Circulation
Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart
Association®
Learn and Live℠

Endothelial Cell Specific Deficiency of Ang II Type 1a Receptors Attenuates Ang II Induced Ascending Aortic Aneurysms in LDL Receptor / 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 and Alan Daugherty

Circ. Res. published online Jan 20, 2011;
DOI: 10.1161/CIRCRESAHA.110.222844
Circulation Research is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
Copyright © 2011 American Heart Association. All rights reserved. Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org

Data Supplement (unedited) at:
http://circres.ahajournals.org/cgi/content/full/CIRCRESAHA.110.222844/DC1

Subscriptions: Information about subscribing to Circulation Research is online at
http://circres.ahajournals.org/subscriptions/

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail:
journalpermissions@lww.com

Reprints: Information about reprints can be found online at
http://www.lww.com/reprints

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

Endothelial Cell–Specific Deficiency of Ang II Type 1a Receptors Attenuates Ang II–Induced Ascending Aortic Aneurysms in LDL Receptor$^{-/-}$ 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_{1a}) receptors and their cellular location in Ang II–induced ascending aortic aneurysms (AAs).**

Methods and Results: **Male LDL receptor$^{-/-}$ 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_{1a} receptor deficiency ablated Ang II–induced ascending AAs ($P<0.001$). To determine the role of AT_{1a} receptors on leukocytes, LDL receptor$^{-/-}$×AT_{1a} receptor$^{+/+}$ or AT_{1a} receptor$^{-/-}$ mice were irradiated and repopulated with bone marrow–derived cells isolated from either AT_{1a} receptor$^{+/+}$ or AT_{1a} receptor$^{-/-}$ mice. Deficiency of AT_{1a} receptors in bone marrow–derived cells had no effect on Ang II–induced ascending AAs. To determine the role of AT_{1a} receptors on vascular wall cells, we developed AT_{1a} receptor floxed mice with depletion on either smooth muscle or endothelial cells using Cre driven by either SM22 or Tek, respectively. AT_{1a} 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_{1a} receptors that are expressed on endothelial cells. (*Circ Res*. 2011;108:00-00.)**

Key Words: ascending aneurysm ■ angiotensin II ■ AT_{1a} receptor ■ Tek-cre

Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION
FIRST PROOF ONLY

Ascending aortic aneurysm (AA) is an asymptomatic expansion of this restricted region in which rupture has catastrophic consequences.[1] 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.[2]

Recent human and experimental studies have inferred a role for angiotensin II (Ang II) in the development of ascending AAs.[3–6] 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.[4] The aortic dilation that is localized to the ascending region is prevented by the administration of the AT_1 receptor antagonist, losartan.[5] Retrospective analysis of pharmacological treatments given to individuals with Marfan syndrome demonstrated that administration of losartan attenuated dilation of the ascending aorta.[3] Prospective evaluations of angiotensin receptor antagonists are currently being performed in populations afflicted with Marfan syndrome.[7]

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_1 receptors in most species, and the AT_{1a} 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.[3] 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

Original received May 7, 2010; revision received January 5, 2011; accepted January 11, 2011. In December 2010, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 14.5 days.

From the Saha Cardiovascular Research Center (D.L.R., J.J.M., A.B., A.P.O., D.A.H., V.S., A.P., A.D.), Graduate Center for Toxicology (A.P.O., LA.C., A.D.), Department of Biostatistics (R.C.), and Graduate Center for Nutritional Sciences (L.A.C., A.D.), University of Kentucky, Lexington.

This manuscript was sent to Kathy Griendling, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

Correspondence to Alan Daugherty, Saha Cardiovascular Research Center, BBSRB, Room 243, University of Kentucky, Lexington, KY 40536-0509. E-mail Alan.Daugherty@uky.edu

© 2011 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org DOI: 10.1161/CIRCRESAHA.110.222844

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

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

aspect of ascending aortas. Using this model as an experimental paradigm of AAs, we determined the role of AT_{1a} 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$^{-/-}$, AT_{1a} receptor$^{-/-}$, SM22-Cre, Tek-Cre, and ROSA26 mice were purchased from The Jackson Laboratory. AT_{1a} 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.[8]

Genotyping

Genotypes of mice were confirmed by PCR of genomic DNA.

Detection of AT_{1a} 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_{1a} receptor mRNA were used in real time PCR, with confirmation of amplicons by visualized detected on agarose gels.

Detection of β-Galactosidase Activity in Tissues

Aortas were removed and β-galactosidase activity was detected by X-gal.

Detection of AT_{1a} 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.[9]

Serum Measurements

Total serum cholesterol concentrations were determined as described previously.[10] 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.[8,11]

Aortic Contractility Studies

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

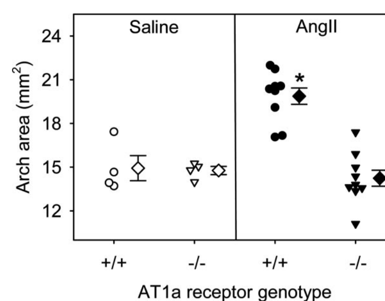

Figure 1. Whole body deficiency of AT_{1a} receptors ablated Ang II–induced ascending AAs. Circles and triangles represent data from individual mice, **diamonds** are means, and **bars** are SEMs. *$P<0.001$, when comparing Ang II vs saline infusion within +/+ group and when comparing +/+ vs −/− genotype 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.[12] 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±SEM. $P<0.05$ was considered statistically significant.

