Angiotensin II Amplifies Macrophage-Driven Atherosclerosis

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Objective—We evaluated the role of angiotensin II (AII) in a marrow-derived macrophage-driven model of atherosclerosis.

Methods and Results—Eight-week-old C57BL/6 wild-type mice were reconstituted with bone marrow harvested from apolipoprotein E-deficient (apoE−/−→apoE+/+) or wild-type for apoE gene (apoE+/+→apoE+/+) mice. At 20 weeks, mice were exposed to either AII (1000 ng/kg per minute subcutaneously) or saline for 2 weeks. Animal did not differ in body weight, blood pressure, cholesterol/triglycerides, or peripheral blood monocyte count. ApoE−/−→apoE+/+ mice exposed to AII had 3-fold greater atherosclerotic area than saline-treated apoE−/−→apoE+/+ mice. By contrast, AII did not affect atherosclerosis in apoE+/+→apoE+/+ mice. Macrophage-positive areas were increased by AII in mice reconstituted with either apoE-deficient or apoE-competent marrow. AII also significantly increased fragmentation of elastin laminae in both apoE−/−→apoE+/+ and apoE+/+→apoE+/+ mice. In vitro, AII caused greater increase in monocyte chemoattractant protein-1–stimulated migration of macrophages harvested from AII-infused versus saline-infused mice.

Conclusion—The current studies reveal that AII has both initiating and sustaining proatherogenic effects; by promoting macrophage migration into the vascular intima, AII is pivotal in initiating atherosclerosis. By promoting elastin breaks, a novel mechanism implicated in migration and proliferation of smooth muscle cells, AII may be pivotal in subsequent development and expansion of atherosclerotic lesion. (Arterioscler Thromb Vasc Biol. 2004;24:1-6.)

Key Words:

Elevation in angiotensin II (AII) amplifies atherosclerosis in animal models, conversely, AII inhibition lessens progression of atherosclerotic lesions in animal models and reduces the number of acute cardiovascular sequelae of atherosclerosis in humans. The underlying mechanisms for these effects remain unclear. AII promotion of atherosclerosis is thought to involve activation of the proinflammatory response of the vessel wall while its inhibition quells this response. Specifically, AII has been shown to stimulate adhesion molecules in vascular endothelium including vascular cell adhesion molecule-1, intercellular adhesion molecule-1, chemotactic factors including monocyte chemoattractant protein-1 (MCP-1), cytokines, and growth factors including IL-1, tumor necrosis factor-α, colony-stimulating factor, platelet-derived growth factor, transforming growth factor-β, as well as reactive species that promote monocyte infiltration and smooth muscle migration into the subendothelial space. These cells then take-up lipids to become foam cells that coalesce into atherosclerotic lesions. The pivotal role of the monocyte/macrophage in the initiation and progression of the atherosclerotic lesion has been demonstrated by studies showing that disruption of cytokines, chemokines, and adhesion molecules that lessen monocyte/macrophage recruitment also abrogate lesion development. Although much of the pro-atherogenesis of AII has been linked to its proinflammatory and oxidative effects on cytokine, chemokine, and adhesion molecule interaction with endothelial cells and smooth muscle cells, little is known about the potential effects and interactions of AII on the monocyte/macrophage component of atherogenesis. We and others have previously shown that reconstitution of wild-type mice with bone marrow cells from apolipoprotein E (apoE)-deficient mice promotes atherosclerosis. Moreover, a significantly larger lesion area was occupied by macrophages in mice reconstituted with apoE-deficient marrow than wild-type marrow. These findings not only illustrated the salient protective role of macrophage apoE but also reiterated the central role of monocyte recruitment in development of the early atherosclerotic lesion. The current studies were designed to examine if AII, which can modulate several processes involved in initiation and progression of atherosclerotic lesion, can influence a macrophage-driven model of atherosclerosis.

