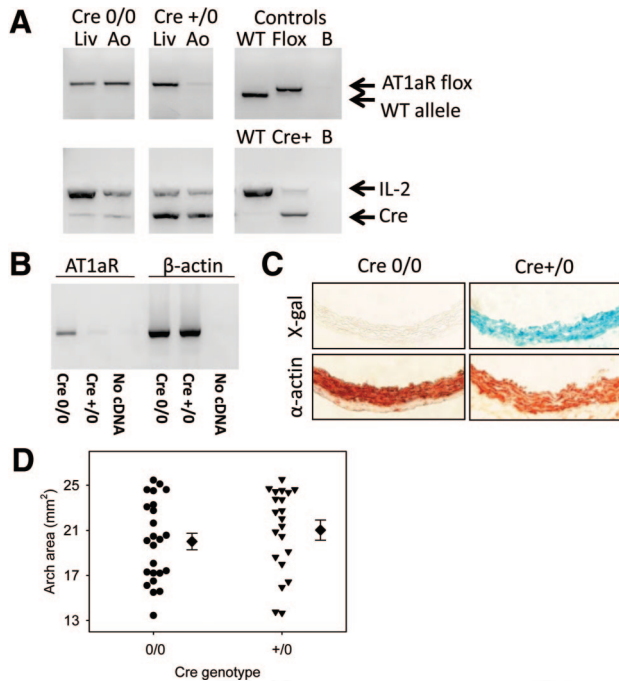


**Figure 3. Ang II-induced aortic contractions were restricted to the infrarenal region.** Regional contractility of rings from the ascending (A), thoracic (B), and infrarenal aorta (C). Aortic rings were contracted during 5-minute incubations with potassium chloride (KCl) (80 mmol/L), 5-HT (1  $\mu$ mol/L), and Ang II (1  $\mu$ mol/L). Contractions are represented as a percentage of the maximal contraction achieved during incubation with elevated KCl (80 mmol/L). **Inverted arrows** indicate the return to Krebs-Henseleit solution containing no drug.

mice expressing Cre under the control of an SMC promoter, SM22. Depletion of exon 3 of the AT<sub>1a</sub> receptor gene was confirmed by PCR using genomic DNA from aortas (Figure 4A). Depletion of AT<sub>1a</sub> receptor mRNA in SMCs was confirmed by RT-PCR (Figure 4B). As with whole body AT<sub>1a</sub> receptor deficiency,<sup>15</sup> the deletion of AT<sub>1a</sub> receptors in SMC had no discernable effect of aortic structure.

We attempted to determine the depletion of AT<sub>1a</sub> receptor protein. However, of the multiple commercial and custom developed antibodies, we were unable to demonstrate an interaction that was specific for the mouse AT<sub>1a</sub> receptor protein in either Western blotting or immunostaining of tissue sections. As an alternative approach to illustrate the uniformity of SM22-Cre-induced gene depletion across the aortic media, we bred SM22-Cre expressing mice to ROSA26 mice. ROSA26 mice have a suppressed  $\beta$ -galactosidase, but this enzyme is present in cells that expressed Cre at some point during lineage development. These studies demonstrated uniform  $\beta$ -galactosidase activity throughout the media of Cre transgenic mice, whereas there was no detectable activity in media extracted from nontransgenic littermates (Figure 4C).

Depletion of AT<sub>1a</sub> receptors in SMCs of LDL receptor<sup>-/-</sup> mice fed a high-fat diet had no significant effect on body weight, serum cholesterol concentrations, or SBP (data not shown). Intimal areas of the aortic arch were similar in both



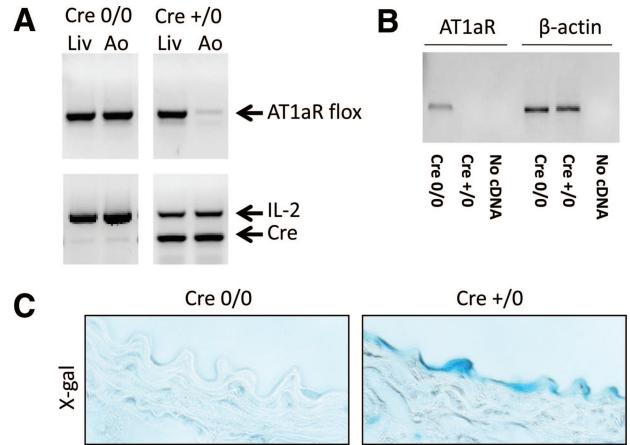
**Figure 4. SMC deficiency of AT<sub>1a</sub> receptor had no effect on Ang II-induced ascending AAs.** Validation of AT<sub>1a</sub> receptor depletion in aortic smooth muscle cells (Ao) by PCR detection of genomic DNA (compared to liver [Liv]) (A) and mRNA (B). C, ROSA26 Cre<sup>+/0</sup> aortas were positive for β-galactosidase activity (blue) in the same regions as positivity for α-actin immunostaining. D, Deficiency of AT<sub>1a</sub> receptors on SMCs had no significant effect on Ang II-induced expansion of ascending aortas as defined by a Mann-Whitney rank sum test. Data are from hemizygous Cre-expressing mice (+/0) compared to Cre-negative littermates (0/0). **Circles and triangles** represent data from individual mice, **diamonds** are means, and **bars** are SEMs.

groups (Figure 4D). These studies demonstrated that SMC-specific AT<sub>1a</sub> receptor deficiency had no effect on Ang II-induced ascending AAs relative to littermate controls.