Results

Whole Body Deficiency of AT_{1a} Receptors Ablated Ascending AAs

We first defined the effects of whole body AT_{1a} receptor deficiency on Ang II–induced ascending AA formation in LDL receptor$^{-/-}$ 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_{1a} receptor$^{+/+}$ and AT_{1a} receptor$^{-/-}$ mice infused with saline. Ang II infusion significantly increased the ascending aortic area in AT_{1a} receptor$^{+/+}$ mice ($P<0.001$). In contrast, there was no significant increase in this region of AT_{1a} receptor deficient mice infused with Ang II (Figure 1).

AT_{1a} Receptor Deficiency in Bone Marrow–Derived Cells Did Not Influence Development of Ascending AAs

Following the demonstration that whole body deficiency of AT_{1a} 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$^{-/-}$ mice that were either AT_{1a} receptor$^{+/+}$ or AT_{1a} receptor$^{-/-}$ were irradiated and repopulated with bone marrow–derived cells harvested from AT_{1a} receptor$^{+/+}$ or AT_{1a} receptor$^{-/-}$ donors. The development of ascending AAs was determined by the AT_{1a} receptor genotype of recipients. Ang II infused into AT_{1a} receptor$^{+/+}$ recipients significantly increased arch

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

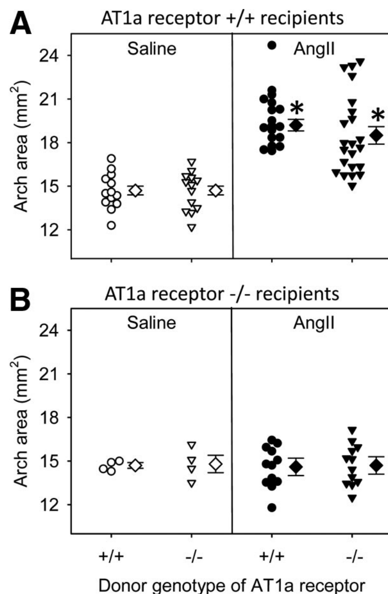

Figure 2. Deficiency of AT_{1a} receptors in bone marrow–derived cells did not affect Ang II–induced ascending AAs. AT_{1a} receptor$^{+/+}$ **(A)** or AT_{1a} receptor$^{-/-}$ **(B)** recipients were irradiated and repopulated with bone marrow–derived cells harvested from either AT_{1a} receptor$^{+/+}$ or AT_{1a} receptor$^{-/-}$ 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_{1a} receptor$^{-/-}$ recipients did not develop ascending AAs (Figure 2B). No significant effects were observed for donor genotype, irrespective of recipient AT_{1a} receptor genotype.

Ang II Failed to Contract SMCs in the Ascending Aorta

Because we failed to detect an effect of leukocytic AT_{1a} 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.[3] 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.[13] 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.[14] These studies failed to provide any evidence of differential contractile responses to Ang II in SMCs of ascending and descending aortas.

AT_{1a} Receptor Deficiency on SMCs Had No Effect on Development of Ascending AAs

To determine whether Ang II interacted directly with AT_{1a} 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

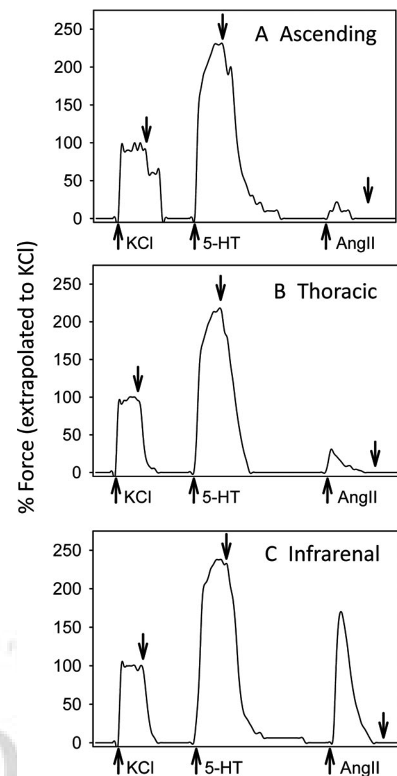

Figure 3. Ang II–induced aortic contractions were restricted to the infrarenal region. Regional contractility of rings from the ascending **(A)**, thoracic **(B)**, and infrarenal aorta **(C)**. Aortic rings were contracted during 5-minute incubations with potassium chloride (KCl) (80 mmol/L), 5-HT (1 μmol/L), and Ang II (1 μmol/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_{1a} receptor gene was confirmed by PCR using genomic DNA from aortas (Figure 4A). Depletion of AT_{1a} receptor mRNA in SMCs was confirmed by RT-PCR (Figure 4B). As with whole body AT_{1a} receptor deficiency,[15] the deletion of AT_{1a} receptors in SMC had no discernable effect of aortic structure.

We attempted to determine the depletion of AT_{1a} 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_{1a} 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 β-galactosidase, but this enzyme is present in cells that expressed Cre at some point during lineage development. These studies demonstrated uniform β-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_{1a} receptors in SMCs of LDL receptor$^{-/-}$ 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