Methods

Mice

C57BL/6 and apoE−/− mice were obtained from Jackson Laboratories (Bar Arbor, Me). All mice were maintained on a 12-hour light/12-hour dark cycle and fed normal mouse chow (RP5015; PMI Feeds Inc, St. Louis, Mo). Animal care and the experimental
procedures were performed in accordance with National Institutes of Health and Vanderbilt University animal care facility guidelines. Group 1 mice were C57BL/6 recipients transplanted with apoE−/− bone marrow (apoE−/− to apoE−/+; n=27); Group 2 mice were C57BL/6 recipients transplanted with C57BL/6 bone marrow (apoE+/+ to apoE−/+; n=18).

**Bone Marrow Transplantation**

Eight-week-old female C57BL/6 mice were used as recipients. They were maintained on acidified water (pH 2.0) containing 100 mg/neomycin and 10 mg/polyoxylin B for 3 days before and 14 days after transplantation. Four hours before transplantation, recipient mice were lethally irradiated with 900 rads from a cesium gamma source. Bone marrow was harvested from femurs of either male C57BL/6 or female apoE−/− mice. Each recipient received 5×10^6 bone marrow cells in 0.2 mL as previously described.12 After transplantation, mice were maintained on regular mouse chow for the next 6 weeks when they were switched to a high-fat diet (ICN) containing 19.5% fat and 1.25% cholesterol that was continued for the next 8 weeks until the end of the study. Identification of cellular genotype of transplanted mice was verified by reverse-transcriptase polymerase chain reaction using total RNA extracted from the recipient bone marrow at euthanization as previously described for apoE−/− and Zfy mice.13–15

**Infusion of AII**

Two weeks before euthanization, all animals were subcutaneously implanted under isoflurane anesthesia with Alzet osmotic minipumps (model 1002; Duract Corporation, Cupertino, Calif). In group 1 (apoE−/− to apoE−/+), 17 mice received AII (1000 ng/min per kg) and 10 were infused with saline. In group II (apoE−/+ to apoE−/+), 10 mice received AII (1000 ng/min per kg) and 8 were infused with saline.

**Determination of Blood Pressure and Serum Lipids**

Systolic blood pressure analysis was determined at the end of AII or saline infusions using the Muramachi Systems (Model MK-2000; Muramachi Kikai Co, Osaka, Japan) automated tail-cuff system in conscious, trained mice. Serum cholesterol and triglycerides were determined as previously described.12

**Quantitation of Arterial Atherosclerotic Lesions**

Fourteen weeks after bone marrow transplantation (after 2 weeks of AII or saline infusion), the hearts and proximal aorta were embedded in OCT and snap-frozen in liquid nitrogen (LN2). Cryosections, 4 μm thick, were sectioned from the proximal aorta beginning at the end of the study (apoE−/−) and Zfy mice.13–15

**Immunostaining**

Serial cryosections 5–μm thick were sectioned from the proximal aorta, fixed in acetone, and incubated with monoclonal rat antibody to mouse macrophages, MOMA-2 (Serotec, Raleigh, NC) and goat anti-smooth muscle α-actin (Santa Cruz Biotechnology, Santa Cruz, Calif). The area stained with MOMA-2 or α-smooth muscle actin were measured using Imaging System KS300 and the percentage of macrophage infiltration or α-smooth muscle actin calculated as a ratio of the macrophage-stained area of the oil-red-O stained area as previously published.12,14,15

**Elastin Fragmentation**

Fragmentation (degradation) of elastin was assessed by counting the number of breaks in aortic elastic laminae stained with Verhoeff–van Gieson as previously described.16 We assessed at least 5 serial sections from each of 4 regions of the aorta including aortic arch, thoracic, suprarenal, and infrarenal areas. The number of breaks was expressed per medial area, which was taken to be the area between the internal and external elastic laminae.