**AT<sub>1a</sub> Receptor Deficiency on Endothelial Cells Attenuated Development of Ascending AAs**

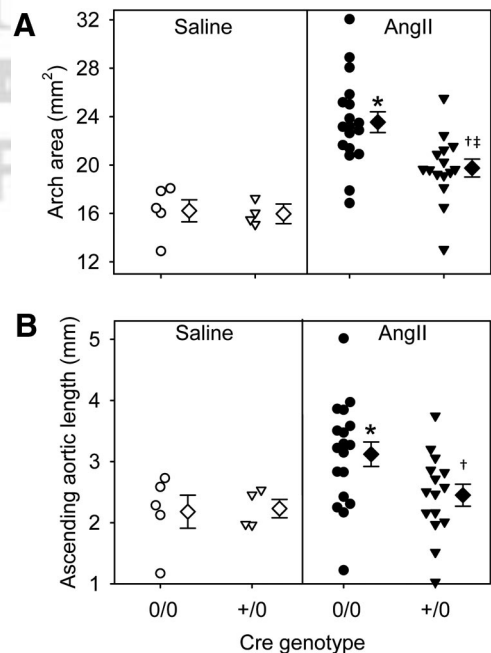
To determine the role of receptor expression on the endothelium, we bred AT<sub>1a</sub> receptor floxed mice to mice expressing Cre under the control of the Tek promoter. Depletion of the AT<sub>1a</sub> receptor from endothelial cells was confirmed by PCR using aortic endothelial cells cultured from Cre<sup>0/0</sup> and Cre<sup>+/0</sup> mice (Figure 5A). Real time PCR confirmed deletion of AT<sub>1a</sub> receptor mRNA in endothelial cells (Figure 5B). In the absence of an AT<sub>1a</sub> receptor antibody to confirm depletion of the receptor protein, Tek Cre expressing mice were bred to ROSA26 mice to examine uniformity of AT<sub>1a</sub> receptor gene depletion of endothelial cells. There was uniform β-galactosidase activity throughout the endothelium of Cre<sup>+/0</sup> mice, whereas there was no detectable activity in endothelium extracted from Cre<sup>0/0</sup> littermates (Figure 5C).

Depletion of AT<sub>1a</sub> receptors in endothelial cells had no significant effect on body weight, serum cholesterol concentrations, MCP-1 concentrations, or SBP (data not shown). However, deficiency in this cell type led to significant reductions in ascending aortic intima area expansion during Ang II infusion ( $P < 0.003$ ; Figure 6A). The reduction was not

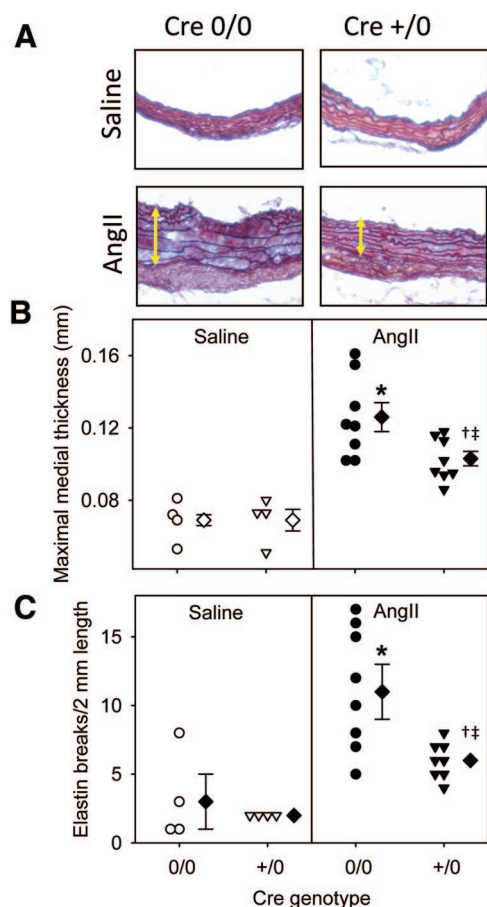


**Figure 5. Validation of AT<sub>1a</sub> receptor depletion in endothelial cells.** Validation was performed by PCR of genomic DNA (A) and mRNA (B) by real-time PCR with amplicons visualized on agarose gels. C, ROSA26 Cre<sup>+/0</sup> aortas were positive for β-galactosidase activity (blue).

complete and intimal area remained significantly increased over saline-infused mice ( $P = 0.035$ ). Using the length of the outer aortic curvature as an aneurysm index ascending aortas of endothelial cell-specific AT<sub>1a</sub> receptor deficient mice elongated less than in the wild type group during Ang II infusion ( $P = 0.014$ ; Figure 6B). Ang II infusion did not promote a significant increase in elongation in Cre<sup>+/0</sup> mice, compared to saline infusion in this strain.



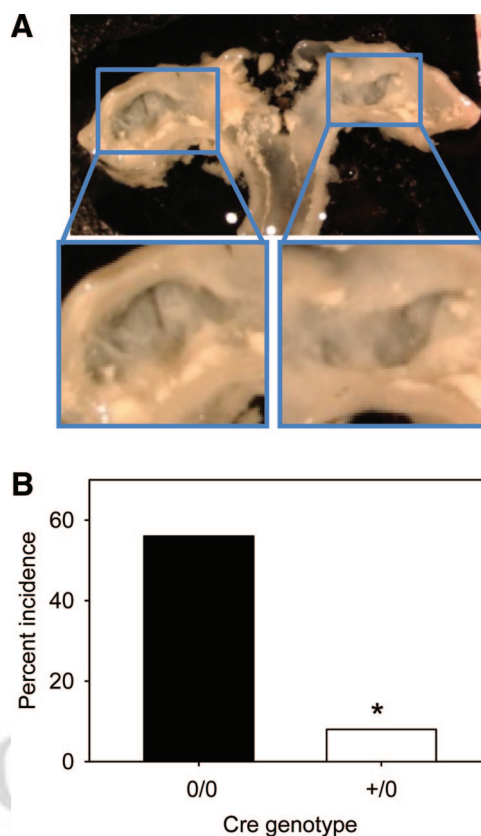
**Figure 6. Endothelial cell-specific deficiency of AT<sub>1a</sub> receptors attenuated Ang II-induced ascending AAs.** A, Intimal areas of ascending aortas. \* $P < 0.001$  comparing saline vs Ang II infusions in 0/0 genotype and comparing saline vs Ang II infusions within genotypes; † $P = 0.035$  comparing saline vs Ang II infusions within +/0 groups; ‡ $P = 0.001$  comparing 0/0 vs +/0 within Ang II infusions by 2-way ANOVA. B, Elongation measurement of outer curvature from aortic root to the brachiocephalic branch of the aorta. \* $P = 0.016$  comparing saline vs Ang II infusions within 0/0; † $P = 0.014$  comparing 0/0 vs +/0 within Ang II infusion.