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

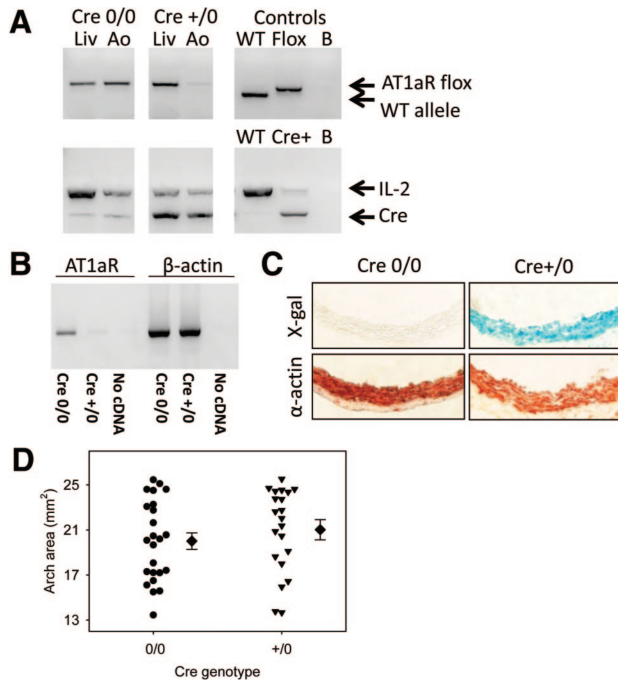

Figure 4. SMC deficiency of AT_{1a} receptor had no effect on Ang II–induced ascending AAs. Validation of AT_{1a} receptor depletion in aortic smooth muscle cells (Ao) by PCR detection of genomic DNA (compared to liver [Liv]) **(A)** and mRNA **(B)**. **C,** ROSA26 Cre+/0 aortas were positive for *β*-galactosidase activity **(blue)** in the same regions as positivity for *α*-actin immunostaining. **D,** Deficiency of AT_{1a} 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_{1a} receptor deficiency had no effect on Ang II–induced ascending AAs relative to littermate controls.

AT_{1a} Receptor Deficiency on Endothelial Cells Attenuated Development of Ascending AAs

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

Depletion of AT_{1a} 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

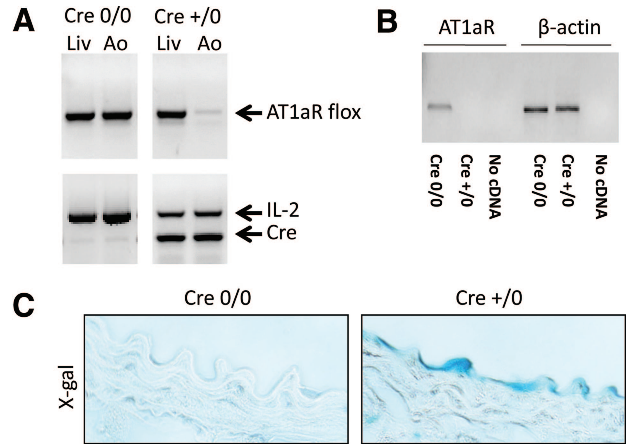

Figure 5. Validation of AT_{1a} receptor depletion in endothelial cells. Validation was performed by PCR of genomic DNA **(A)** and mRNA **(B)** by real-time PCR with amplicons visualized on agarose gels. **C,** ROSA26 $Cre^{+/0}$ aortas were positive for *β*-galactosidase activity **(blue)**.

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_{1a} 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 $Cre^{+/0}$ mice, compared to saline infusion in this strain.

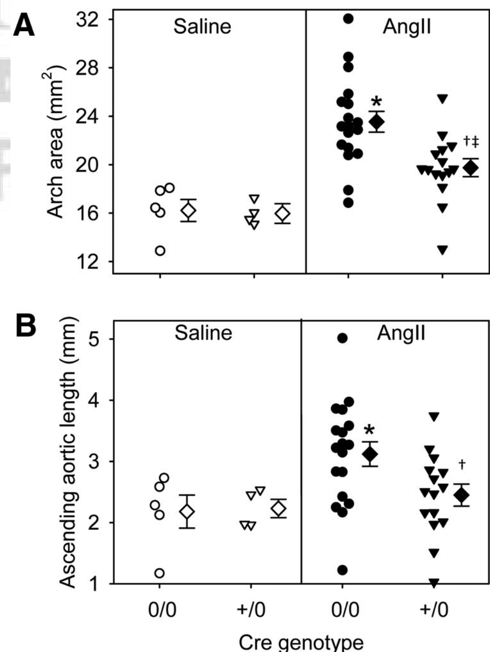

Figure 6. Endothelial cell–specific deficiency of AT_{1a} receptors attenuated Ang II–induced ascending AAs. A, Intimal areas of ascending aortas. *$P<0.001$ comparing saline vs Ang II infusions in 0/0 genotype and comparing saline vs Ang II infusions within genotypes; †$P=0.035$ comparing saline vs 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.

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

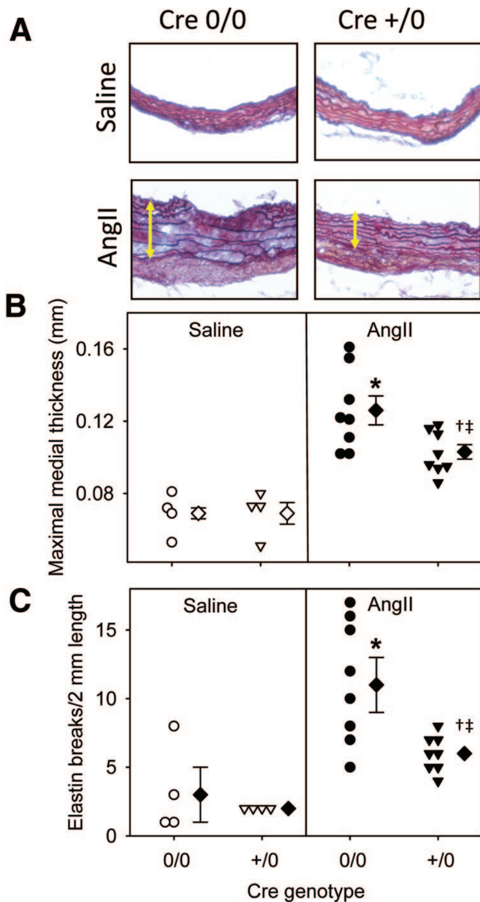

Figure 7. Depletion of AT_{1a} receptors in endothelium reduced medial thickness and elastin breaks. A, Representative photomicrograph of saline and Ang II infused AAs in Tek $Cre^{0/0}$ and $Cre^{+/0}$ mice stained with Movat's pentachrome. **B,** Medial thickness was measured from internal to external 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,[6] 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 $Cre^{0/0}$ 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 $Cre^{0/0}$ littermates ($P=0.002$; Figure 7C).

