Contributions of Leukocyte Angiotensin-Converting Enzyme to Development of Atherosclerosis

Xiaofeng Chen, Hong Lu, Mingming Zhao, Katsuya Tashiro, Lisa A. Cassis, Alan Daugherty

Objective—This study determined the role of angiotensin-converting enzyme (ACE) on the development of angiotensin I–induced atherosclerosis and the contribution of leukocyte-specific expression of this enzyme.

Approach and Results—To define the contribution of ACE-dependent activity to angiotensin II synthesis in atherosclerotic development, male low-density lipoprotein receptor−/− mice were fed a fat-enriched diet and infused with either angiotensin I or angiotensin II. The same infusion rate of these peptides had equivalent effects on atherosclerotic development. Coinfusion of an ACE inhibitor, enalapril, ablated angiotensin I–augmented atherosclerosis but had no effect on angiotensin II–induced lesion development. ACE protein was detected in several cell types in atherosclerotic lesions, with a predominance in macrophages. This cell type secreted angiotensin II, which was ablated by ACE inhibition. To study whether leukocyte ACE contributed to atherosclerosis, irradiated male low-density lipoprotein receptor−/− mice were repopulated with bone marrow–derived cells from either ACE−/− or ACE+/− mice and fed the fat-enriched diet for 12 weeks. Chimeric mice with ACE deficiency in bone marrow–derived cells had modestly reduced atherosclerotic lesions in aortic arches but had no effects in aortic roots.

Conclusions—ACE mediates angiotensin I–induced atherosclerosis, and ACE expression in leukocytes modestly contributes to atherosclerotic development in hypercholesterolemic mice. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: angiotensins • angiotensin-converting enzyme inhibitors • atherosclerosis • hypercholesterolemia • macrophages • renin

R

ecent studies have demonstrated an evolving complexity of the renin–angiotensin system with a diversity of bioactive peptides that are derived from a multitude of enzymes.1 Angiotensin-converting enzyme (ACE) is considered the major enzyme responsible for converting the inactive decapeptide, angiotensin I (AngI), to the bioactive octapeptide, angiotensin II (AngII). AngII is a major bioactive component of the renin–angiotensin system and has been demonstrated as a crucial mediator for atherosclerotic lesion development.2 Direct evidence that AngII promotes atherosclerosis has been gained through chronic subcutaneous infusion of AngII into either apolipoprotein E−/− or low-density lipoprotein (LDL) receptor−/− mice.3,5 Conversely, pharmacological inhibition of AngII actions through AT1 receptor blockade profoundly decreases experimental atherosclerosis.4–6 Furthermore, genetic depletion of AT1a receptors markedly reduces hypercholesterolemia or AngII infusion–induced atherosclerosis.5,10 It is clear that AngII, either produced endogenously or administered exogenously, is a robust stimulus for atherosclerotic lesion formation. However, it is unclear whether the AngII precursor, AngI, augments lesion formation through an ACE-dependent mechanism.

The activity of ACE is considered the major systemic mechanism of converting AngI to AngII. However, several other enzymes also have dipeptidyl carboxylypeptidase activity within local tissue environments to convert AngI into AngII.11–13 These include chymase and cathepsin G. In addition, the activity of ACE is not exclusive to AngI conversion because it also cleaves many other peptides, including bradykinin, substance P, and N-acetyl-seryl-aspartyl-lysyl-proline. Given the promiscuity of substrates for ACE, the use of ACE inhibitors to inhibit the renin–angiotensin system pharmacologically in hypercholesterolemia-induced atherosclerosis does not indicate a definitive role for AngI to AngII conversion as the primary mechanism regulating lesion formation. ACE expression is most abundant in endothelial cells. However, it is also expressed in several different cell types of atherosclerotic lesions, such as smooth muscle cells and macrophages, in both animal and human atherosclerotic lesions.10,14,15 Macrophages have the potential to be a local source for AngII,
given the predominance of this cell type in lesions and the ability to synthesize this octapeptide through a renin-dependent mechanism. However, the role of ACE in macrophage AngII production and lesion formation has not been defined.