**Figure 7. Depletion of AT<sub>1a</sub> receptors in endothelium reduced medial thickness and elastin breaks.** **A**, Representative photomicrograph of saline and Ang II infused AAs in Tek Cre<sup>0/0</sup> and Cre<sup>+ / 0</sup> mice stained with Movat's pentachrome. **B**, Medial thickness was measured from internal to external lamina. **Circles and triangles** represent data from individual mice, **diamonds** are means, and **bars** are SEMs. \* $P < 0.001$  when comparing saline vs Ang II infusion within 0/0; † $P = 0.003$  when comparing saline vs Ang II infusion within + / 0; ‡ $P = 0.01$  when comparing 0/0 vs + / 0 within Ang II infusion. **C**, Elastin breaks were counted in each section. **Circles and triangles** represent data from individual mice, **diamonds** are means, and **bars** are SEMs. \* $P < 0.001$  when comparing saline vs Ang II infusion within 0/0; † $P = 0.044$  when comparing saline vs Ang II infusion within + / 0; ‡ $P = 0.002$  when comparing 0/0 vs + / 0 within Ang II infusions.

Tissue sections from the ascending aorta were stained with Movat's pentachrome (Figure 7A). As described previously,<sup>6</sup> these sections confirmed the increased media thickness with a major expansion of intraelastin spaces being present in the adventitial aspect of the media. Ang II infusion into Cre<sup>0/0</sup> mice resulted in medial thickening with a blue-green stain that is indicative of glycosaminoglycan and proteoglycan deposition (Figure 7A and 7B). Tek-driven expression of Cre resulted in significant attenuation of Ang II-induced expansion of medial thickness ( $P = 0.01$ ; Figure 7B). Elastin breaks with a random occurrence were present throughout the ascending aortic media following Ang II-infusion. As with the medial thickness, this was significantly attenuated in the endothelial-specific Cre expressing mice relative to Cre<sup>0/0</sup> littermates ( $P = 0.002$ ; Figure 7C).

Many of AT<sub>1a</sub> receptor wild type also exhibited ulceration in ascending aortas (Figure 8A). Endothelial-specific AT<sub>1a</sub>



**Figure 8. Endothelial cell-specific deficiency of AT<sub>1a</sub> receptors reduced the incidence of ascending aortic ulcers.** **A**, Example of an ulcerated ascending aorta. **B**, Incidence of ulceration in ascending aortas of Ang II-infused groups. \* $P = 0.004$  by Fisher's exact test.

receptor deficiency led to a significantly reduced incidence of ulceration in ascending aortas ( $P = 0.004$ ; Figure 8B).

## Discussion

Numerous publications have demonstrated that Ang II infusion into mice leads to development of AAAs.<sup>16,17</sup> More recently, it has been observed that Ang II infusion into mice also leads to pronounced aortic dilation and dissections that are highly restricted to the ascending aortic region.<sup>6,18</sup> Although Ang II generates aortic aneurysms in both regions, the pathology of ascending AAs demonstrates many distinctions, including concentric dilation without local medial rupture and medial thickening that is most pronounced on the adventitial aspect.<sup>6,19</sup> This ascending aortic pathology that evolves during Ang II infusion bears many similarities to a model of Marfan syndrome in which mice express a mutant allele of fibrillin-1.<sup>5</sup>

In the present study, whole body deficiency of AT<sub>1a</sub> receptors ablated development of Ang II-induced ascending AAs. To define the cell type expressing AT<sub>1a</sub> receptors that contributed to development of ascending AAs, a combination of approaches was taken using bone marrow transplantation and cell-specific conditional deficiencies. These approaches studied 3 major cell types in Ang II-induced ascending AAs: leukocytes, SMCs, and endothelial cells. Using these approaches, we were unable to detect any effect of AT<sub>1a</sub> receptor deficiency in leukocytes or SMCs on development of

Ang II–induced ascending AAs. In contrast, depletion of AT<sub>1a</sub> receptors in endothelium attenuated development of Ang II–induced ascending AAs.

Many of the physiological and pathological effects of Ang II are mediated by stimulation of AT<sub>1</sub> receptors.<sup>20</sup> In rodents, chromosomal duplication has led to expression of 2 subtypes of AT<sub>1</sub> receptors, termed a and b.<sup>21</sup> Although AT<sub>1b</sub> receptor expression is more restricted compared to AT<sub>1a</sub> receptors, aortic tissue contains both subtypes with a predominance of the b subtype.<sup>22,23</sup> Only the b subtype is involved in Ang II–induced contractile activity that has been previously demonstrated to be restricted to the infrarenal region.<sup>24,25</sup> Despite the presence of functional AT<sub>1b</sub> receptors in aorta, AT<sub>1a</sub> receptors plays an essential role in Ang II–induced ascending AAs based on the ablation of the pathology in AT<sub>1a</sub> receptor deficient mice. It may be presumed that minor amino acid differences between AT<sub>1a</sub> and AT<sub>1b</sub> receptor subtypes lead to differential stimulation of signaling pathways that differentiate Ang II–induced contractile versus pathological processes.<sup>26</sup>