**Determination of Macrophage Migrating Activity**

Peritoneal macrophages were collected from additional wild-type mice or from angiotensin receptor type 1-deficient (Agtr1−/−) mice generated in our laboratory17 that were infused with AII (1000 ng/min per kg) (n=4 each genotype) or saline (n=4 each genotype) for 2 weeks as described. Four days before euthanization, mice underwent peritoneal injection of 3% thioglycollate and were harvested as previously described.18 Ex vivo studies of these cells exposed in vivo to AII or saline were performed in 96-well microchemotaxis chamber in which the upper compartment was separated from the lower compartment by an uncoated polycarbonate filter (5-μm pore diameter; Neuroprobe, Cabin John, Md). The cells were suspended in DMEM without fetal bovine serum and added to the upper compartment at a density of 5×10^4 cells per well. Chemottractant 0.1 μg/mL of MCP-1 (recombinant murine JE; Peprotech Inc, Rocky Hill, NJ) was added to the lower compartment. Migrated cells adhering to the lower surface of the membrane were counted manually under the microscope. Quadruplicate wells were used for each experimental condition, and ≥3 fields (×400) were counted for each well.

**3H-Cholesterol Efflux Studies in Macrophages**

Cholesterol efflux in ex vivo macrophages harvested from wild-type (n=10) or apoE−/− (n=4) mice infused with AII (1000 ng/min per kg) or saline for 14 days was determined by a modified procedure of Lin.19 Fractionation of lipoproteins from fasted normolipidemic human volunteers was performed by raising the density of serum to 1.019 g/mL for very-low-density lipoprotein, 1.040 for low-density lipoprotein, and 1.210 for high-density lipoprotein. Acetylated-density lipoprotein was prepared by repeated addition of acetic anhydride (Sigma, St. Louis, Mo). Quantitative analysis of lesions was performed using Imaging System KS300 (Release 2.0; Kontron Elektronik) on at least 15 sections from each animal12–15 by an operator who was blinded to group assignment.

**Statistical Analysis**

Results are expressed as mean±SEM. Statistical difference was assessed by a single-factor variance (analysis of variance [ANOVA]) followed by unpaired t test as appropriate. Nonparametric data were compared by Mann–Whitney U test. P<0.05 was considered to be significant.

**Whole Animal Studies**

Polymerase chain reaction analysis of bone marrow DNA extracted from recipient mice 14 weeks after transplantation showed complete replacement with apoE-deficient (apoE−/− to apoE−/+ or Zfy (apoE−/+ male → apoE−/+ female) marrow indicating that the recipient marrow population had been reconstituted by donor bone marrow. Body weight was comparable in the 2 groups before AII or saline infusion and increased similarly over the length of the study (apoE−/− to apoE−/+ AII weighed 17.2±0.4 grams plus saline weighed 18.1±0.5 grams, P=NS;
apoE+/+ → apoE+/+ + AII weighed 18.3±0.3 grams and saline weighed 18.3±0.3 grams, \( P=NS \).

Serum lipids were not affected by exposure to AII in mice reconstituted with either bone marrow. At euthanization, apoE−/−→apoE+/+ AII-infused mice had serum cholesterol of 178.7±11.2 mg/dL and triglyceride of 107.5±10.5 mg/dL, whereas in saline-infused mice, cholesterol level was 172.7±22.3 mg/dL (\( P=NS \)) and triglyceride was 103.9±5.0 mg/dL (\( P=NS \)). Similarly, at euthanization, apoE+/+ → apoE+/+ plus AII had serum cholesterol of 175.6±19.7 mg/dL and triglyceride of 94.8±7.4 mg/dL, not different than in saline-infused mice (cholesterol of 184.8±5.7 mg/dL and triglyceride of 104.1±11.0 mg/dL, \( P=NS \)).