To define the role of ACE-mediated AngII production in experimental atherosclerosis, we initially determined effects of inhibition of this enzyme on lesion formation during infusion of AngI, the direct AngII precursor. ACE protein within atherosclerotic lesions was present in several cell types. Because some of the ACE within atherosclerotic lesions colocalized with CD68-immunostained cells, we explored the role of the enzyme in this cell type in vitro for AngII production and abundance of chemokines that modulate atherosclerosis. To determine the contribution of leukocyte expression of ACE to the development of atherosclerosis, we transplanted ACE−/− or ACE−/− bone marrow–derived cells into irradiated LDL receptor−/− mice. Although whole-body ACE inhibition profoundly reduced atherosclerosis in both the aortic root and arch regions, depletion of leukocyte ACE only modestly reduced atherosclerotic lesion in aortic arches, but had no effect in aortic roots.

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

Results
ACE-Dependent Effects on Atherosclerosis Were Attributed to Conversion of AngI to AngII
Many studies have demonstrated that chronic AngII infusion augments atherosclerosis. To determine whether ACE contributes to atherosclerosis in an AngII-dependent mechanism, male LDL receptor−/− mice were infused with AngI (the direct precursor of AngII) alone or confused with an ACE inhibitor, enalapril, for 4 weeks. The purpose of using exogenous infusion of AngI versus AngII was to dissect the effect clearly of ACE in the AngI–AngII axis on the development of atherosclerosis. Chronic infusion of AngI in the absence or presence of enalapril did not affect body weight and plasma cholesterol concentrations (Table II in the online-only Data Supplement) in LDL receptor−/− mice. AngI infusion led to pronounced increases in systolic blood pressure that were ablated by coinfusion of AngII and enalapril (Figure 1B). AngI infusion led to a 20% increase in systolic blood pressure that were ablated by coinfusion of AngII and enalapril for 4 weeks. The purpose of using exogenous infusion of AngI versus AngII was to dissect the effect clearly of ACE in the AngI–AngII axis on the development of atherosclerosis. Chronic infusion of AngI in the absence or presence of enalapril did not affect body weight and plasma cholesterol concentrations (Table II in the online-only Data Supplement) in LDL receptor−/− mice. AngI infusion led to pronounced increases in systolic blood pressure that were ablated by coinfection of enalapril (Table II in the online-only Data Supplement). AngI infusion increased percent atherosclerotic lesion area in both the aortic arch and the aortic root regions, whereas ACE inhibition abolished AngI-induced increases of percent lesion area in the aortic arch (Figure 1A) and mean lesion area in aortic roots (Figure I in online-only Data Supplement). There was no difference in lesion sizes between mice infused with vehicle and mice infused with both AngI and enalapril (Figure 1A and Figure I in online-only Data Supplement).

ACE acts on multiple substrates, and a previous study reported that ACE inhibition reduced atherosclerosis through an AngII-independent mechanism. Therefore, we determined whether ACE inhibition also reduced atherosclerosis in AngII-infused mice. Male LDL receptor−/− mice were infused with AngI, AngII, or coinfused with both AngII and enalapril for 4 weeks. Mice from the 3 groups had comparable body weight, systolic blood pressure, plasma cholesterol concentration, and low-density lipoprotein cholesterol distribution (data not shown). AngI infusion had an equivalent effect as AngII infusion on percent lesion area in the aortic arch. However, in contrast to ACE inhibition in AngII-infused mice, ACE inhibition did not alter AngII-induced augmentation of atherosclerotic lesions (Figure 1B).

Macrophages Secreted AngII Through an ACE-Dependent Mechanism
We have detected all classical components in the generation of AngII in mouse atherosclerotic lesions, which were primarily associated with macrophages. Among the renin–angiotensin system components, ACE immunoreactivity was ubiquitous in all cell types within atherosclerotic lesions and medial layers of the aorta (Figure 2 and Figure II in the online-only Data Supplement). Because ACE was abundant in macrophages of atherosclerotic lesions, and this cell type is predominant in atherosclerotic lesions, we determined whether cultured macrophages secreted AngII into culture media via an ACE-dependent mechanism. As determined by a high-performance liquid chromatographic resolution of angiotensin peptides followed by radioimmunoassay, AngII was secreted from cultured mouse macrophages. The secretion of AngII was abolished in cells incubated with an ACE inhibitor, captopril (Figure 3A).