Leukocytes are prominent components in both Ang II–induced AAAs and ascending AAs.<sup>6,19</sup> Evaluation for a role of Ang II acting directly on leukocytes was performed in AAAs using bone marrow transplantation to create mice that are chimeric for AT<sub>1a</sub> receptors.<sup>8</sup> Although this approach did not distinguish the cells types that arise from the bone marrow, macrophages are the most dominant cell type from this origin in Ang II–induced AAAs.<sup>18,19,27</sup> Similarly, macrophages are the most abundant leukocytes in Ang II–induced ascending AAs. Repopulation of irradiated mice demonstrated no effect of AT<sub>1a</sub> receptors on donor cells to development of Ang II–induced AAAs.<sup>8</sup> The present study also failed to demonstrate a role for AT<sub>1a</sub> receptors on donor cells in promoting Ang II–induced ascending AAs. Furthermore, whereas functional AT<sub>1</sub> receptors have been identified on mouse macrophages,<sup>28</sup> we were unable to demonstrate a role for AT<sub>1a</sub> receptors on this cell type in directly influencing development of ascending AAs.

Ang II is known to exert region-specific effects on aortic SMCs in physiological and pathological responses.<sup>14,29</sup> We have demonstrated previously that the mechanism of Ang II–induced SMC-rich medial thickening *in vivo* differs in the ascending aorta relative to all other regions.<sup>15</sup> This difference may be a consequence of the different developmental origin of SMCs throughout the aortic tree.<sup>30</sup> In the present study, we initially sought to demonstrate whether any regional differences in SMC responses to Ang II can be detected *ex vivo*. Previous studies have demonstrated that contractile responses to Ang II differ between aortic regions, with minimal contraction in the descending thoracic aorta compared to the infrarenal region.<sup>14,25</sup> Responses may differ in the ascending aorta because SMCs in this region have a different developmental origin from the descending aorta, with the potential for different functional characteristics.<sup>31,32</sup> However, similar to the descending aorta, we were also unable to detect any Ang II–induced SMC contraction in rings isolated from the ascending aorta, despite this region contracting to KCl and 5-HT. Therefore, Ang II does not have demonstrable effects on a physiological function of SMCs in this aortic area.

To directly determine the role of Ang II stimulation of SMCs in the pathological process of ascending AAs development, we generated AT<sub>1a</sub> receptor floxed mice to develop cell-specific deficient mice. The floxed sites were located either side of exon 3 that is responsible for the entire translatable portion of the protein. AT<sub>1a</sub> receptor deficiency on SMCs was created by breeding mice that were hemizygous for Cre driven by the SM22 promoter.<sup>33</sup> This Cre expression strategy has been used previously to determine the role of SMCs in experimental AAAs, but has not been used to determine ascending AAs.<sup>33–35</sup> The normal mouse aorta not only has different developmental origins of SMCs, but the media may also contain other cell types, such as fibroblasts and myofibroblasts.<sup>36</sup> Despite this potential heterogeneity, studies in which SM22 driven Cre was expressed in ROSA26 mice demonstrated the uniformity of gene depletion across the media that coincided with  $\alpha$ -actin immunostaining. Although we were unable to validate spatial distribution of AT<sub>1a</sub> receptor protein expression, the combined evidence from the SM22-Cre-ROSA26 mice and the absence of AT<sub>1a</sub> receptor mRNA in aortic SMCs is consistent with an effective depletion. In mice with this depletion, we were unable to detect any difference in Ang II–induced ascending AAs. This is consistent with the lack of direct effects of Ang II on SMCs to initiate and propagate AAs.

Endothelial function has a profound effect on vascular physiology and pathology and endothelial cells express angiotensin II receptors.<sup>37</sup> To determine a role of Ang II stimulation on endothelial cells in the development of Ang II–induced ascending AAs, we bred AT<sub>1a</sub> receptor floxed mice to those expressing Cre under the control of the Tek promoter. Use of the Tek promoter has the potential complication of deleting genes in cells of myeloid origin, in addition to endothelial. Given that myeloid and endothelial cells are derived from a common precursor cell of the hemangioblast, any genes expressed at this stage of development would have a broader spectrum of effects on these different cell types.<sup>38</sup> However, because we were unable to demonstrate any effect of AT<sub>1a</sub> receptor expression on bone marrow–derived cells, the attenuation of Ang II–induced ascending AAs in Tek-driven Cre mice was not confounded by deletion of the receptor in myeloid cells.

Although deficiency of AT<sub>1a</sub> receptors in endothelium attenuated the development of Ang II–induced ascending AAs, the effect was not as profound as the complete ablation of disease in mice with whole body AT<sub>1a</sub> receptor deficiency. In contrast, deficiency of AT<sub>1a</sub> receptors in SMCs had no discernible effect on the development of aneurysms. Furthermore, bone marrow transplantation studies failed to reveal any effect of leukocyte AT<sub>1a</sub> receptor deficiency on aneurysm development. Therefore, of the 3 major cell types present in aneurysmal tissue, only depletion of AT<sub>1a</sub> receptors in the endothelium reduced aneurysmal disease, but not as dramatically as whole body deficiency. One potential explanation is a technical shortcoming that the activity of Cre was not sufficient to delete the floxed exon 3. It would have been preferable to demonstrate deletion by determination of changes in AT<sub>1a</sub> receptor protein. Unfortunately, we were unable to validate that commercially available antibodies authentically stained the AT<sub>1a</sub> receptor protein. We were also

unable to develop antibodies that specifically recognize this protein. Therefore, the index of deletion was dependent on measurement of mRNA abundance. In agreement with previous studies using Cre under the control of SM22 Cre,<sup>33,34,39</sup> we demonstrated a highly effective deletion of the floxed components of the gene. Given the inability to isolate mRNA from the endothelial cells harvested from the ascending aorta, we performed this analysis on endothelial cells isolated from lung. Findings in these cells were directly complemented by studies using Tek expressing mice in the ROSA26 background. Analyses of sections from ascending aortas of these mice demonstrated a uniform expression of  $\beta$ -galactosidase in the endothelium. Therefore, expression of Cre in either endothelial or smooth muscle selective manner appears to have been effective in removing the exon that translates the entire functional protein.

