

# Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart  
Association®



*Learn and Live* SM

## **Bone Marrow Transplantation Reveals That Recipient AT1a Receptors Are Required to Initiate Angiotensin II–Induced Atherosclerosis and Aneurysms**

Lisa A. Cassis, Debra L. Rateri, Hong Lu and Alan Daugherty

*Arterioscler. Thromb. Vasc. Biol.* 2007;27;380-386; originally published online Dec 7, 2006;

DOI: 10.1161/01.ATV.0000254680.71485.92

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

Copyright © 2007 American Heart Association. All rights reserved. Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://atvb.ahajournals.org/cgi/content/full/27/2/380>

Data Supplement (unedited) at:

<http://atvb.ahajournals.org/cgi/content/full/01.ATV.0000254680.71485.92/DC1>

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at

<http://atvb.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](mailto:journalpermissions@lww.com)

Reprints: Information about reprints can be found online at

<http://www.lww.com/reprints>

# Bone Marrow Transplantation Reveals That Recipient AT1a Receptors Are Required to Initiate Angiotensin II–Induced Atherosclerosis and Aneurysms

Lisa A. Cassis, Debra L. Rateri, Hong Lu, Alan Daugherty

**Objective**—Angiotensin II (AngII) infusion into hypercholesterolemic mice accelerates atherosclerosis and promotes formation of abdominal aortic aneurysms (AAAs). The purpose of this study was to define whether AngII interacts with receptors on infiltrating versus resident cells in promoting vascular pathologies.

**Methods and Results**—Male LDL receptor<sup>-/-</sup> mice, that were either AT1a receptor <sup>+/+</sup> or <sup>-/-</sup>, were fed a fat enriched diet and infused with either saline or AngII. AngII-induced augmentation of atherosclerosis and formation of AAAs was ablated in AT1a receptor<sup>-/-</sup> mice. Bone marrow transplantation studies were performed to determine the role of AT1a receptors expressed on infiltrating cells. AT1a receptor <sup>+/+</sup> and <sup>-/-</sup> mice were irradiated and repopulated with bone marrow–derived stem cells of either genotype. These 4 groups of chimeric mice were infused with either saline or AngII. Repopulation of irradiated AT1a receptor <sup>+/+</sup> mice with <sup>-/-</sup> bone marrow–derived cells resulted in modest reductions in AngII-induced atherosclerosis. Unexpectedly, AT1a receptor–deficient recipient mice were dramatically protected from AngII-induced vascular pathologies, irrespective of donor genotype.

**Conclusion**— AngII promotes vascular pathology via AT1a receptors. AT1a receptors expressed on infiltrating cells exert modest regulation of AngII-induced atherosclerosis. However, the presence of this receptor in resident tissue is required for the initiation of AngII-induced atherosclerosis and AAAs. (*Arterioscler Thromb Vasc Biol.* 2007;27:380-386.)

**Key Words:** angiotensin receptors ■ atherosclerosis ■ aneurysms

Angiotensin II (AngII) infusion into either LDL receptor<sup>-/-</sup> or apoE<sup>-/-</sup> mice leads to acceleration of atherogenesis and the development of abdominal aortic aneurysms (AAAs).<sup>1-4</sup> AngII-induced atherosclerosis is characterized by intimal infiltration of leukocytes that become engorged with lipid. In contrast, AngII induction of AAAs is characterized by medial destruction, macrophage infiltration, thrombus formation, and vascular remodeling.<sup>5</sup> Thus, AngII provides a common stimulus for atherosclerosis and AAAs, but the development of these pathologies appears to occur via distinct mechanisms.<sup>6</sup> The specific receptor regulating the formation of AngII-induced atherosclerosis has not been determined. A role for AT1 receptors in AngII-induced AAAs has been demonstrated by inhibition with losartan, although the contribution of AT1a versus AT1b receptors has not been defined.<sup>7</sup>

Atherosclerosis and AAAs display distinct histological features during initiation and progression, but have the common feature of macrophage infiltration throughout the progression of both of these vascular diseases. AT1 receptors have been detected on macrophages.<sup>8,9</sup> AngII promotes several potentially atherogenic effects on cultured macrophages,

including enhanced expression of 12/15 lipoxygenase, production of peroxide, promotion of LDL oxidation, reduced cholesterol efflux, and augmented cholesterol synthesis.<sup>8,10-13</sup> AT1 receptors are also present on resident cells of the arterial wall, including endothelium and smooth muscle cells. Incubation of AngII with cultured endothelial cells promotes the atherogenic mechanism of increased leukocyte adhesion that is associated with increased expression of several adhesion molecules, such as E-selectin and vascular cell adhesion molecule (VCAM)-1.<sup>14-16</sup> AngII also stimulates several potential atherogenic mechanisms in smooth muscle cells, including secretion of MCP-1 and increased production of reactive oxygen species.<sup>17,18</sup> In contrast, the AngII-induced mechanisms in these cell types that relate to the development of AAAs have not been as extensively characterized.

Because infiltration of leukocytes occurs in AngII-induced atherosclerotic lesions and AAAs, we hypothesized that AngII interacts with a specific receptor on infiltrating leukocytes to initiate the formation of vascular diseases. In initial studies, we defined the effects of AT1a receptor deficiency on the development of AngII-induced atherosclerosis and AAAs. After definition of the angiotensin receptor subtype mediating

Original received December 16, 2005; final version accepted November 20, 2006.

From the Graduate Center for Nutritional Sciences (L.A.C., A.D.) and the Cardiovascular Research Center (D.L.R., H.L., A.D.), University of Kentucky, Lexington.

Correspondence to Alan Daugherty or Lisa Cassis, Wethington Building, Room 521, University of Kentucky, Lexington, KY 40536-0200. E-mail Alan.Daugherty@uky.edu or cassis@uky.edu

© 2007 American Heart Association, Inc.

*Arterioscler Thromb Vasc Biol.* is available at <http://www.atvbaha.org>

DOI: 10.1161/01.ATV.0000254680.71485.92

AngII-induced atherosclerosis and/or AAA formation, we used bone marrow transplantation to determine the role of the angiotensin receptor in donor versus recipient cells in the formation of these vascular pathologies. The transplantation protocol was validated to ensure that chimeric mice reproducibly expressed the donor phenotype in bone marrow-derived cells. Using this validated protocol, we performed these studies on mice in which both wild-type and receptor-deficient recipients were irradiated and repopulated with bone marrow-derived cells that were either receptor wild-type or deficient. Creation of these 4 groups of chimeric mice enabled distinction of the location of angiotensin receptor effects to the donor versus the recipient genotype.

## Methods

### Mice, Diet, and Osmotic Mini Pumps

For whole animal deficiency studies, AT1a receptor $-/-$  males (The Jackson Laboratory (Bar Harbor, Me), B6.129P2-Agtr1a<sup>tm1Unc</sup>, Stock #002682) were mated to LDL receptor $-/-$  females (The Jackson Laboratory, B6.129S7-Ldlr<sup>tm1Her</sup>, Stock #002207). All mice were backcrossed 10 times into a C57BL/6 background. Resultant F1 heterozygous mice were bred. Male littermates were screened for LDL receptor deficiency and AT1a receptor genotype. C57BL/6.SJL (Ptpcr<sup>a</sup>; Stock #002014) mice were also obtained from the Jackson Laboratory. All mice were maintained in a barrier facility and fed normal mouse laboratory diet until placed on an experimental protocol.