AngII stimulates synthesis of an inflammatory chemokine, monocyte chemoattractant protein-1 (MCP-1), which plays a crucial role in the development of atherosclerosis by recruiting monocytes probably through interaction with CCR2. To determine whether ACE deficiency influenced
MCP-1 expression in macrophages, we examined abundance of MCP-1 mRNA and secreted protein in cultured peritoneal macrophages and bone marrow–derived cells harvested from ACE+/+ and ACE−/− mice. ACE deficiency profoundly reduced mRNA abundance (Figure 3B) and protein secretion (Figure 3C) of MCP-1 in peritoneal macrophages. Reductions of MCP-1 mRNA and protein abundance were also observed in cultured macrophages derived from bone marrow cells in ACE−/− mice (Figure III in the online-only Data Supplement).

ACE Deficiency in Bone Marrow–Derived Cells Modestly Reduced Atherosclerosis

Irradiated male LDL receptor−/− mice were repopulated with bone marrow–derived cells from either ACE+/+ or ACE−/− mice. Successful repopulation of donor cells was confirmed by ACE genotyping of genomic DNA from bone marrow–derived cells of recipient mice after termination (Figure 4A) and mRNA analyses of peritoneal macrophages from these recipient mice (Figure IV in the online-only Data Supplement).

Recipient mice repopulated with ACE+/+ versus ACE−/− bone marrow–derived cells had no significant difference in body weight, systolic blood pressure, serum ACE activity, plasma renin concentrations, plasma cholesterol concentrations (Table), and plasma lipoprotein cholesterol distributions (Figure V in the online-only Data Supplement). ACE−/− mice are anemic as a result of a combination of increased plasma N-acetyl-seryl-aspartyl-lysyl-proline and absence of plasma AngII.20 However, we did not find any abnormality of red blood cell number, hemoglobin, or hematocrit in mice repopulated with ACE−/− bone marrow–derived cells (Table). In addition, leukocyte (total and subpopulations) and platelet numbers were not different between the 2 chimeric groups.

Atherosclerotic lesions were quantified on the intimal surface of aortic arches and throughout the aortic root (representative images shown in Figures VI and VII in the online-only Data Supplement). ACE deficiency in bone marrow–derived cells led to a significant reduction of percent lesion area in aortic arches (Figure 4B) but no significant changes of mean lesion area in aortic roots (Figure 4C and Figure VIII in the online-only Data Supplement).

Discussion

In the present study, we provide direct evidence that ACE-dependent conversion of AngI into AngII plays an important role in atherosclerosis, as demonstrated by direct infusion of exogenous AngI versus AngII with or without enalapril, an ACE inhibitor. ACE protein was present in many cell types of atherosclerotic lesions, including macrophages, the predominant infiltrating cell type in experimental atherosclerotic lesions. Production of AngII in macrophages, a main leukocyte type, was abolished with ACE inhibition. Abundance of an inflammatory chemokine, MCP-1, implicated in lesion formation was also reduced in ACE-deficient macrophages.
A major novel finding of this study is that ACE deficiency in bone marrow–derived cells reduced atherosclerosis in aortic arches but not in aortic roots of low-density lipoprotein receptor−/− mice. The presence of wild-type or disrupted ACE allele in bone marrow–derived cells of chimeric mice was confirmed by polymerase chain reaction. Percent lesion area in aortic arches was measured using an en face method in chimeric mice repopulated with bone marrow–derived cells from ACE+/+ (n=15) and ACE−/− (n=13) mice. Lesion area in aortic roots was measured on serial cross sections in mice repopulated with bone marrow–derived cells from ACE+/+ (n=11) and ACE−/− (n=10) mice. Triangles represent values of individual mice, circles represent means, and bars are SEM. *P<0.05 by Student t test.