Another explanation of the different extent of whole body versus cell-specific AT<sub>1a</sub> receptor deficiency on ascending AAs may be the involvement of other cell types. There is emerging evidence for a role of fibroblasts and myofibroblasts in the development of experimental aneurysms by exposure to calcium chloride or Ang II infusion.<sup>18,36,40</sup> These cell types have been detected in both the media and adventitia of mice. Future studies in mice expressing Cre under a fibroblast-specific promoter will provide insight into the role of this cell type.

Depletion of AT<sub>1a</sub> receptors in endothelial cells reduced the development of Ang II-induced ascending AAs to a similar extent to that previously described in mice deficient in CCR2, the cognate receptor for MCP-1. With both deficiencies, there was a decrease in lumen area and medial thickness. Ang II can stimulate the release of MCP-1 from endothelial cells.<sup>41</sup> Therefore, it is interesting to speculate that Ang II stimulation of endothelial AT<sub>1a</sub> receptor promotes MCP-1 release that permeates the media because of blood pressure. This could promote leukocyte recruitment via the adventitial aspect of the aorta with the subsequent elaboration of elastolytic enzymes. The recent availability of MCP-1 floxed mice will permit the testing of this hypothesis.<sup>42</sup>

In summary, we demonstrate that endothelial-specific deficiency of AT<sub>1a</sub> receptors markedly attenuates the development of Ang II-induced AAs. Future studies will define the nature of the mediator released from the endothelium that promotes this localized disease.

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### Disclosures

None.

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## Novelty and Significance

### What Is Known?

- Ang II infusion promotes profound aortic dilation that is restricted to the ascending portion.
- AT<sub>1b</sub> receptors have functional effects in aortic tissue.

### What New Information Does This Article Contribute?

- Whole body deficiency of AT<sub>1a</sub> receptors ablates the development of ascending AAs.
- Depletion of AT<sub>1a</sub> receptors on bone marrow-derived cells or SMCs has no effect on Ang II-induced ascending AAs.
- Depletion of AT<sub>1a</sub> receptors on endothelial cells markedly attenuates ascending aortic dilation.

Studies in humans have inferred a role of Ang II in aneurysms localized to the ascending aorta. A mouse model of Marfan syndrome has recently been shown to be responsive to admin-

istration of the AT<sub>1</sub> receptor antagonist losartan. Furthermore, we have demonstrated recently that infusion of Ang II promotes aneurysms that are highly localized to the ascending portion of the aorta. However, there is no information on the specific angiotensin receptor that mediates the aortic dilation or the cellular localization of the responsive receptor. To provide this information, we performed studies using mice with whole body AT<sub>1a</sub> receptor deficiency, bone marrow transplantation to generate mice with leukocyte-specific AT<sub>1a</sub> receptor deficiencies, and newly developed floxed mice to generate SMC or endothelial-specific AT<sub>1a</sub> receptor deficient mice. Using these mice, we determined the requirement of AT<sub>1a</sub> receptor stimulation for generation of ascending AAs. Studies in mice with cell-specific deficiencies demonstrated a major contribution of AT<sub>1a</sub> receptor stimulation in the endothelium. These studies will provide a basis for determining how endothelial cells promote this disease.

**SUPPLEMENTAL MATERIAL**

**Endothelial Cell-specific Deficiency of AngII Type 1a Receptors Attenuates  
AngII-Induced Ascending Aortic Aneurysms in LDL Receptor  $-/-$  Mice**

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**Short title:** Endothelial AT1a receptor deficiency decreases aneurysms

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## METHODS AND MATERIALS

### Mice

The following mice were purchased from the Jackson Laboratory: LDL receptor -/- (B6.129S7-Ldlr<sup>tm1Her</sup>/J, C57BL/6 N10, Stock #002207); AT1a receptor -/- (B6.129P2-Agtr1a<sup>tm1Unc</sup>/J, C57BL/6 N10, Stock #002682); SM22-Cre (Stock Tg(Tagln-cre)1Her/J, N = unknown, Stock #004746); Tek-Cre [B6.Cg-Tg(Tek-Cre)12Flv/J, C57BL/6 N10, Stock # 004128], and ROSA26 [B6.129S4-Gt(ROSA)26Sor<sup>tm1Sor</sup>/J, N5, Stock #003474]. AT1a receptor floxed (f/f) mice were generated directly in ES cells of C57BL/6 mice by InGenious Targeting Laboratory. The construct placed lox sites on either side of exon 3 that contains the entire translatable region for AT1a receptors (Online Figure I).<sup>1,2</sup>

AT1a receptor -/- male mice were bred to LDL receptor -/- females to generate heterozygous progeny. The F2 progeny were screened for developing breeding pairs of AT1a receptor +/+ or -/- x LDL receptor -/- mice.

For Cre expression studies, ROSA26 females were bred to SM22-Cre and Tek-Cre male mice.