To induce hypercholesterolemia, male mice (8 weeks of age) were fed a diet supplemented with saturated fat (milk fat 21%) and cholesterol (0.15%; Harlan Teklad; Diet #TD88137). One week after initiation of fat feeding, saline or AngII (1,000 ng/kg/min) was administered subcutaneously via Alzet osmotic minipumps (Model 2004) as described previously.<sup>2</sup> All studies were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

### Genotyping by Polymerase Chain Reaction

PCR screening for AT1a and LDL receptors was performed as described previously.<sup>19</sup>

### Bone Marrow Transplantation

This procedure was performed as described previously.<sup>20–22</sup> Mice were maintained on antibiotic water (sulfratrim, 4  $\mu$ g/mL) for one week before irradiation. Recipient mice were irradiated with a total of 900 Rads from a cesium source that was delivered in two doses within 3 to 4 hours. Bone marrow-derived cells for CD45.1, AT1a receptor +/+ or  $-/-$   $\times$  LDL receptor $-/-$  mice were obtained from the tibias and femurs of donor mice and were injected into the tail vein of 8-week-old irradiated recipient mice ( $1 \times 10^7$  cells per mouse). Mice were maintained on antibiotic water for 4 weeks after irradiation, then placed on regular water. For mice used in vascular pathology studies, six weeks after irradiation, the mice were fed a diet enriched in saturated fat and cholesterol (Teklad #TD88137) for 5 weeks. Osmotic mini-pumps containing drugs were implanted 1 week after the initiation of fat feeding.

### Detection of CD45 Allelic Variants

CD45 allelic variants were detected on blood leukocytes and peritoneal macrophages with fluorescence-activated-cell sorter (FACS) using fluorescently labeled monoclonal antibodies (BD Pharmingen catalog # 553772 for CD45.2 and # 553776 for CD45.1). Cell counts in whole blood were determined using Beckman Coulter Counter.

### Measurement of Serum Components

Serum cholesterol concentrations were determined by a commercially available enzymatic assay kit (Wako Chemicals). Lipoprotein cholesterol distribution was performed by size exclusion chromatography as described previously.<sup>2</sup>

Serum autoantibodies titers to modified lipoproteins were determined as described previously, with data expressed as the ratio of chromagen development for LDL compared with malondialdehyde-modified LDL coated plates.<sup>23</sup>

### Blood Pressure Measurements

Systolic blood pressure was measured on conscious, restrained mice using the Visitech tail cuff system, as described previously.<sup>2</sup>

### Quantification of Atherosclerosis and AAA

Atherosclerosis was quantified on the aortic arch as described previously.<sup>23,24</sup> The maximum width of the abdominal aorta was measured using computerized morphometry (Image-Pro). Aneurysm incidence was quantified based on a definition of an external width of the suprarenal aorta that was increased by 50% or greater compared with aortas from saline-infused mice.<sup>25</sup>

### Tissue Composition

Aortic tissues were sectioned and histologically stained using Movat pentachrome and immunostained for macrophages as described previously.<sup>26</sup>

### Statistics

Data were analyzed with two way ANOVA using SigmaStat. Data were tested for use of parametric or nonparametric post hoc analysis, and multiple comparisons were performed using Tukey or Holm-Sidak tests as appropriate for the data. Percent incidence of AAAs was analyzed by Fishers exact test.  $P < 0.05$  values were considered to be statistically significant. Differences that attained statistical significance are represented in Tables and Figures. All data are represented as means  $\pm$  SEM.

## Results

### Deficiency of AT1a Receptors Prevents AngII-Induced Atherosclerosis and AAAs

To determine the contribution of AT1a receptors in AngII-induced atherosclerosis and AAA formation, male LDL receptor $-/-$  mice that were either AT1a receptor +/+ or  $-/-$  were fed a fat-enriched diet and infused with either saline or AngII (1000 ng/kg/min) for 28 days. AT1a receptor genotype had no effect on either body weight or total serum cholesterol during the study (Table 1). Systolic blood pressure was not significantly different in saline-infused AT1a receptor +/+ mice compared with  $-/-$  mice. However, AngII infusion significantly increased systolic pressure in AT1a receptor +/+ mice, while not significantly increasing AT1a receptor $-/-$  mice (Table 1).

Atherosclerosis was quantified on the intimal surface of the aortic arch. In saline-infused AT1a receptor $-/-$  mice, lesion area was significantly decreased compared with saline-infused AT1a receptor +/+ mice ( $P < 0.02$ ). AngII infusion significantly increased atherosclerosis in the en face measurement of lesion size in AT1a receptor +/+ mice (Figure 1). AngII-induced elevations in the extent of atherosclerosis were totally ablated in AT1a receptor  $-/-$  mice (Figure 1).

The suprarenal aortic width of saline-infused LDL receptor $-/-$  mice was  $0.90 \pm 0.03$  mm for both AT1a receptor +/+ and  $-/-$  mice (Figure 2A). Neither genotype of saline-infused mice developed AAAs (Figure 2B). AngII

**TABLE 1. Effects of AngII Infusion Into LDL Receptor<sup>-/-</sup> Mice That Are Wild-Type or Deficient in AT1a Receptors**

Infusion	AT1a Receptor Genotype	No.	Body weight (g)	Cholesterol (mg/dL)	Systolic Blood Pressure (mm Hg)
Saline	+/+	4	27.8±2.5	975±172	126±9
	-/-	4	27.9±1.0	1060±103	111±8
AngII	+/+	9	27.0±0.5	1163±67	149±6*
	-/-	9	27.6±0.8	1066±117	125±8

Body weight and serum cholesterol were measured at the end of study. Systolic blood pressure was measured daily during the last week of the study and values represent the mean of the last week. Values are represented as mean±SEM. \**P*=0.004 for comparison of AT1a receptor +/+ genotypes infused with saline vs AngII.

infusion into AT1a receptor wild-type mice led to a considerable expansion of suprarenal aortic width (2.48±0.39 mm, *P*<0.01). In contrast, infusion of AngII into AT1a receptor<sup>-/-</sup> mice failed to promote any change in aortic width (0.89±0.03 mm; Figure 2A). Accordingly, AngII-infusion into AT1a receptor +/+ mice led to an AAA incidence of 67%, whereas AAAs were totally absent in AT1a receptor<sup>-/-</sup> mice (Figure 2B).

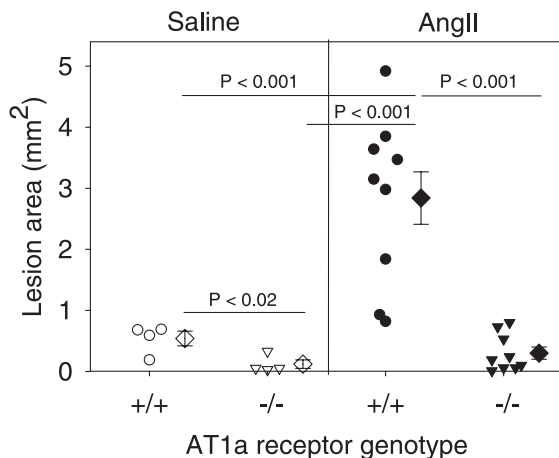
We determined serum titers of autoantibodies against MDA-LDL as an index of AT1a receptor deficiency on a measurement of systemically detectable oxidant stress. Neither the AT1a receptor genotype or the infusion of AngII significantly influenced these titers (supplemental Figure IA).