Many of AT_{1a} receptor wild type also exhibited ulceration in ascending aortas (Figure 8A). Endothelial-specific AT_{1a} receptor deficiency led to a significantly reduced incidence of ulceration in ascending aortas ($P=0.004$; Figure 8B).

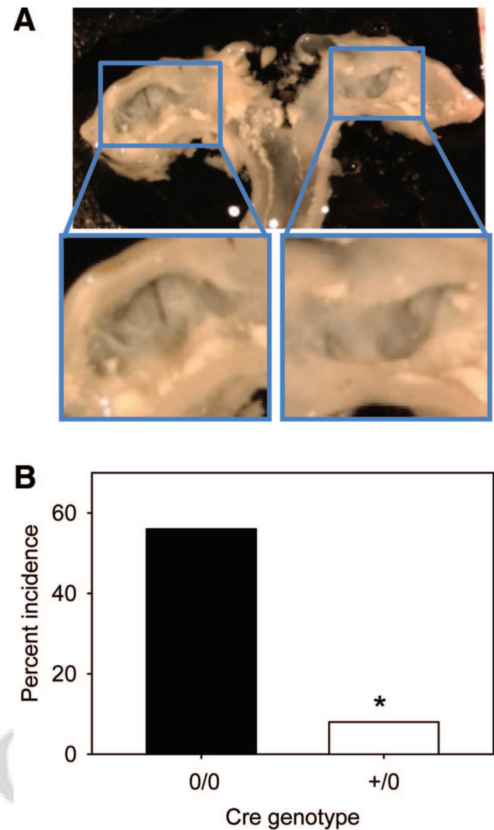

Figure 8. Endothelial cell–specific deficiency of AT_{1a} receptors reduced the incidence of ascending aortic ulcers. A, Example of an ulcerated ascending aorta. **B,** Incidence of ulceration in ascending aortas of Ang II–infused groups. *$P=0.004$ by Fisher's exact test.

Discussion

Numerous publications have demonstrated that Ang II infusion into mice leads to development of AAAs.[16,17] 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.[6,18] 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.[6,19] 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.[5]

In the present study, whole body deficiency of AT_{1a} receptors ablated development of Ang II–induced ascending AAs. To define the cell type expressing AT_{1a} 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_{1a} receptor deficiency in leukocytes or SMCs on development of

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

Ang II–induced ascending AAs. In contrast, depletion of AT_{1a} 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_1 receptors.[20] In rodents, chromosomal duplication has led to expression of 2 subtypes of AT_1 receptors, termed a and b.[21] Although AT_{1b} receptor expression is more restricted compared to AT_{1a} receptors, aortic tissue contains both subtypes with a predominance of the b subtype.[22,23] Only the b subtype is involved in Ang II–induced contractile activity that has been previously demonstrated to be restricted to the infrarenal region.[24,25] Despite the presence of functional AT_{1b} receptors in aorta, AT_{1a} receptors plays an essential role in Ang II–induced ascending AAs based on the ablation of the pathology in AT_{1a} receptor deficient mice. It may be presumed that minor amino acid differences between AT_{1a} and AT_{1b} receptor subtypes lead to differential stimulation of signaling pathways that differentiate Ang II–induced contractile versus pathological processes.[26]

Leukocytes are prominent components in both Ang II–induced AAAs and ascending AAs.[6,19] 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_{1a} receptors.[8] 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.[18,19,27] Similarly, macrophages are the most abundant leukocytes in Ang II–induced ascending AAs. Repopulation of irradiated mice demonstrated no effect of AT_{1a} receptors on donor cells to development of Ang II–induced AAAs.[8] The present study also failed to demonstrate a role for AT_{1a} receptors on donor cells in promoting Ang II–induced ascending AAs. Furthermore, whereas functional AT_1 receptors have been identified on mouse macrophages,[28] we were unable to demonstrate a role for AT_{1a} 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.[14,29] 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.[15] This difference may be a consequence of the different developmental origin of SMCs throughout the aortic tree.[30] 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.[14,25] 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.[31,32] 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_{1a} 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_{1a} receptor deficiency on SMCs was created by breeding mice that were hemizygous for Cre driven by the SM22 promoter.[33] 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.[33–35] 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.[36] 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 α-actin immunostaining. Although we were unable to validate spatial distribution of AT_{1a} receptor protein expression, the combined evidence from the SM22-Cre-ROSA26 mice and the absence of AT_{1a} 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.[37] To determine a role of Ang II stimulation on endothelial cells in the development of Ang II–induced ascending AAs, we bred AT_{1a} 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.[38] However, because we were unable to demonstrate any effect of AT_{1a} 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_{1a} 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_{1a} receptor deficiency. In contrast, deficiency of AT_{1a} 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_{1a} receptor deficiency on aneurysm development. Therefore, of the 3 major cell types present in aneurysmal tissue, only depletion of AT_{1a} 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_{1a} receptor protein. Unfortunately, we were unable to validate that commercially available antibodies authentically stained the AT_{1a} receptor protein. We were also

Circulation Research FIRST PROOF ONLY

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

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,[33,34,39] 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 β-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_{1a} 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.[18,36,40] 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_{1a} 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.[41] Therefore, it is interesting to speculate that Ang II stimulation of endothelial AT_{1a} 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.[42]

In summary, we demonstrate that endothelial-specific deficiency of AT_{1a} 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.