Male SM22-Cre mice were subsequently bred to female LDL receptor -/- mice for several generations. Male SM22-Cre +/0 x LDL receptor -/- mice C57BL/6 N10 were identified by speed congenic screening (Jackson Laboratory). Male Tek-Cre mice were bred to female LDL receptor -/- mice to generate Tek-cre +/0 mice in an LDL receptor -/- background. To develop cell-specific deficiency of AT1a receptors, female AT1a receptor homozygous (f/f) mice were bred to male AT1a receptor f/f mice that were hemizygous (+/0) to either SM22-Cre or Tek-Cre, in an LDL receptor -/- background. Final breeding pairs were male AT1a receptor f/f Cre +/0 bred to female AT1a receptor f/f x Cre 0/0. Both Cre 0/0 and +/0 littermates were used for the experiments.

All animal procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

### Genotyping

Primers used for genotyping the mice are detailed in Online Table I. DNA was isolated from tail and liver. For aortic smooth muscle cells and endothelial cells, tissues were dissected. Aortas were digested in collagenase and adventitia was removed. The aorta was cut open and the endothelial cells were removed. Medial smooth muscle cell DNA was isolated. For endothelial cells, lung tissue was digested with collagenase and endothelial cells were isolated from the mixture using CD31-magnetic beads. All DNA was isolated using a Dneasy Kit (Qiagen). IL-2 primers were used as an internal control for the Cre PCR.

### Aortic SMC RNA isolation

Aortas from AT1a receptor f/f mice that were either deficient (0/0) or hemizygous (+/0) for Cre under the control of the SM22 promoter were excised immediately. After grossly visible adventitial tissue was removed, aortas were incubated with collagenase type I (1 mg/ml, Worthington; Cat #: LS004194) in Dulbecco's Modified Eagle Medium (DMEM) for 20 minutes at 37°C. This process permitted adventitia to be efficiently stripped off using sterile forceps. Aortas were cut open longitudinally and endothelia were removed by gently rubbing of intimal surfaces using a sterile DMEM-soaked cotton swabs. RNA was extracted from aortic SMCs using RNeasy fibrous tissue mini kits (Qiagen).

### Endothelial cell RNA isolation

Lungs from AT1a receptor f/f mice that were either deficient (0/0) or hemizygous (+/0) for Cre under the control of the Tek promoter were dissected, minced, and incubated with collagenase type I (2 mg/ml) at 37°C for 45 minutes. Digested lung tissues were passed through cell strainers (70 µm) and centrifuged (400 g for 8 minutes at 4°C). Cells were resuspended in cold PBS (2 ml) without Mg<sup>2+</sup>/Ca<sup>2+</sup> with 0.1% BSA (0.1%wt/vol), containing rat anti-mouse CD31 IgG linked to sheep anti-rat IgG coated Dynabeads (30 µl). After incubation for 10 minutes at room temperature, a magnet was used to separate endothelial cells. Selected cells were washed with PBS and resuspended in endothelial cell growth medium. Cells were plated and cultured for 3 days. Alexa fluor-488 labeled acetylated LDL (AcLDL 10 µg/ml, Molecular probe; Cat # L-23380) was incubated with cells for 4 hours at 37°C. Cells were then trypsinized, washed, and resuspended in PBS containing FBS (1% FBS wt/vol). Fluorescence-activated cell sorting (Aria II, Becton Dickinson) was performed to sort cells positive for Alexa Fluor-488 AcLDL. RNA was isolated from positive cells using an SV Total RNA kit (Promega) following the manufacturer's instructions.

#### **Detection of AT1a receptor mRNA by Real time PCR**

RNA from aortic SMCs or lung endothelial cells was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, cat # 170-8891). Reverse transcriptase (RT)-PCR reactions were performed as described previously.<sup>3</sup> Real time PCR was performed using Eva-green reagent in a CFX96 cyler (Bio-Rad). mRNA abundance was compared to β-actin. Primer sequences are listed in Online Table II. The primer pair for AT1a receptor mRNA failed to generate a detectable amplicon in RNA isolated from AT1a receptor deficient mice, indicating no cross reaction with AT1b receptor mRNA. Amplicon sizes for AT1a receptors and β-actin were 238 and 220 bp, respectively. Samples containing either no template or no RT reactions were used as negative controls.

#### **Detection of β-Galactosidase activity in tissues**

Frozen tissues were sectioned (10 µm thickness). Sections were fixed in formalin for 1 hour, then rinsed three times with buffer (100 mM sodium phosphate, pH7.3; 2mM MgCl<sub>2</sub>; 0.01% sodium deoxycholate; 0.02% NP-40). Sections were then incubated 4 hours to overnight in X-Gal staining buffer (above rinse buffer with additions of 5mM potassium ferricyanide; 5 mM potassium ferrocyanide; 1 mg/ml X-gal).

#### **Detection of AT1a receptor protein**

To verify the deletion of the AT1a receptor in floxed mice, we have acquired and developed several antibodies.

We have used the following commercially available AT1 receptor antibodies: Santa Cruz: sc-1173 (N-terminal extracellular domain; residues #1-51); sc-579 (residues #306-359); and sc-31181 (C-terminal cytoplasmic domain; residues #300-350). Abcam: ab18801 (residues #341-355).

In addition to these commercially available antibodies, we have also developed several chicken IgYs against peptides from sequences of mouse AT1 receptor subtypes. The following peptides were synthesized from several regions of the mouse AT1a and AT1b receptors. Since both are expressed in the aorta, a useable antibody would need to be able to discriminate between these 2 subtypes. The selection of peptides was based on predicted immunogenicity based on an Immunogenicity Algorithm model. Peptide selection was also based on regions in which sequences

differed between the two subtypes. The following peptides were synthesized.