**Recipient AT1a Receptor Genotype Is the Major Determinant of Vascular Disease in Chimeric Mice Infused with AngII**

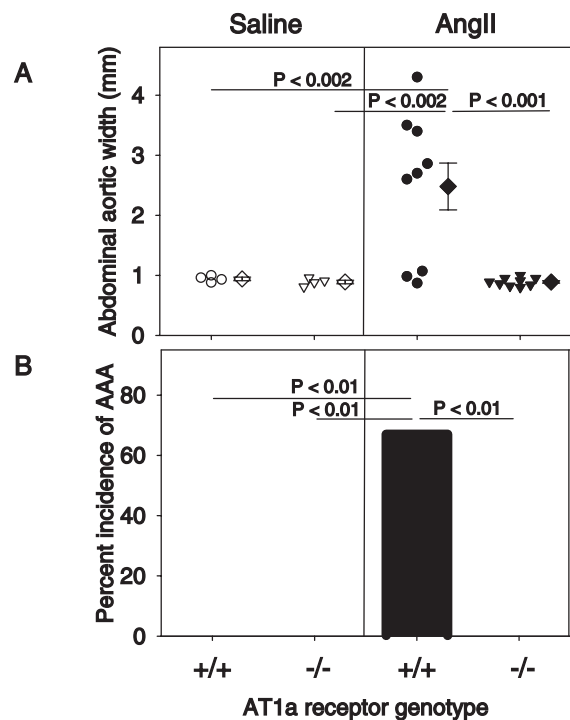
Having established the critical role of AT1a receptors in the development of AngII-induced vascular diseases, we used bone marrow transplantation to define the role of infiltrating cells expressing this receptor. To determine the efficacy of the protocol used for bone marrow transplantation in subsequent studies, we took advantage of congenic mice expressing allelic variants of the common leukocyte antigen, CD45.

Irradiation leads to a rapid reduction in the number of circulating leukocytes. Subsequent to the introduction of bone marrow-derived cells, the donor cells rapidly and consistently become the predominant phenotype in the blood (supplemental Figure II). Leukocytes recovered from lavage of the peritoneal space also predominantly express the donor phenotype 8 weeks after injection (88.7±0.7% express donor CD45.1). Having demonstrated the high repopulation, the same protocol was used for all subsequent studies.

After having validated the irradiation and repopulation procedure, studies were performed in which both AT1a receptor +/+ and -/- mice were irradiated and repopulated with cells from AT1a receptor +/+ and -/- mice. All recipient and donor mice were LDL receptor-deficient. At the termination of the study, bone marrow was harvested and



**Figure 1.** Whole body deficiency of AT1a receptor ablates AngII-induced atherosclerosis. Atherosclerotic lesion size was measured on aortic arch intimal surfaces. Circles (AT1a receptor +/+) and inverted triangles (-/-) represent individual mice, diamonds represent means, and bars are SEM. Saline-infused mice are represented by open symbols and AngII-infused by closed symbols.



**Figure 2.** Whole body deficiency of AT1a receptor ablates AngII-induced AAAs. A, Measurements of maximal external width of abdominal aortas. Circles (AT1a receptor +/+) and inverted triangles (-/-) represent individual mice, diamonds represent means, and bars are SEM. Saline-infused mice are represented by open symbols and AngII-infused by closed symbols. B, Percent incidence of AAAs based on the criterion described in the Methods.

**TABLE 2. Effects of AngII-Infusion Into LDL Receptor<sup>-/-</sup> Mice That Were Chimeric for AT1a Receptors**

Infusion	AT1a Receptor Genotype		No.	Body Weight (g)	Cholesterol (mg/dl)	Leukocytes (cells $\times 10^3/\mu$ L)	Systolic Blood Pressure (mm Hg)
	Recipient	Donor					
Saline	+/+	+/+	4	32.4 $\pm$ 0.8	1659 $\pm$ 58	9.0 $\pm$ 1.6	141 $\pm$ 6
		-/-	4	28.4 $\pm$ 1.3	1445 $\pm$ 75	7.6 $\pm$ 1.1	139 $\pm$ 6
	-/-	+/+	4	32.5 $\pm$ 1.7	1591 $\pm$ 284	8.5 $\pm$ 0.9	119 $\pm$ 6
		-/-	4	28.6 $\pm$ 1.4	1345 $\pm$ 126	8.7 $\pm$ 0.8	127 $\pm$ 5
AngII	+/+	+/+	13	29.2 $\pm$ 1.3	1580 $\pm$ 105	9.2 $\pm$ 0.8	168 $\pm$ 5*
		-/-	12	27.4 $\pm$ 1.3	1698 $\pm$ 89	7.0 $\pm$ 0.8	169 $\pm$ 6*
	-/-	+/+	12	32.1 $\pm$ 1.3	1604 $\pm$ 132	8.2 $\pm$ 1.1	124 $\pm$ 7
		-/-	13	29.4 $\pm$ 0.9	1550 $\pm$ 188	8.4 $\pm$ 1.3	126 $\pm$ 4

Body weight, serum cholesterol, and blood leukocytes were measured at the end of study. Systolic blood pressure was measured daily during the last week of study. Values are represented as mean  $\pm$  SEM. \* $P < 0.01$  for comparison of AngII-infused mice of AT1a receptor +/+ vs -/- recipient genotype.

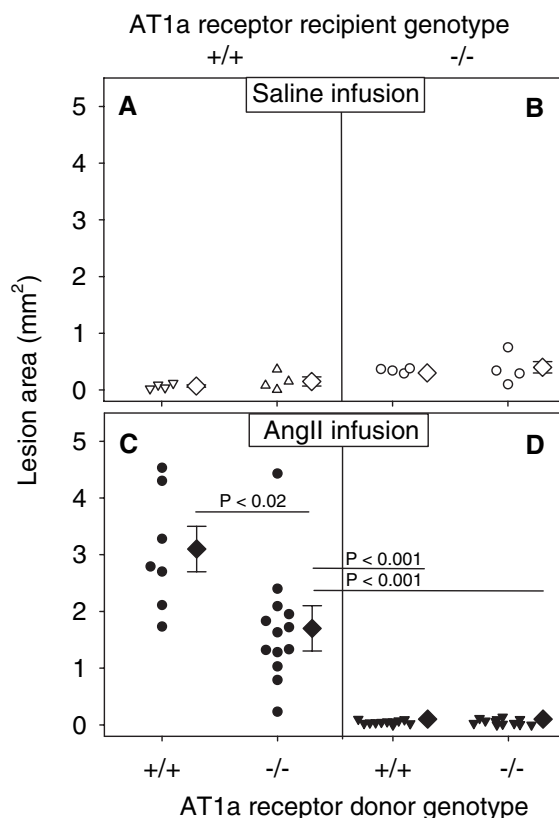
PCR analysis performed. These analyses demonstrated that mice had the expected genotype after irradiation and repopulation (supplemental Figure III). These 4 chimeric groups determined whether the AT1a receptors involved in AngII-induced atherosclerosis and AAAs were expressed on cells of recipient or donor origin.

The AT1a receptor genotype of the recipient or donor had no effect on body weight or serum cholesterol in groups infused with either saline or AngII (Table 2). Blood leukocytes numbers were equivalent in all groups and were unaffected by recipient or donor AT1a receptor genotype during saline or AngII infusion (Table 2). During saline infusion, there was no statistically significant difference in systolic blood pressure, irrespective of the AT1a receptor genotype of the recipient or donor. AngII infusion significantly increased blood pressure in recipient AT1a receptor +/+, but not -/- mice. The genotype of AT1a receptor on the donor cells had no significant effect on systolic blood pressure (Table 2).