Acknowledgments

We appreciate the skilled editorial assistance of Dr Hong Lu (University of South Carolina).

Sources of Funding

This work was supported by NIH grant HL08100.

Disclosures

None.

References

1. Hiratzka LF, Bakris GL, Beckman JA, Bersin RM, Carr VF, Casey DE Jr, Eagle KA, Hermann LK, Isselbacher EM, Kazerooni EA, Kouchoukos NT, Lytle BW, Milewicz DM, Reich DL, Sen S, Shinn JA, Svensson LG, Williams DM. 2010 ACCF/AHA/AATS/ACR/ASA/SCA/SCAI/SIR/STS/SVM Guidelines for the Diagnosis and Management of Patients With Thoracic Aortic Disease: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines, American Association for Thoracic Surgery, American College of Radiology, American Stroke Association, Society of Cardiovascular Anesthesiologists, Society for Cardiovascular Angiography and Interventions, Society of Interventional Radiology, Society of Thoracic Surgeons, and Society for Vascular Medicine. *Circulation*. 2010;121:e266–e369.
2. Elefteriades JA, Farkas EA. Thoracic aortic aneurysm clinically pertinent controversies and uncertainties. *J Am Coll Cardiol*. 2010;55:841–857.
3. Brooke BS, Habashi JP, Judge DP, Patel N, Loeys B, Dietz HC III. Angiotensin II blockade and aortic-root dilation in Marfan's syndrome. *N Engl J Med*. 2008;358:2787–2795.
4. Pannu H, Tran-Fadulu V, Papke CL, Scherer S, Liu Y, Presley C, Guo D, Estrera AL, Safi HJ, Brasier AR, Vick GW, Marian AJ, Raman CS, Buja LM, Milewicz DM. MYH11 mutations result in a distinct vascular pathology driven by insulin-like growth factor 1 and angiotensin II. *Hum Mol Genet*. 2007;16:3453–3462.
5. Habashi JP, Judge DP, Holm TM, Cohn RD, Loeys BL, Cooper TK, Myers L, Klein EC, Liu G, Calvi C, Podowski M, Neptune ER, Halushka MK, Bedja D, Gabrielson K, Rifkin DB, Carta L, Ramirez F, Huso DL, Dietz HC. Losartan, an AT1 antagonist, prevents aortic aneurysm in a mouse model of Marfan syndrome. *Science*. 2006;312:117–121.
6. Daugherty A, Rateri DL, Charo IF, Owens AP, Howatt DA, Cassis LA. Angiotensin II infusion promotes ascending aortic aneurysms: attenuation by CCR2 deficiency in apoE−/− mice. *Clin Sci (Lond)*. 2010;118:681–689.
7. Lacro RV, Dietz HC, Wruck LM, Bradley TJ, Colan SD, Devereux RB, Klein GL, Li JS, Minich LL, Paridon SM, Pearson GD, Printz BF, Pyeritz RE, Radojewski E, Roman MJ, Saul JP, Stylianou MP, Mahony L. Rationale and design of a randomized clinical trial of beta-blocker therapy (atenolol) versus angiotensin II receptor blocker therapy (losartan) in individuals with Marfan syndrome. *Am Heart J*. 2007;154:624–631.
8. Cassis LA, Rateri DL, Lu H, Daugherty A. Bone marrow transplantation reveals that recipient AT1a receptors are required to initiate angiotensin II-induced atherosclerosis and aneurysms. *Arterioscler Thromb Vasc Biol*. 2007;27:380–386.
9. Daugherty A, Rateri D, Hong L, Balakrishnan A. Measuring blood pressure in mice using volume pressure recording, a tail-cuff method. *J Vis Exp*. 2009;1291.
10. Roselaar SE, Kakkanathu PX, Daugherty A. Lymphocyte populations in atherosclerotic lesions of apoE−/− and LDL receptor−/− mice. Decreasing density with disease progression. *Arterioscler Thromb Vasc Biol*. 1996;16:1013–1018.
11. Lu H, Rateri DL, Feldman DL Jr, RJ, Fukamizu A, Ishida J, Oesterling EG, Cassis LA, Daugherty A. Renin inhibition reduces hypercholesterolemia-induced atherosclerosis in mice. *J Clin Invest*. 2008;118:984–993.
12. Henriques TA, Huang J, D'Souza SS, Daugherty A, Cassis LA. Orchidectomy, but not ovariectomy, regulates angiotensin II-induced vascular diseases in apolipoprotein E-deficient mice. *Endocrinology*. 2004;145:3866–3872.
13. Babamusta F, Rateri DL, Moorleghen JJ, Howatt DA, Li XA, Daugherty A. Angiotensin II infusion induces site-specific intra-laminar hemorrhage in macrophage colony-stimulating factor-deficient mice. *Atherosclerosis*. 2005;186:282–290.
14. Zhou Y, Dirksen WP, Babu GJ, Periasamy M. Differential vasoconstrictions induced by angiotensin II: the role of AT1 and AT2 receptors in isolated C57BL/6J mouse blood vessels. *Am J Physiol Heart Circ Physiol*. 2003;285:H2797–H2803.
15. Owens AP III, Subramanian V, Moorleghen JJ, Guo Z, McNamara CA, Cassis LA, Daugherty A. Angiotensin II induces a region-specific hyperplasia of the ascending aorta through regulation of inhibitor of differentiation 3. *Circ Res*. 2010;106:611–619.
16. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J Clin Invest*. 2000;105:1605–1612.
17. Deng GG, Martin-McNulty B, Sukovich DA, Freay A, Halks-Miller M, Thinnes T, Loskutoff DJ, Carmeliet P, Dole WP, Wang YX. Urokinase-type plasminogen activator plays a critical role in angiotensin II-induced abdominal aortic aneurysm. *Circ Res*. 2003;92:510–517.
18. Tieu BC, Lee C, Sun H, Lejeune W, Recinos A III, Ju X, Spratt H, Guo DC, Milewicz D, Tilton RG, Brasier AR. An adventitial IL-6/MCP1 amplification loop accelerates macrophage-mediated vascular inflammation leading to aortic dissection in mice. *J Clin Invest*. 2009;119:3637–3651.