AT1aR (residues #1-18):	CZMALNSSTEDGIKRIQDDC
AT1aR (residues #173-190)	ENTNITVCAFHYESRNST.
AT1aR (residues #223-237):	CZKKAYEIQKNKPRNDD
AT1aR (residues #342-355):	SDNMSSAAKKPASC
AT1bR (residues #223-237):	CZKKAYKIQKNTPRNDD
AT1bR (residues #342-355):	SDNMSSSARKSAYC

Peptides were conjugated to KLH and injected into chickens (Aves Lab, Oregon). IgYs were purified by affinity column chromatography. Unfractionated IgYs were incubated on Sephadex columns containing beads conjugated with the immunizing peptide. Studies of specificity were performed by comparing any signal obtained by the affinity purified antibody to both pre-immune IgY and the fraction that was not retained by affinity columns. All antibodies had strong reactivity against the immunizing peptide.

For all these antibodies, we were not able to demonstrate any specific interaction to the AT1a or AT1b receptors by either Western blotting or immunostaining of tissue sections.

### **Diet and AngII infusion**

Male mice (8 -12 weeks) were fed a saturated fat enriched diet (milk fat, 21% wt/wt, cholesterol 0.2% wt/wt; Harlan Teklad, catalog #TD.88137) ad libitum started one week prior to pump implantation that was continued throughout 28 day pump infusion. After 1 week of feeding, mini-osmotic pumps (Durect Corp; Model 2004) containing saline or AngII (1,000 ng/kg/min; Sigma or Bachem) were implanted subcutaneously on the right flank of the mice.

### **Systolic blood pressure measurements**

One week prior to pump implantation and during the last week of osmotic mini-pump infusion, SBP was measured using a non-invasive tail cuff method (Kent Scientific or Visitech) as described previously.<sup>4</sup> Briefly, mice were measured on a heated platform the same time each day for 5 consecutive days. Twenty measurements per mouse per day were taken. Only mice that measured 50% (10 measurements) with a SD < 30 mmHg were accepted.

### **Total serum cholesterol and MCP-1 concentration measurements**

Mice were overdosed with anesthesia (ketamine/xylazine; 90 and 10 mg/kg respectively). Blood was drawn by ventricular puncture. Serum was separated by centrifugation and cholesterol was measured enzymatically (Wako Chemicals; Cholesterol E; Cat #439-17501). MCP-1 concentration was measured by ELISA (R&D Systems; Mouse JE/MCP-1 ELISA Kit; Cat #SMJE00).

### **Bone marrow transplantation**

Male mice (8 weeks old) were given antibiotic water (Sulfa-Trim) for one week prior to and 4 weeks after irradiation.<sup>5,6</sup> Bone marrow was flushed from femurs of donor mice (1 donor mouse for 5 recipient mice). Cells were counted and diluted in DMEM + 2% FBS for a final concentration of  $1 \times 10^6$  cells/100  $\mu$ l. Recipient mice were irradiated with 2 doses of 450 rads, 3 hours apart. Donor bone marrow cells ( $1 \times 10^6$  cells for each) were injected into tail veins of recipients after the second irradiation. AngII infusion was performed 5 weeks after the donor cell transfer. This interval enables repopulation of blood-borne leukocytes with donor cells.<sup>7</sup> At experimental termination, bone marrow from the recipients was harvested, DNA was isolated, and chimerism was

confirmed by PCR for gene of interest (Online Figure II).

### **Aortic contractility studies**

Mice were anesthetized with ketamine/xylene. Aortas from 8 male C57BL/6 mice were perfused with phosphate-buffered saline (PBS) via the left ventricle and then removed. Adventitia was carefully dissected free. Measurement of contractile activity was performed using aortic rings as described previously.<sup>8</sup> Ascending (3 mm), middle thoracic (4 mm), and infrarenal (4 mm) aortic segments were mounted by passing two tungsten wires through the arterial lumen while immersed in Krebs Henseleit solution. Tension (1 gm) was maintained continuously and recorded on a Micro-Med instrument. After 30 minutes for equilibration, tissues were incubated for 5 minutes with potassium chloride (80 mM, Sigma), 5-HT (1  $\mu$ M, Sigma), and AngII (1  $\mu$ M, Sigma) with 30 minutes of recovery between each agonist.

### **Measurements and pathohistology of ascending aortic aneurysms**

After exsanguination, saline was perfused through the left ventricle of the heart. Aortas were removed from heart to iliac bifurcation, and placed in formalin (10% wt/vol) overnight. Adventitia was cleaned from the aortas. Aortas were cut open longitudinally from the arch inner curvature to the iliac bifurcation, and also from the outer curvature to the subclavian branch. Aortas were pinned and photographed using a Nikon Digital Camera (DXM1200). Intimal areas of ascending aortas were measured from the ascending aorta to 3 mm distal from the subclavian branch using Image-Pro Plus software (Media Cybernetics). Elongation was defined by length of the aortic outer curvature from the emergence of the aorta to the branch of the brachiocephalic artery.

The presence of ulceration in the ascending aortic regions was defined by observers who were blinded to the experimental design. An example of an ascending aortic ulceration is provided in Figure 8.

Ascending aortas were placed in OCT and sectioned in sets of 10 slides serially with 9 sections/slide by a cryostat. One from the 10 slides was stained with Movat's pentachrome (Poly Scientific). Measurements were performed to determine the medial thickness and the frequency of elastin breaks. To quantify medial thickness, every section from each slide was measured perpendicular from the internal to external elastic lamina. For frequency of elastin breaks, these were counted by two observers who were blinded to the identity of the sections.