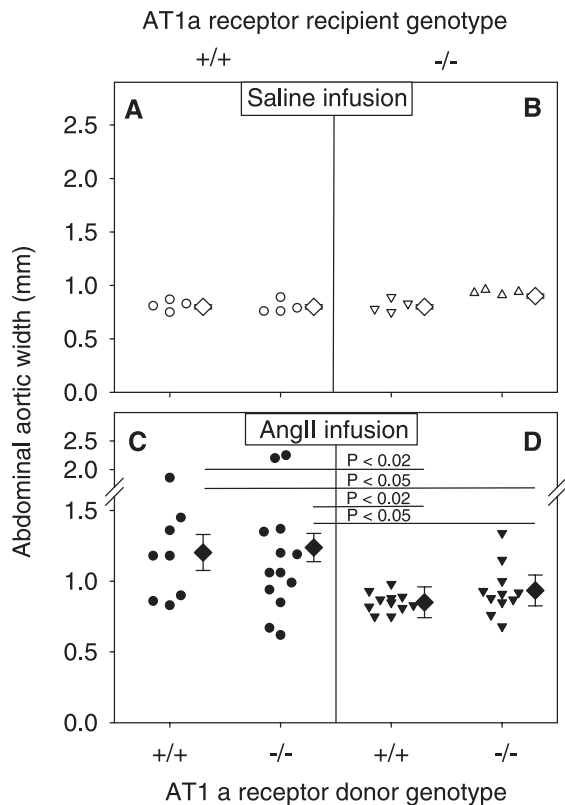
Mice infused with saline had minimal atherosclerosis irrespective of the recipient or donor AT1a receptor genotype (Figure 3A and 3B). Infusion of AngII into AT1a receptor +/+ recipient mice repopulated with AT1a receptor +/+ bone marrow-derived cells augmented the size of atherosclerotic lesions compared with saline-infused groups. The size of AngII-induced atherosclerotic lesions was significantly attenuated in AT1a receptor +/+ mice repopulated with AT1a receptor -/- cells (Figure 3C). The characteristics of lesions from both groups were equivalent, being predominantly macrophage containing (data not shown). In accord with the experiments described earlier, minimal atherosclerotic lesions were present in the arch of AngII-infused AT1a receptor -/- mice repopulated with AT1a receptor -/- cells. Surprisingly, minimal atherosclerotic lesions formed during AngII infusion in AT1a receptor -/- mice repopulated with AT1a receptor +/+ cells (Figure 3D).

The width of the suprarenal aorta was not significantly different during saline infusion, irrespective of the AT1a receptor genotype of the recipient or donor cells, and no AAAs were detected (Figure 4A and 4B). During AngII-infusion, the maximal aortic width increased significantly in both AT1a receptor +/+ recipient groups, and there was no

difference based on the genotype of the donor bone marrow (Figure 4C and 4D). In contrast, in AT1a receptor -/- recipients, aortic width did not increase compared with saline, regardless of AT1a receptor donor genotype. Infusion of AngII into AT1a receptor +/+ mice repopulated with cells from +/+ mice resulted in a 58% AAA incidence. The



**Figure 3.** Development of AngII-induced atherosclerosis in chimeric mice requires AT1a receptor presence in the recipient mice. A and B represent saline infused mice in recipient mice of AT1a receptor +/+ (A) or -/- (B) background repopulated with the indicated genotype of donor bone marrow-derived cells. C and D represent AngII-infused mice in AT1a receptor +/+ (C) and -/- (D) recipient mice. Circles (AT1a receptor +/+ donors) and inverted triangles (AT1a receptor -/- donors) represent lesion sizes in individual mice, diamonds represent means, and bars are SEM.



**Figure 4.** Development of AngII-induced AAAs in chimeric mice requires AT1a receptor presence in the recipient mice. A and B represent saline infused mice in recipient mice of AT1a receptor +/+ (A) or -/- (B) background repopulated with the indicated genotype of donor bone marrow-derived cells. C and D represent AngII-infused mice in AT1a receptor +/+ (C) and -/- (D) recipient mice. Circles (AT1a receptor +/+ donors) and inverted triangles (AT1a receptor -/- donors) represent abdominal aortic width in individual mice, diamonds represent means, and bars are SEM.

incidence of AngII-induced AAAs was not significantly altered by repopulating AT1a receptor -/- cells into AT1a receptor +/+ recipients. Conversely, in AngII-infused AT1a receptor -/- recipients, AAAs did not develop, irrespective of the AT1a receptor genotype of the donor cells. Histological analysis revealed that aneurysmal tissue had characteristics similar to those we have described previously.<sup>5</sup> The appearance of tissue in the suprarenal aorta of AngII-infused mice that had AT1a receptor -/- genotypes was indistinguishable from tissue obtained from saline-infused wild type mice (supplemental Figure IV).

No effect was noted of the recipient or donor genotype on serum titers of MDL-LDL autoantibodies in both saline and AngII-infused mice (supplemental Figure IB).

### Discussion

In the present study, we used gene-targeted mice to demonstrate that the AT1a receptor subtype was responsible for the development of AngII-induced atherosclerosis. The dependence of AT1a receptors on the development of AngII-induced atherosclerosis is similar to previous findings demonstrating a major role for the AT1a receptor in hypercholesterolemia-induced atherosclerosis.<sup>19,27</sup> The effect

of AT1a receptor deficiency in markedly attenuating hypercholesterolemia-induced atherosclerosis has been attributed to the upregulation of the endogenous renin-angiotensin system.<sup>19</sup> In addition, these results extend previous findings, because early reports did not demonstrate an effect of losartan administration on AngII-induced atherosclerosis.<sup>7</sup> This lack of effect of losartan in our previous studies was probably attributable to the extensive atherosclerosis that was already present in 11-month-old apoE -/- mice. In the current study, the effect of exogenous AngII infusion was examined in LDL receptor -/- mice at 8 weeks of age that were fed a saturated fat enriched diet for 5 weeks. Thus, the effect of AT1a receptor deficiency on AngII-induced atherosclerosis was examined against a low level of preexisting atherosclerosis. Results from this and previous studies demonstrate a critical role for the AT1a receptor subtype in mediating the effects of both endogenous and exogenous AngII.

The present study also demonstrated that deletion of the AT1a receptor subtype ablated formation of AngII-induced AAAs. Previous studies in our laboratory demonstrated that pharmacological antagonism of the AT1 receptor by losartan administration totally ablated AngII-induced aneurysm formation in ApoE -/- mice.<sup>7</sup> However, because AT1 receptor antagonists cannot discriminate between AT1a and AT1b receptor subtypes, it was unclear which receptor subtype was responsible for the effects of losartan. Both the AT1a and the AT1b receptor subtypes are expressed in the aortas of mice, with greater preponderance of the AT1b receptor particularly in the abdominal region of the aorta.<sup>28</sup> Functional studies demonstrated that the AT1b receptor mediates the contractile response of the abdominal aorta to AngII, as contractile responses were maintained in aortic segments from AT1a receptor-deficient mice.<sup>28</sup> Our results demonstrate a critical role for the AT1a receptor subtype in AngII-induced AAA formation. Thus, regardless of the greater preponderance of AT1b receptor expression in the abdominal aorta, it is the presence of AT1a receptors which mediates AngII-induced AAAs.

In this study, AngII-induced atherosclerosis and AAA formation were abolished in AT1a receptor-deficient mice that did not exhibit a pressor response to infusion of AngII. Thus, the lack of hypertension in AT1a receptor-deficient mice infused with AngII may have contributed to reductions in atherosclerosis or AAA formation. Previous studies demonstrated that AngII exacerbates atherosclerosis<sup>3,29</sup> and results in AAA formation<sup>1,2,29</sup> at infusion doses that do not appreciably elevate systolic pressure. Similarly, infusion of norepinephrine at a dose that elevated blood pressure to a similar extent as AngII did not increase atherosclerosis in ApoE -/- mice,<sup>3</sup> or result in AAA formation.<sup>29</sup> Recent studies demonstrate that administration of the AT1 receptor antagonist, telmisartan, to ApoE -/- mice at a dose that did not lower blood pressure suppressed atherosclerotic lesion formation.<sup>30</sup> Collectively, these results suggest that reductions in blood pressure in AT1a receptor-deficient mice were not the primary mechanism for ablation of AngII-induced atherosclerosis and/or AAA formation in AT1a receptor-deficient mice.