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

19. Saraff K, Babamusta F, Cassis LA, Daugherty A. Aortic dissection precedes formation of aneurysms and atherosclerosis in angiotensin II-infused, apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. 2003;23:1621–1626.
20. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol*. 2007;292:C82–C97.
21. Sasamura H, Hein L, Krieger JE, Pratt RE, Kobilka BK, Dzau VJ. Cloning, characterization, and expression of two angiotensin receptor (AT-1) isoforms from the mouse genome. *Biochem Biophys Res Comm*. 1992;185:253–259.
22. Zhou Y, Chen Y, Dirksen WP, Morris M, Periasamy M. AT1b receptor predominantly mediates contractions in major mouse blood vessels. *Circ Res*. 2003;93:1089–1094.
23. Daugherty A, Rateri DL, Lu H, Inagami T, Cassis LA. Hypercholesterolemia stimulates angiotensin peptide synthesis and contributes to atherosclerosis through the AT1A receptor. *Circulation*. 2004;110: 3849–3857.
24. Zhou Y, Dirksen WP, Chen Y, Morris M, Zweier JL, Periasamy M. A major role for AT1b receptor in mouse mesenteric resistance vessels and its distribution in heart and neuroendocrine tissues. *J Mol Cell Cardiol*. 2005;38:693–696.
25. Cassis LA, Huang J, Gong MC, Daugherty A. Role of metabolism and receptor responsiveness in the attenuated responses to Angiotensin II in mice compared to rats. *Regul Pept*. 2004;117:107–116.
26. Burson JM, Aguilera G, Gross KW, Sigmund CD. Differential expression of angiotensin receptor 1A and 1B in mouse. *Am J Physiol*. 1994;267: E260–E267.
27. Gavrila D, Li WG, McCormick ML, Thomas M, Daugherty A, Cassis LA, Miller FJ Jr, Oberley LW, Dellsperger KC, Weintraub NL. Vitamin E inhibits abdominal aortic aneurysm formation in angiotensin II-infused apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. 2005; 25:1671–1677.
28. Scheidegger KJ, Butler S, Witztum JL. Angiotensin II increases macrophage-mediated modification of low density lipoprotein via a lipoxygenase-dependent pathway. *J Biol Chem*. 1997;272:21609–21615.
29. Wang Y, Ait-Oufella H, Herbin O, Bonnin P, Ramkhelawon B, Taleb S, Huang J, Offenstadt G, Combadiere C, Renia L, Johnson JL, Tharaux PL, Tedgui A, Mallat Z. TGF-beta activity protects against inflammatory aortic aneurysm progression and complications in angiotensin II-infused mice. *J Clin Invest*. 2010;120:422–432.
30. Majesky MW. Developmental basis of vascular smooth muscle diversity. *Arterioscler Thromb Vasc Biol*. 2007;27:1248–1258.
31. Jiang X, Rowitch DH, Soriano P, McMahon AP, Sucov HM. Fate of the mammalian cardiac neural crest. *Development*. 2000;127:1607–1616.
32. Li P, Pashmforoush M, Sucov HM. Retinoic acid regulates differentiation of the secondary heart field and TGFbeta-mediated outflow tract septation. *Dev Cell*. 2010;18:480–485.
33. Boucher P, Gotthardt M, Li WP, Anderson RGW, Herz J. LRP: role in vascular wall integrity and protection from atherosclerosis. *Science*. 2003; 300:329–332.
34. Subramanian V, Golledge J, Ijaz T, Bruemmer D, Daugherty A. Pioglitazone-induced reductions in atherosclerosis occur via smooth muscle cell-specific interaction with PPAR{gamma}. *Circ Res*. 2010; 107:953–958.
35. Hamblin M, Chang L, Zhang H, Yang K, Zhang J, Chen YE. Vascular smooth muscle cell peroxisome proliferator-activated receptor-gamma deletion promotes abdominal aortic aneurysms. *J Vasc Surg*. 2010;52: 984–993.
36. Jones JA, Beck C, Barbour JR, Zavadzkas JA, Mukherjee R, Spinale FG, Ikonomidis JS. Alterations in aortic cellular constituents during thoracic aortic aneurysm development: myofibroblast-mediated vascular remodeling. *Am J Pathol*. 2009;175:1746–1756.
37. Savoia C, Schiffrin EL. Inhibition of the renin angiotensin system: implications for the endothelium. *Curr Diab Rep*. 2006;6:274–278.
38. Wang L. Endothelial and hematopoietic cell fate of human embryonic stem cells. *Trends Cardiovasc Med*. 2006;16:89–94.
39. Frutkin AD, Shi H, Otsuka G, Leveen P, Karlsson S, Dichek DA. A critical developmental role for tgfbr2 in myogenic cell lineages is revealed in mice expressing SM22-Cre, not SMMHC-Cre. *J Mol Cell Cardiol*. 2006;41:724–731.
40. Tieu BC, Ju X, Lee C, Sun H, Lejeune W, Recinos A III, Brasier AR, Tilton RG. Aortic adventitial fibroblasts participate in angiotensin-induced vascular wall inflammation and remodeling. *J Vasc Res*. 2010; 48:261–272.
41. Mateo T, Abu Nabah YN, Abu Taha M, Mata M, Cerda-Nicolas M, Proudfoot AE, Stahl RA, Issekutz AC, Cortijo J, Morcillo EJ, Jose PJ, Sanz MJ. Angiotensin II-induced mononuclear leukocyte interactions with arteriolar and venular endothelium are mediated by the release of different CC chemokines. *J Immunol*. 2006;176:5577–5586.
42. Takahashi M, Galligan C, Tessarollo L, Yoshimura T. Monocyte chemoattractant protein-1 (MCP-1), not MCP-3, is the primary chemokine required for monocyte recruitment in mouse peritonitis induced with thioglycollate or zymosan A. *J Immunol*. 2009;183:3463–3471.