### **Statistics**

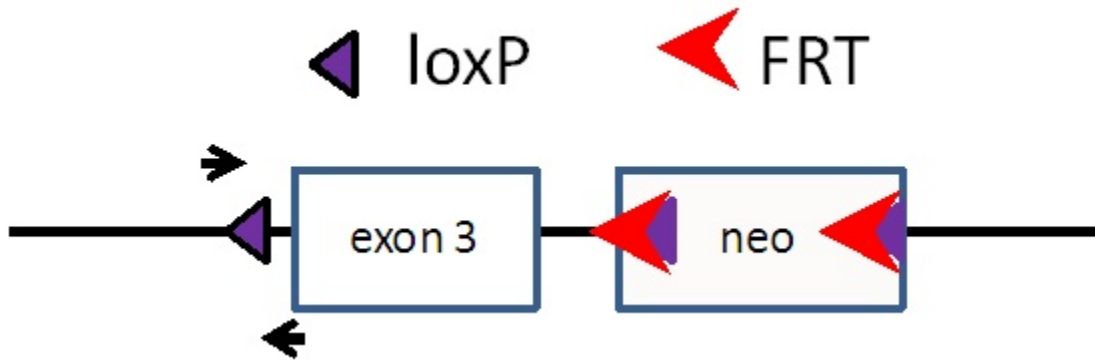
Data were analyzed by Student's t test for 2 group comparisons that conformed to the constraints of parametric analysis. Two group comparisons of non parametric data were analyzed by Mann Whitney test. Percent incidence was analyzed by Fisher Exact test. Multiple group analyses were performed using ANOVA with Holm Sidak for post hoc analysis. All data are represented as means  $\pm$  SEM.  $P < 0.05$  was considered statistically significant.

**Table I.** Primers for genotyping of mice.

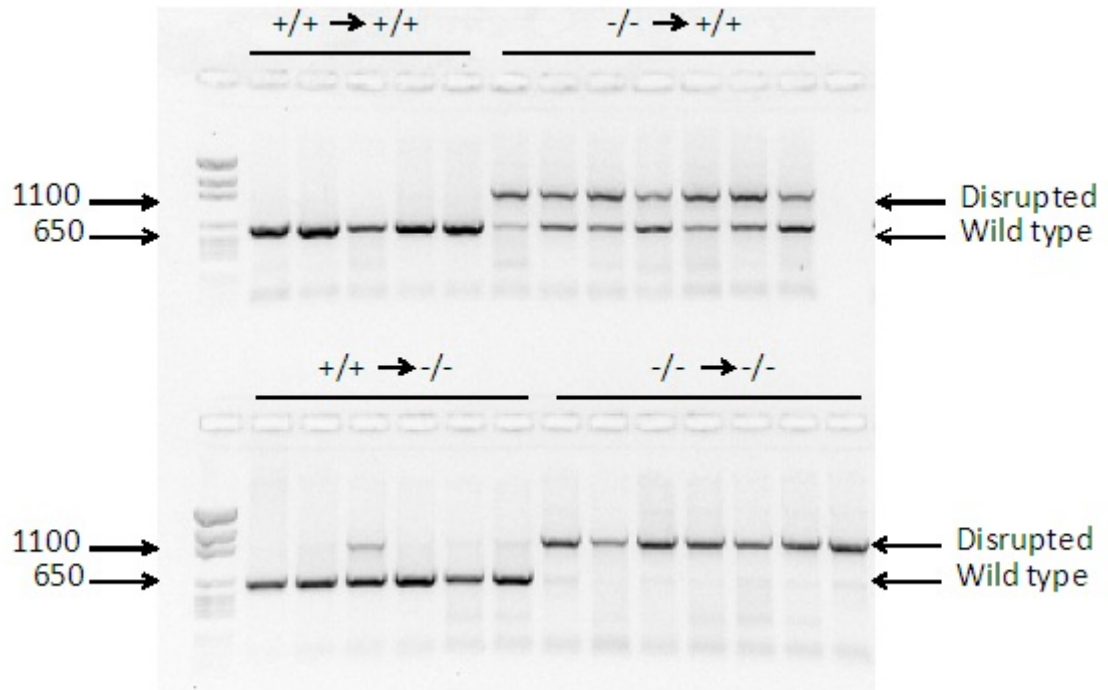
<b>Gene</b>	<b>Primers for genomic PCR</b>	<b>Product size (bp)</b>
AT1a receptor	5'-AAATGGCCCTTAACTCTTCTACTG 5'-ATTAGGAAAGGGAACAGGAAGC	WT = 650 Disrupted = 1100
AT1a receptor flox	5'-TGTTGCATCTACATCCTG 5'-TCTAAAGAAACCTCATGAAC	WT = 202 flox = 262
Cre	5'-ACCTGAAGATGTTTCGCGATT 5'-CGGCATCAACGTTTTCTTTT	WT = none Cre+ = 182
IL-2	5'-CTAGGCCACAGAATTGAAAGATCT 5'-AGTAGGTGGAAATTCTAGCATCATCC	IL2 = 324
LDL receptor	5'-AGGTGAGATGACAGGAGATC 5'-AGGATGACTTCCGATGCCAG 5'-GCAGTGCTCCTCATCTGACTTG	WT = 383 Disrupted = 800

**Table II.** Primers for reverse transcriptase (RT)-PCR and real time PCR.

<b>Gene</b>	<b>Primers for RT-PCR and real time PCR</b>	<b>Product size (bp)</b>
AT1a receptor	5'-ACTCACAGCAACCCTCCAAG 5'-ATCACCACCAAGCTGTTTCC	238
$\beta$ -actin	5'-CGTGGGCCGCCCTAGGCAACCA 5'-TTGGCCTTAGGGTTCAGGGGGG	220



**Figure I.** Construct map of floxed exon 3 of the AT1a receptor. Black arrows indicate the location of the primers used to genotype for the presence of the 1<sup>st</sup> flox site.



**Figure II.** Genotyping of bone marrow from chimeric mice after the termination. Both AT1a receptor  $+/+$  and  $-/-$  recipient mice were irradiated and repopulated with cells harvested from bone marrow of donor AT1a receptor  $+/+$  and  $-/-$  mice, as indicated on the top of each gel. Amplicons for the wild type allele are 650 bp and the disrupted allele are 1100 bp.

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