Both AngII-induced atherosclerosis and AAAs are characterized by complex changes of many cell types that are both resident and infiltrates of the arterial wall. The results from whole body AT1a receptor-deficient mice demonstrate the dominant role of this protein, but do not indicate which AT1a receptor expressing cell types are interacting with AngII to generate the vascular disease. One commonly used experimental mode of discriminating the role of infiltrating cells has been use of bone marrow cell transplantation into irradiated recipients.<sup>31,32</sup> To assist in interpretation of this experiment, we irradiated both AT1a receptor +/+ and -/- mice and repopulated with bone marrow-derived cells from these two genotypes, creating 4 groups of chimeric mice. All the donor mice were LDL receptor-deficient to circumvent the possibility of its presence influencing the development of atherosclerosis, even though this is unlikely in LDL receptor-/- recipients.<sup>33-35</sup> If bone marrow-derived stem cells are critical to AngII-induced vascular disease, then reductions in disease with transplantation of AT1a-/- donors into +/+ recipients should be balanced by restoration of disease when +/+ donors are transplanted into -/- recipients. Using this approach, our results clearly demonstrate that AT1a receptors in the recipient are required for the initiation of AngII-induced vascular diseases, as absence of vascular pathology in AT1a receptor-/- recipients could not be overcome by AT1a receptor expression in bone marrow-derived stem cells. In AT1a receptor +/+ recipients, the repopulation with AT1a receptor-/- cells led to some attenuation of AngII-induced atherosclerosis. Thus, it appears that AT1a receptors on the recipient are required for the initiating events in the development of atherosclerosis. However, AT1a receptor expression on donor cells can exert some modulation of the development of lesion formation, but are insufficient to initiate the disease.

Although the absence of AT1a receptors on recipients had similar effects on development of both AngII-induced atherosclerosis and AAAs, this may have occurred via independent mechanisms. AngII-induced atherosclerosis is characterized by intimal accumulation of lipid-laden macrophages, in which the endothelium is a major regulator in the attraction and adherence of monocytes.<sup>2</sup> In contrast, the earliest detectable cellular event in AngII-induced AAAs is a medial accumulation of macrophages, which may be regulated by smooth muscle cells.<sup>5</sup> This localization may be attributable to local effects of AngII on smooth muscle cells to promote monocyte chemotaxis. Regulation of the extracellular proteolytic environment of smooth muscle cells is known to regulate AAA production.<sup>36</sup> The development of AT1a receptor floxed mice to use in conjunction with cell-specific *Cre* transgenic mice will enable further refinement of the cell type(s) expressing AT1a receptors that modulate the development of these diverse AngII-induced vascular diseases.

In conclusion, the present study demonstrated that the AT1a receptor subtype on resident host cells mediates AngII-induced vascular disease. However, AT1a receptors on bone marrow-derived cells may modulate AngII-induced atherosclerosis. Future studies will define the relative role of AT1a receptors on specific vascular wall cells in AngII-induced atherosclerosis and AAA formation.

## Acknowledgments

We acknowledge the excellent technical assistance of Deborah Howatt and Jessica Moorleghen.

## Sources of Funding

This work was supported by grants from the National Institutes of Health (RO1 HL62846 and HL58205).

## Disclosures

None.

## References

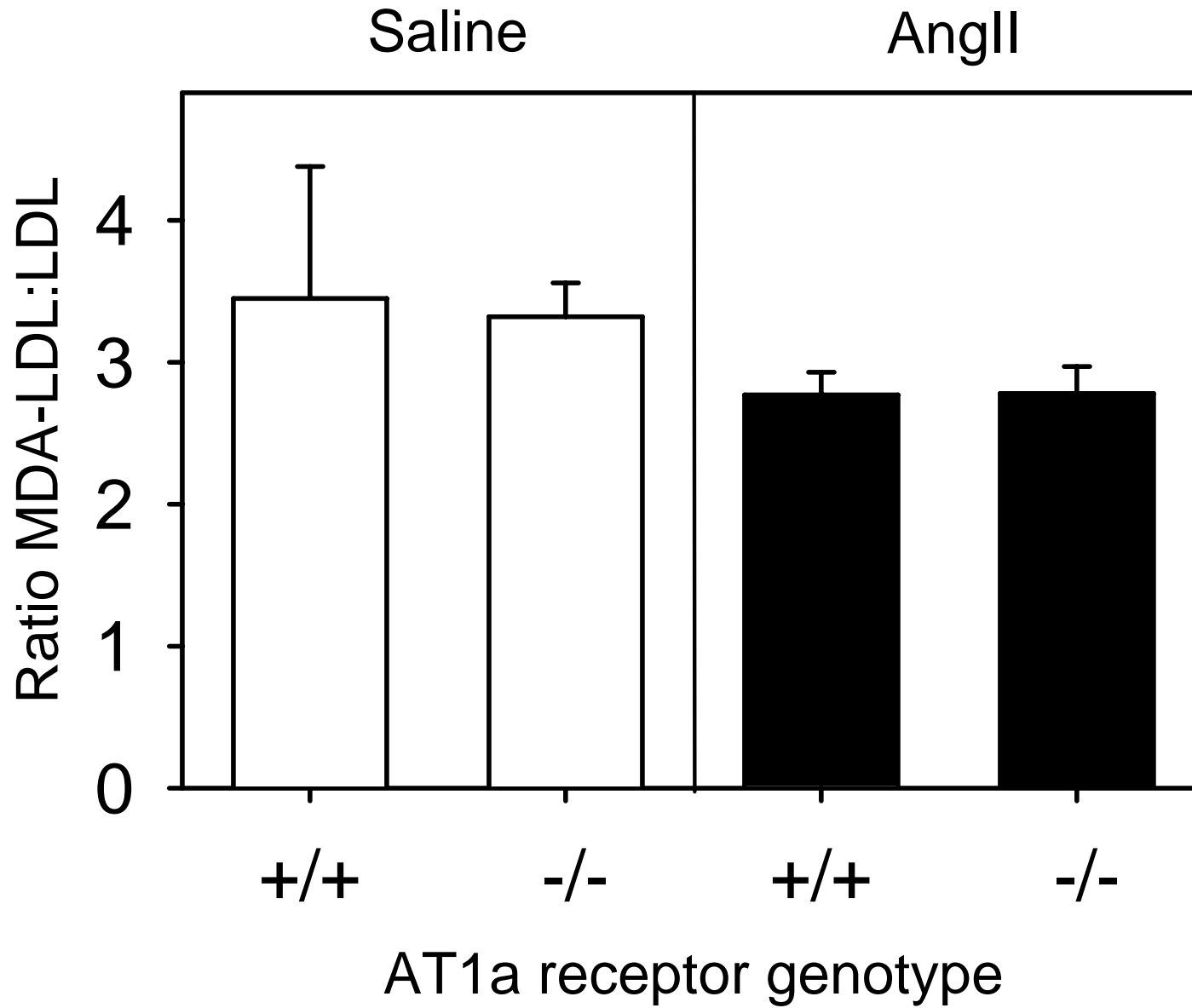
- Daugherty A, Cassis L. Chronic angiotensin II infusion promotes atherogenesis in low density lipoprotein receptor -/- mice. *Ann NY Acad Sci.* 1999;892:108-118.
- Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J Clin Invest.* 2000;105:1605-1612.
- Weiss D, Kools JJ, Taylor WR. Angiotensin II-induced hypertension accelerates the development of atherosclerosis in ApoE-deficient mice. *Circulation.* 2001;103:448-454.
- Wang YX, Martin McNulty B, Freay AD, Sukovich DA, Halks Miller M, Li WW, Vergona R, Sullivan ME, Morser J, Dole WP, Deng GG. Angiotensin II increases urokinase-type plasminogen activator expression and induces aneurysm in the abdominal aorta of apolipoprotein E-deficient mice. *Am J Pathol.* 2001;159:1455-1464.
- 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.
- Henriques TA, Huang J, D'Souza SS, Daugherty A, Cassis LA. Orchiectomy, but not Ovariectomy, Regulates Angiotensin II-Induced Vascular Diseases in Apolipoprotein E Deficient Mice. *Endocrinology.* 2004;145:3866-3872.
- Daugherty A, Manning MW, Cassis LA. Antagonism of AT2 receptors augments Angiotensin II-induced abdominal aortic aneurysms and atherosclerosis. *Br J Pharmacol.* 2001;134:865-870.
- 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.
- Okamura A, Rakugi H, Ohishi M, Yanagitani Y, Takiuchi S, Moriguchi K, Fennessy PA, Higaki J, Ogihara T. Upregulation of renin-angiotensin system during differentiation of monocytes to macrophages. *J Hyperten.* 1999;17:537-545.
- Yanagitani Y, Rakugi H, Okamura A, Moriguchi K, Takiuchi S, Ohishi M, Suzuki K, Higaki J, Ogihara T. Angiotensin II type 1 receptor-mediated peroxide production in human macrophages. *Hypertension.* 1999;33:335-339.
- Keidar S, Attias J, Heinrich R, Coleman R, Aviram M. Angiotensin II atherogenicity in apolipoprotein E deficient mice is associated with increased cellular cholesterol biosynthesis. *Atherosclerosis.* 1999;146:249-257.
- Keidar S, Kaplan M, Hoffman A, Aviram M. Angiotensin II stimulates macrophage-mediated oxidation of low density lipoproteins. *Atherosclerosis.* 1995;115:201-215.
- Kaplan M, Aviram M, Knopf C, Keidar S. Angiotensin II reduces macrophage cholesterol efflux: A role for the AT-1 receptor but not for the ABC1 transporter. *Biochem Biophys Res Commun.* 2002;290:1529-1534.
- Kim JA, Berliner JA, Nadler JL. Angiotensin II increases monocyte binding to endothelial cells. *Biochem Biophys Res Commun.* 1996;226:862-868.
- Grafe M, Auch-Schwelk W, Zakrzewicz A, Regitz-Zagrosek V, Bartsch P, Graf K, Loebe M, Gaehtgens P, Fleck E. Angiotensin II-induced leukocyte adhesion on human coronary endothelial cells is mediated by E-selectin. *Circ Res.* 1997;81:804-811.
- Pueyo ME, Gonzalez W, Nicoletti A, Savoie F, Arnal JF, Michel JB. Angiotensin II stimulates endothelial vascular cell adhesion molecule-1 via nuclear factor-kappa B activation induced by intracellular oxidative stress. *Arterioscler Thromb Vasc Biol.* 2000;20:645-651.
- Chen XL, Tummala PE, Olbrych MT, Alexander RW, Medford RM. Angiotensin II induces monocyte chemoattractant protein-1 gene expression in rat vascular smooth muscle cells. *Circ Res.* 1998;83:952-959.