FIRST PROOF ONLY

Novelty and Significance

What Is Known?

- Ang II infusion promotes profound aortic dilation that is restricted to the ascending portion.
- AT_{1b} receptors have functional effects in aortic tissue.

What New Information Does This Article Contribute?

- Whole body deficiency of AT_{1a} receptors ablates the development of ascending AAs.
- Depletion of AT_{1a} receptors on bone marrow–derived cells or SMCs has no effect on Ang II–induced ascending AAs.
- Depletion of AT_{1a} 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 administration of the AT_1 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_{1a} receptor deficiency, bone marrow transplantation to generate mice with leukocyte-specific AT_{1a} receptor deficiencies, and newly developed floxed mice to generate SMC or endothelial-specific AT_{1a} receptor deficient mice. Using these mice, we determined the requirement of AT_{1a} receptor stimulation for generation of ascending AAs. Studies in mice with cell-specific deficiencies demonstrated a major contribution of AT_{1a} receptor stimulation in the endothelium. These studies will provide a basis for determining how endothelial cells promote this disease.

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

CIRCRES/2010/222844/R3

SUPPLEMENTAL MATERIAL

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

Debra L. Rateri,[1] Jessica J. Moorleghen,[1] Anju Balakrishnan,[1] A. Phillip Owens III,[1,2]

Deborah A. Howatt,[1] Venkateswaran Subramanian,[1] Aruna Poduri,[1] Richard Charnigo,[3]

Lisa A. Cassis,[2,4] Alan Daugherty[1,2,4]

Saha Cardiovascular Research Center[1]
Graduate Center for Toxicology[2]
Department of Biostatistics[3]
Graduate Center for Nutritional Sciences[4]
University of Kentucky
Lexington, KY

Short title: Endothelial AT1a receptor deficiency decreases aneurysms

Address for Correspondence: Alan Daugherty
Saha Cardiovascular Research Center
BBSRB, Room 243
University of Kentucky
Lexington, KY 40536-0509
Telephone: (859) 323-3512
Fax: (859) 257-3235
E-mail: Alan.Daugherty@uky.edu

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

METHODS AND MATERIALS

Mice

The following mice were purchased from the Jackson Laboratory: LDL receptor -/- (B6.129S7-Ldlr^{tm1Her}/J, C57BL/6 N10, Stock #002207); AT1a receptor -/- (B6.129P2-Agtr1a^{tm1Unc}/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<tm1Sor>/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).[1,2]

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

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

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^{2+}/Ca^{2+} 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.[3] Real time PCR was performed using Eva-green reagent in a CFX96 cycler (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 $MgCl_2$; 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 ferricyaninde; 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

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

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.[4] 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.[5,6] 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 x 10^6 cells/100 µl. Recipient mice were irradiated with 2 doses of 450 rads, 3 hours apart. Donor bone marrow cells (1 x 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.[7] At experimental termination, bone marrow from the recipients was harvested, DNA was isolated, and chimerism was

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

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.[8] 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 μM, Sigma), and AngII (1 μM, 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 ± SEM. $P<0.05$ was considered statistically significant.

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

Table I. Primers for genotyping of mice.

Gene	Primers for genomic PCR	Product size (bp)
AT1a receptor	5'-AAATGGCCCTTAACTCTTCTACTG 5'-ATTAGGAAAGGGAACAGGAAGC	WT = 650 Disrupted = 1100
AT1a receptor flox	5'-TGTTGCATCTACATCCTG 5'-TCTAAAGAAACCTCATGAAC	WT = 202 flox = 262
Cre	5'-ACCTGAAGATGTTCGCGATT 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

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

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

Gene	Primers for RT-PCR and real time PCR	Product size (bp)
AT1a receptor	5’-ACTCACAGCAACCCTCCAAG 5’-ATCACCACCAAGCTGTTTCC	238
β-actin	5'-CGTGGGCCGCCCTAGGCAACCA 5'-TTGGCCTTAGGGTTCAGGGGGG	220

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

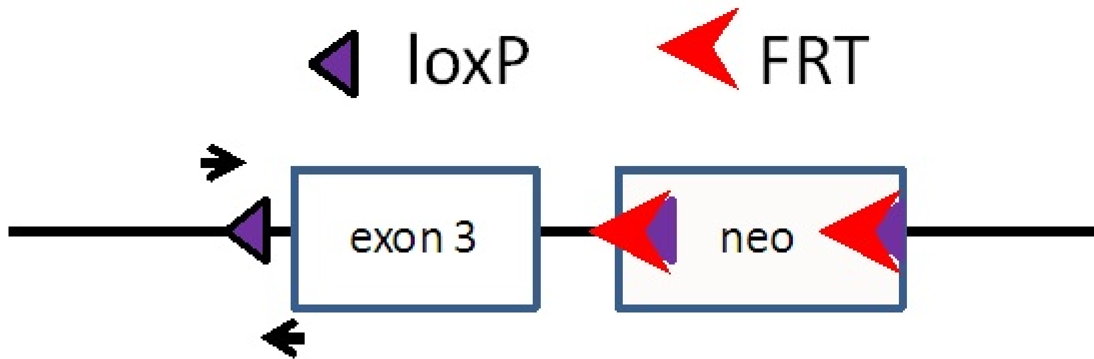

Figure I. Construct map of floxed exon 3 of the AT1a receptor. Black arrows indicate the location of the primers used to genotype for the presence of the 1st flox site.