The systemic blood pressure was not different between mice exposed to AII versus saline. In apoE−/−→apoE+/+ plus AII, the blood pressure was 118.7±2.8 mm Hg compared with 116.8±3.8 in saline-infused mice (\( P=NS \)). In apoE+/+ → apoE+/+ plus AII, the blood pressure was 112.5±2.8 mm Hg compared with 113.0±2.2 in saline-infused mice (\( P=NS \)). At euthanization, the peripheral white blood cell, neutrophil, monocyte, and red blood cell counts were similar in all groups except for the total white blood cell count in apoE+/+ → apoE+/+ mice exposed to AII. In apoE−/−→apoE+/+ plus AII, white blood cell, neutrophil, monocyte, and red blood cell counts were 11.4±1.9×10⁶/μL, 0.3±0.1%, 2.6±0.5%, and 10.4±0.4×10⁶/μL, whereas, in saline-infused mice these values were 14.3±2.1 (\( P=NS \)), 0.3±0.1 (\( P=NS \)), 2.3±0.3 (\( P=NS \)), and 9.6±0.5 (\( P=NS \)). In apoE+/+ → apoE+/+ plus AII, white blood cell, neutrophil, monocyte, and red blood cell counts were 19.0±2.0×10⁶/μL, 0.4±0.1%, 2.9±0.3%, and 10.6±0.4×10⁶/μL, whereas in saline-infused mice, these values were 10.6±1.2 (\( P<0.05 \) versus apoE+/+ → apoE+/+ + AII), 0.1±0.03 (\( P=NS \)), 2.4±0.5 (\( P=NS \)), and 10.1±0.3 (\( P=NS \)). There were no apparent infections or other apparent reasons for this increased white cell count in the apoE+/+ → apoE+/+ mice infused with AII.

**Atherosclerosis**

Two weeks of AII infusion did not change the extent of atherosclerotic lesions in wild-type bone marrow-reconstituted mice. Thus, at the end of AII infusion, the mean lesion area of 3862±1725 μm² was not different from mice infused with saline (3723±1387 μm², \( P=NS \); Figure 1). By contrast, apoE−/−→apoE+/+ plus AII had 3-times greater atherosclerotic lesions in the proximal aorta than apoE−/−→apoE+/+ mice exposed to saline. Mean lesion area in AII-infused apoE−/−→apoE+/+ mice was 1917±6550 versus 6099±1647 μm² in apoE−/−→apoE+/+ animals infused with saline (\( P=0.003 \)). The proximal aortic lesion after AII in apoE−/−→apoE+/+ animals was also significantly greater than in either AII-exposed (1917±6550 versus 3862±1725 μm², \( P=0.0002 \)) or saline-exposed (1917±6550 versus 3723±1387 μm², \( P<0.001 \)) apoE+/+ → apoE+/+ mice. These observations indicate that AII exposure with high-fat diet amplifies atherosclerotic progression in mice reconstituted with apoE-deficient bone marrow, but not in mice reconstituted with wild-type bone marrow.

**Atherosclerotic Lesion Characteristics**

To analyze the effects of AII exposure on macrophages in the lesions, immunohistochemical staining for macrophages was performed and revealed more staining in apoE−/−→apoE+/+ plus AII than saline (90.8±6.6 MOMA positivity of total lipid stained area versus 57.7±4.8, \( P=0.030 \); Figure 2A). All also caused more macrophage-positive area in lesions of apoE+/+ → apoE+/+ mice (67.5%±13.9 of total lipid area stained macrophage positive versus 20.6±10.2, \( P=0.019 \)). Thus, AII exposure caused increased vascular macrophage infiltration regardless of the type of marrow that was transplanted.
All increase in fragmentation of the elastic laminae also did not depend on the origin of the donor bone marrow. Compared with apoE⁻/⁻ → apoE⁺/+ mice infused with saline, apoE⁻/⁻ → apoE⁺/+ plus AII had 60% more breaks in elastin laminae ($P=0.009$; Figure 2B). Similarly, all increased elastin breaks in apoE⁺/+ → apoE⁺/+ mice, which increased by 30% above the saline-infused apoE⁺/+ → apoE⁺/+ group ($P=0.049$). This elastolytic effect was not confined to any region, but rather uniform destruction along the length of the aorta. In view of these findings that AII causes elastin breaks, and the recent recognition that elastin degradation per se can stimulate smooth muscle cell migration and proliferation, we assessed α-smooth muscle actin in AII versus saline-exposed apoE⁻/⁻ → apoE⁺/+ mice. However, comparison of α-smooth muscle actin immunostaining in apoE⁻/⁻ → apoE⁺/+ plus AII versus saline showed no difference at this early stage of the atherosclerotic lesion.