18. Griending KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res.* 1994;74:1141–1418.
19. 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.
20. King VL, Szilvassy SJ, Daugherty A. Interleukin-4 deficiency decreases atherosclerotic lesion formation in a site-specific manner in female LDL receptor<sup>-/-</sup> mice. *Arterioscler Thromb Vasc Biol.* 2002;22:456–461.
21. Whitman SC, Rateri DL, Szilvassy SJ, Yokoyama W, Daugherty A. A depletion of natural killer cell function decreases atherosclerosis in low-density lipoprotein receptor null mice. *Arterioscler Thromb Vasc Biol.* 2004;24:1049–1054.
22. Whitman SC, Rateri DL, Szilvassy SJ, Cornicelli JA, Daugherty A. Macrophage-specific expression of class A scavenger receptors in LDL receptor<sup>-/-</sup> mice decreases atherosclerosis and changes spleen morphology. *J Lipid Res.* 2002;43:1201–1208.
23. Daugherty A, Pure E, Delfel-Butteiger D, Chen S, Leferovich J, Roselaar SE, Rader DJ. The effects of total lymphocyte deficiency on the extent of atherosclerosis in apolipoprotein E<sup>-/-</sup> mice. *J Clin Invest.* 1997;100:1575–1580.
24. Daugherty A, Whitman SC. Quantification of atherosclerosis in mice. *Methods Mol Biol.* 2003;209:293–309.
25. Wang YX, Cassis LA, Daugherty A. Angiotensin II-induced abdominal aortic aneurysms. Xu Q, ed. *A Handbook of Mouse Models for Cardiovascular Disease.* Chichester, UK: John Wiley & Sons. 125–136.
26. Roselaar SE, Kakkanathu PX, Daugherty A. Lymphocyte populations in atherosclerotic lesions of apoE<sup>-/-</sup> and LDL receptor<sup>-/-</sup> mice. Decreasing density with disease progression. *Arterioscler Thromb Vasc Biol.* 1996;16:1013–1018.
27. Wassmann S, Hilgers S, Laufs U, Bohm M, Nickenig G. Angiotensin II type 1 receptor antagonism improves hypercholesterolemia-associated endothelial dysfunction. *Arterioscler Thromb Vasc Biol.* 2002;22:1208–1212.
28. 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.
29. Ayabe N, Babaev VR, Tang Y, Tanizawa T, Fogo AB, Linton MF, Ichikawa I, Fazio S, Kon V. Transiently heightened angiotensin II has distinct effects on atherosclerosis and aneurysm formation in hyperlipidemic mice. *Atherosclerosis.* 2005;184:312–321.
30. Takaya T, Kawashima S, Shinohara M, Yamashita T, Toh R, Sasaki N, Inoue N, Hirata KI, Yokoyama M. Angiotensin II type 1 receptor blocker telmisartan suppresses superoxide production and reduces atherosclerotic lesion formation in apolipoprotein E-deficient mice. *Atherosclerosis.* 2006;186:402–410.
31. Linton MF, Atkinson JB, Fazio S. Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. *Science.* 1995;267:1034–1037.
32. Boisvert WA, Spangenberg J, Curtiss LK. Treatment of severe hypercholesterolemia in apolipoprotein E-deficient mice by bone marrow transplantation. *J Clin Invest.* 1995;96:1118–1124.
33. Linton MF, Babaev VR, Gleaves LA, Fazio S. A direct role for the macrophage low density lipoprotein receptor in atherosclerotic lesion formation. *J Biol Chem.* 1999;274:19204–19210.
34. Boisvert WA, Spangenberg J, Curtiss LK. Role of leukocyte-specific LDL receptors on plasma lipoprotein cholesterol and atherosclerosis in mice. *Arterioscler Thromb Vasc Biol.* 1997;17:340–347.
35. Herijgers N, van Eck M, Groot PHE, Hoogerbrugge PM, van Berkel TJC. Low density lipoprotein receptor of macrophages facilitates atherosclerotic lesion formation in C57B1/6 mice. *Arterioscler Thromb Vasc Biol.* 2000;20:1961–1967.
36. 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.

## Online Figure Legends

- Figure I** *Titers of autoantibodies against MDA-modified LDL in LDL receptor -/- mice of AT1a +/+ or -/- genotype.* Serum (1:1,000 dilution) was incubated with wells coated with LDL or MDA-LDL and titers of IgG were determined. Data are presented as the ratio of chromagen development in (A) native mice and (B) irradiated mice repopulated with bone marrow derived cells of the indicated AT1a receptor genotype. Histograms represent the mean data from at least 5 individual mice, and bars are SEM. There were no significant differences between genotypes or infusions.
- Figure II** *Relative percent of blood-borne leukocytes immunostained for CD45 allelic variants after the repopulation of irradiated mice.* C57BL/6 mice (CD45.2) were irradiated and repopulated with  $1 \times 10^7$  bone marrow derived donor cells harvested from a congenic strain expressing CD45.1. Blood was collected at the indicated intervals and FACS performed with isoform specific antibodies. Immunostaining of leukocytes for the donor cells (CD45.1) are represented by the closed circles, and recipient cells (CD45.2) are represented by open circles. Each point represents the mean of determination on at least 5 mice and bars are SEM.
- Figure III** *PCR genotyping of bone marrow of chimeric mice created by the bone marrow transplantation procedure.* PCR on bone marrow derived DNA yielded an amplicon of 650 bp for the wild type AT1a receptor and ~1.1 kb for the disrupted gene.
- Figure IV** *Tissue sections of suprarenal aortas from chimeric mice.* Tissue sections are shown of the suprarenal aorta of irradiated AT1a receptor +/+ or -/- mice that were repopulated with bone marrow derived cells from AT1a receptor +/+ and -/-mice. The chimeric mice were infused with AngII (1,000 ng/kg/min) for 28 weeks. The tissue sections were stained with Movat's pentachrome. Photos are examples that were taken from the site of maximal dilation for each group.