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

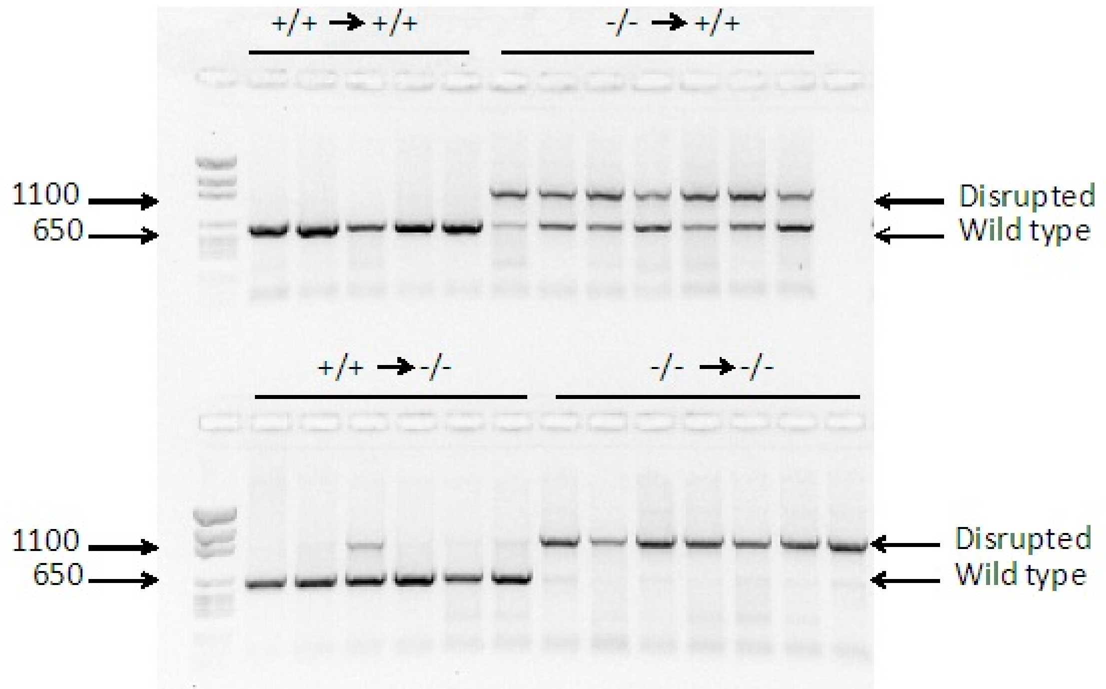

Figure II. Genotyping of bone marrow from chimeric mice after the termination. Both AT1a receptor +/+ and -/- recipient mice were irradiated and repopulated with cells harvested from bone marrow of donor AT1a receptor +/+ and -/- mice, as indicated on the top of each gel. Amplicons for the wild type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

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011

REFERENCES

1. Takeuchi K, Alexander RW, Nakamura Y, Tsujino T, Murphy TJ. Molecular structure and transcriptional function of the rat vascular AT1a angiotensin receptor gene. *Circ Res.* 1993; 73: 612-621.
2. Elton TS, Martin MM. Angiotensin II type 1 receptor gene regulation: transcriptional and posttranscriptional mechanisms. *Hypertension.* 2007; 49: 953-961.
3. Daugherty A, Rateri DL, Lu H, Inagami T, Cassis LA. Hypercholesterolemia stimulates angiotensin peptide synthesis and contributes to atherosclerosis through the AT1A receptor. *Circulation.* 2004; 110: 3849-3857.
4. Daugherty A, Rateri D, Hong L, Balakrishnan A. Measuring blood pressure in mice using volume pressure recording, a tail-cuff method. *J Vis Exp.* 2009; 1291.
5. Wang M, Lee E, Song W, Ricciotti E, Rader DJ, Lawson JA, Pure E, FitzGerald GA. Microsomal prostaglandin E synthase-1 deletion suppresses oxidative stress and angiotensin II-induced abdominal aortic aneurysm formation. *Circulation.* 2008; 117: 1302-1309.
6. Lu H, Rateri DL, Feldman DL, Jr RJ, Fukamizu A, Ishida J, Oesterling EG, Cassis LA, Daugherty A. Renin inhibition reduces hypercholesterolemia-induced atherosclerosis in mice. *J Clin Invest.* 2008; 118: 984-993.
7. Cassis LA, Rateri DL, Lu H, Daugherty A. Bone marrow transplantation reveals that recipient AT1a receptors are required to initiate angiotensin II-induced atherosclerosis and aneurysms. *Arterioscler Thromb Vasc Biol.* 2007; 27: 380-386.
8. Babamusta F, Rateri DL, Moorleghen JJ, Howatt DA, Li XA, Daugherty A. Angiotensin II infusion induces site-specific intra-laminar hemorrhage in macrophage colony-stimulating factor-deficient mice. *Atherosclerosis.* 2005; 186: 282-290.

Downloaded from circres.ahajournals.org at University of Kentucky--Lexington on January 21, 2011