### Macrophage Migrating Activity

In the absence of MCP-1, there was no difference in migration between macrophages harvested from mice infused with saline or AII (Figure 3). Exposure of macrophages from saline-infused mice to MCP-1 caused a significant increase in the chemotactic response, from 6.6 ± 0.3% at 6 hours and 21.1 ± 0.4% at 24 hours in cells from AII-exposed mice versus 4.7 ± 0.2% ($P<0.05$) and 16.8 ± 0.3% ($P<0.05$) in cells from saline-exposed mice. Macrophage from apoE⁻/⁻ mice exposed to AII or saline had similar efflux at these time points, ie, 5.3 ± 0.9% at 6 hours and 15.5 ± 1.8% at 24 hours in cells from AII-exposed apoE⁻/⁻ mice versus 5.3 ± 0.9% ($P=NS$) and 14.9 ± 1.3% ($P=NS$) in saline-exposed apoE⁻/⁻ mice.

### Discussion

Although AII has been shown to contribute to atherosclerosis in genetically engineered hyperlipidemic mouse models and cholesterol-fed susceptible strains of rabbits and primes, the current studies reveal for the first time to our knowledge that AII amplifies macrophage-driven atherosclerosis. The results show that even short-term 2-week exposure to AII amplifies the proatherogenic effects imparted by disruption of local macrophage production of apoE. Thus, compared with apoE⁻/⁻ reconstituted mice infused with saline, apoE⁻/⁻ reconstituted mice infused with AII had 3-times as much atherosclerosis. This proatherogenic effect occurred in the absence of changes in systemic blood pressure, serum lipids, or peripheral blood cell counts between the AII-infused and saline-infused mice. Notably, 2 weeks of AII in a hyperlipidemic environment did not amplify atherosclerotic lesions in mice reconstituted with wild-type marrow. These findings reiterate the pivotal role for macrophage apoE, although they do not rule out the possibility that a more persistent elevation of AII on the background of a hyperlipidemic environment promotes atherosclerosis even in the absence of macrophages null for apoE. Such synergy between hyperlipidemia and AII may be especially germane if combined with other risk factors, such as elevation in blood pressure. Indeed, 14 weeks of atherogenic diet in hypertensive transgenic mice carrying both the human renin and angiotensinogen genes caused 4-times as much atherosclerosis than identically fed mice without activated renin-angiotensin system. In some circumstances, even transiently heightened AII sets up injurious processes. Thus, 2-week exposure of apoE-deficient hyperlipidemic mice to AII, which did not affect the extent of atherosclerotic lesions during the time of infusion, caused a dramatic increase in atherosclerosis months after cessation of exposure to AII. Together these observations suggest that AII amplifies atherosclerosis in various circumstances including hyperlipidemia, hypertension, and even in the setting of apoE-deficient macrophages. Interestingly, even though the short-term exposure to AII in the current study did not increase lipid deposition in vessels of mice reconstituted with wild-type marrow, AII encouraged intimal infiltration of both apoE-deficient and wild-type monocytes, suggesting that AII affects accumulation of monocytes and/or modulates the monocyte–vessel interaction.