# Online Figure IA

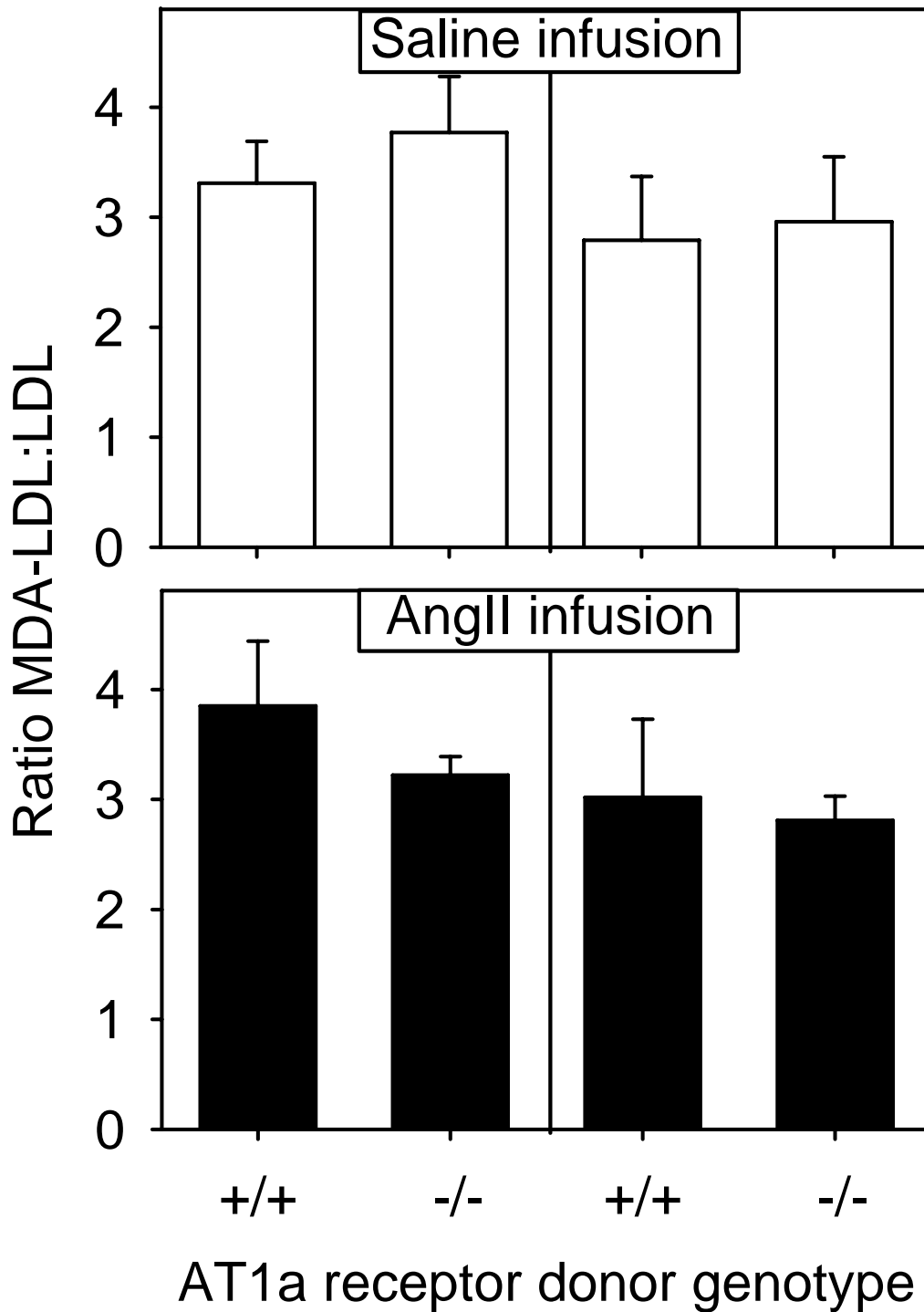


# Online Figure IB

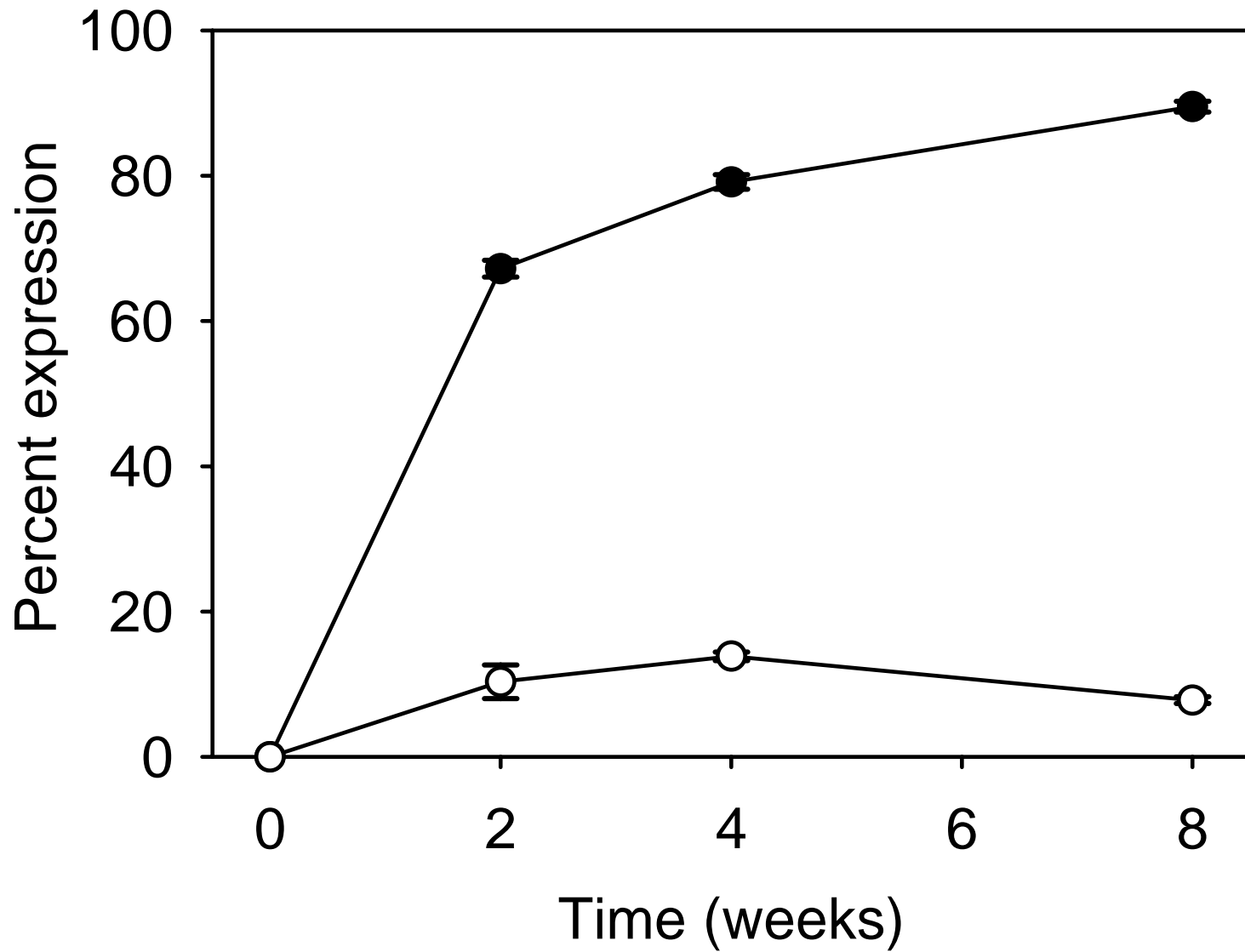
AT1a receptor recipient genotype

+/+

-/-



# Online Figure II



# Figure III

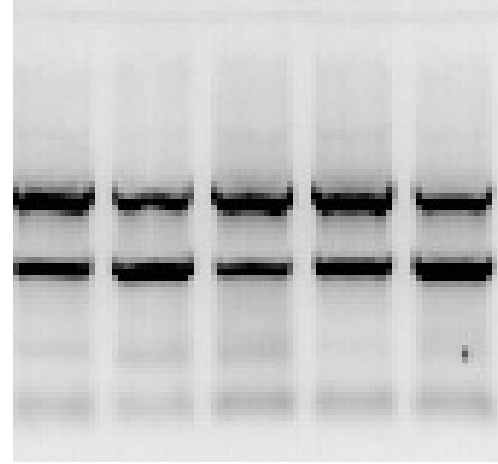
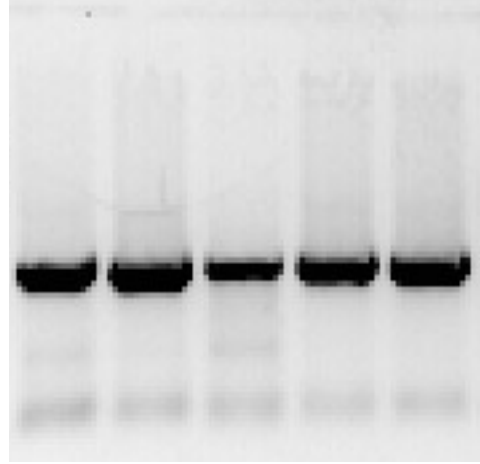
AT1a receptor donor genotype

$+/+$

$-/-$

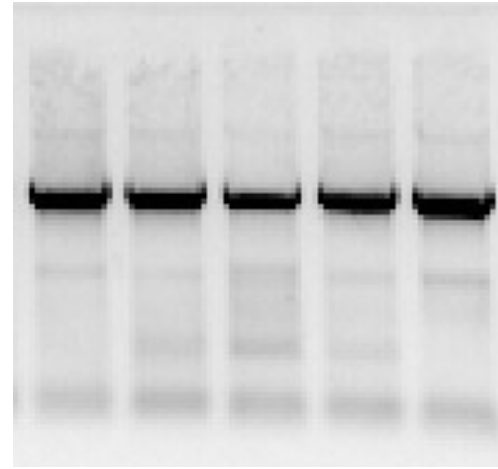
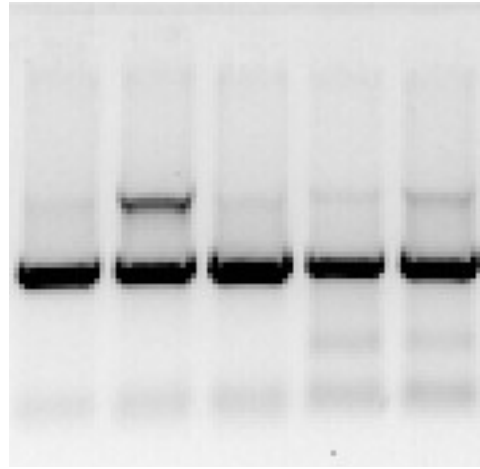
AT1a receptor recipient genotype

$+/+$



← Disrupted  
← Wild type

$-/-$



← Disrupted  
← Wild type

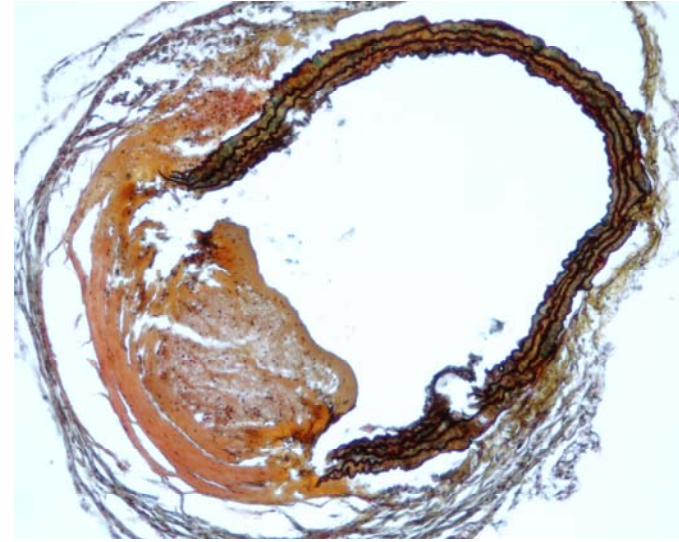
# Online Figure IV

AT1a receptor recipient genotype

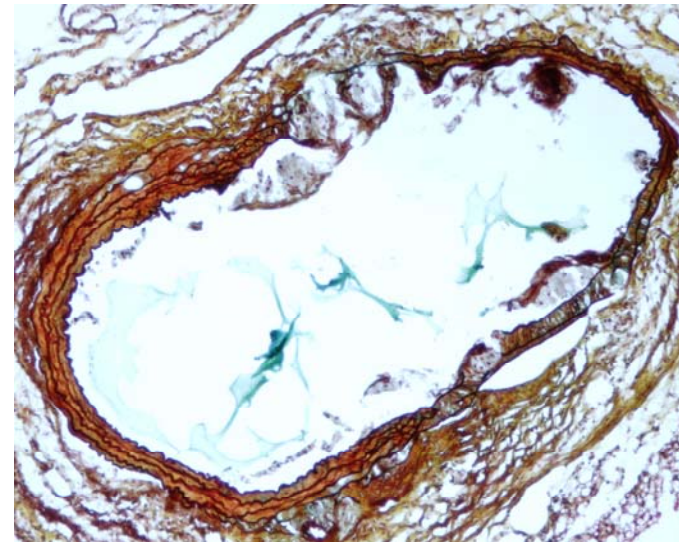
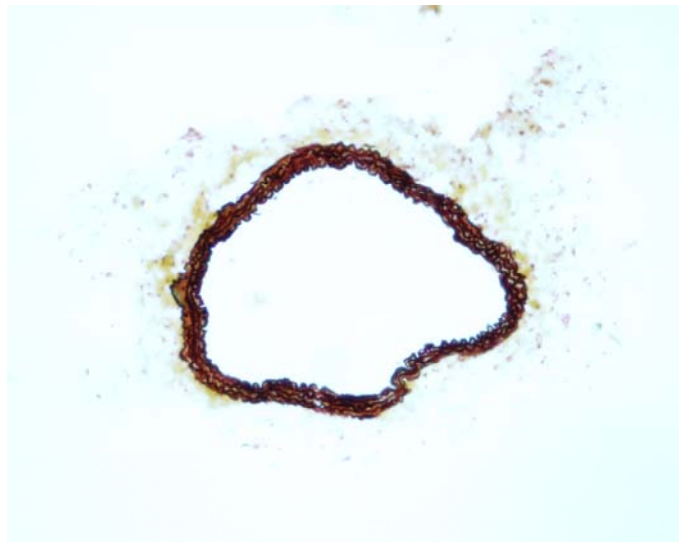
-/-

+/+

-/-



+/+



AT1a receptor donor genotype