Recruitment of monocytes is one of the earliest events in initiation of atherosclerotic lesion. Disruption in processes for

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**Figure 3.** Legend text.
monocyte recruitment abrogates initiation and progression of atherosclerosis, whereas neointimal formation and plaque progression is enhanced by processes that promote macrophage infiltration.\textsuperscript{9,10,21,22} The present studies show that AII promotes infiltration of macrophages. Thus, although 50\% of the lesion in apoE\textsuperscript{−/−} mice is occupied by macrophages, \textasciitilde 90\% of the lesion area of apoE\textsuperscript{−/−}→ apoE\textsuperscript{+/+} mice exposed to AII is macrophage-positive (Figure 2A). These findings indicate that factors that promote accumulation of abnormal macrophages unable to secrete apoE lead to more foam cell formation and promote atherosclerotic lesions. AII enhanced monocyte infiltration into the vessel wall not only in mice reconstituted with apoE-deficient marrow but also in mice reconstituted with wild-type marrow, although in the absence of the proatherogenic macrophages, increase in atherosclerosis did not ensue. Thus, apoE\textsuperscript{−/+}→ apoE\textsuperscript{+/+} exposed to AII had significantly more macrophage-positive area than apoE\textsuperscript{−/+}→ apoE\textsuperscript{+/+} mice infused with saline. Because oil-red-O staining was similar between these 2 groups of mice, it is possible that more of the lipids were contained within the macrophages. The association between AII and macrophage infiltration has been made in other circumstances, including renal fibrosis and in ischemic heart disease.\textsuperscript{23,24} The potentially injurious effect of AII has been linked to augmentation of inflammatory cytokines such as tumor necrosis factor-\textalpha, IL-6, and chemokines such as MCP-1 and CCR2 in the arterial wall, kidney, and heart. We and others have shown the pivotal effect of AII on MCP-1 and CCR2 in atherogenesis such that in vivo exposure to AII potentiates macrophage migration and that this response is mediated by the AGTR-1 on the macrophage.\textsuperscript{25} The increased elastolysis was associated with increased atherosclerosis only in the presence of proatherogenic apoE\textsuperscript{−/−} macrophages, suggesting that in the setting of wild-type macrophages, the AII-induced vascular damage is not sufficient to establish atherosclerosis. Nonetheless, in view of the recent recognition of elastolysis as a novel mechanism to stimulate migration and proliferation of smooth muscle cells,\textsuperscript{16,29} we hypothesize that as the lesion progresses, AII-induced elastin breaks will augment influx of smooth muscle cells from the tunica media into the intima. Determination of \alpha-actin–positive cells in the current study found no difference between AII-exposed and saline-exposed mice reconstituted with marrow, further underscoring the fact that the lesion is composed primarily of macrophage-generated foam cells and not smooth muscle cells. By contrast, preliminary studies in apoE-deficient mice with established atherosclerosis treated with the angiotensin receptor type 1 antagonist, losartan, for 3 months have dramatically less atherosclerosis, fewer individual lesions, fewer elastin breaks, and fewer \alpha-actin–positive cells in the intima of these lesions.\textsuperscript{30} It is noteworthy that antagonism of AII actions ameliorated ruptured arterial internal lamina in the Brown Norway rat, independently of blood pressure.\textsuperscript{31} Similarly, angiotensin inhibition prevented the increase in serum and aortic elastolytic activity in cholesterol-fed rabbits.\textsuperscript{32} These data point to a key role of intact versus injured elastin in maintaining vascular integrity.

In summary, these findings indicate that AII promotes macrophage migration into the vascular intima, which in the setting of disrupted macrophage function encourages formation of foam cells. In addition, AII causes elastin break that may be an important, novel mechanism to facilitate influx of smooth muscle cells, which expands atherosclerotic lesions.

Acknowledgments

This work was supported in part by grants from National Institutes of Health DK44757 (V.K.), DK37868 (I.I.), HL 53989 (M.F.L.), HL 65709, and 57986 (S.F.), The Lipid, Lipoprotein, and Atherosclerosis Core of the Vanderbilt Mouse Metabolic Phenotyping Center (National Institutes of Health DK59637–01), and the Clinical Nutrition Research Unit. The authors acknowledge the expert technical assistance of Cathy ZhiQi Xu and Youming Zhang.

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Arterioscler Thromb Vasc Biol. published online September 16, 2004; Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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