Using an infusion approach to deliver AngI versus AngII enabled us to address the role clearly of ACE in the conversion of AngI to AngII on the development of atherosclerosis. The specificity of AngI to AngII conversion through ACE as the mechanism for acceleration of atherosclerosis is evident from lack of effect of ACE inhibition to influence atherosclerosis induced by infusing AngII directly. These findings support that antiatherosclerotic effects of ACE inhibition are attributed to direct inhibition of AngII generation. In contrast to the present study, a previous study reported that enalapril reduced atherosclerosis in carotid arteries of apolipoprotein E−/− mice through ACE-independent mechanisms. In addition to differences of mouse strain and location of atherosclerosis quantified between the 2 studies, a major difference is the specificity of AngI to AngII conversion through ACE as the mechanism for acceleration of atherosclerosis is evident from lack of effect of ACE inhibition to influence atherosclerosis induced by infusing AngII directly. These findings support that antiatherosclerotic effects of ACE inhibition are attributed to direct inhibition of AngII generation. In contrast to the present study, a previous study reported that enalapril reduced atherosclerosis in carotid arteries of apolipoprotein E−/− mice through ACE-independent mechanisms. In addition to differences of mouse strain and location of atherosclerosis quantified between the 2 studies, a major difference is the specificity of AngI to AngII conversion through ACE as the mechanism for acceleration of atherosclerosis is evident from lack of effect of ACE inhibition to influence atherosclerosis induced by infusing AngII directly. These findings support that antiatherosclerotic effects of ACE inhibition are attributed to direct inhibition of AngII generation. In contrast to the present study, a previous study reported that enalapril reduced atherosclerosis in carotid arteries of apolipoprotein E−/− mice through ACE-independent mechanisms. In addition to differences of mouse strain and location of atherosclerosis quantified between the 2 studies, a major difference is the specificity of AngI to AngII conversion through ACE as the mechanism for acceleration of atherosclerosis is evident from lack of effect of ACE inhibition to influence atherosclerosis induced by infusing AngII directly. These findings support that antiatherosclerotic effects of ACE inhibition are attributed to direct inhibition of AngII generation. In contrast to the present study, a previous study reported that enalapril reduced atherosclerosis in carotid arteries of apolipoprotein E−/− mice through ACE-independent mechanisms.

Table. Comparison of Recipient Mice Repopulated With Bone Marrow–Derived Cells From ACE+/+ vs ACE−/− Mice

<table>
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<tr>
<th>Genotype of Donor</th>
<th>ACE+/+</th>
<th>ACE−/−</th>
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<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>35±1</td>
<td>36±1</td>
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<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>121±3</td>
<td>116±3</td>
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<tr>
<td>Plasma cholesterol, mg/dL</td>
<td>1,049±60</td>
<td>1,187±42</td>
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<tr>
<td>Serum ACE activity, nmole His-Leu/min</td>
<td>164±7.7</td>
<td>149±5.2</td>
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<tr>
<td>Plasma renin, ng/mL per 30 min</td>
<td>8.2±1.2</td>
<td>7.1±1.7</td>
</tr>
<tr>
<td>WBC, 10^9 cells/μL</td>
<td>3.6±0.6</td>
<td>3.8±0.7</td>
</tr>
<tr>
<td>Monocytes, 10^6 cells/μL</td>
<td>0.18±0.04</td>
<td>0.20±0.05</td>
</tr>
<tr>
<td>RBC, 10^6 cells/μL</td>
<td>8.4±0.2</td>
<td>8.5±0.1</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>9.2±0.2</td>
<td>9.4±0.2</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>35.2±0.6</td>
<td>35.3±0.6</td>
</tr>
<tr>
<td>Platelets, 10^11/μL</td>
<td>669±30</td>
<td>584±43</td>
</tr>
</tbody>
</table>

Values are represented as mean±SEM. Comparisons were performed using an unpaired 2-tailed Student t test for non-normally distributed continuous variables and Mann–Whitney U test for non-normally distributed variables. No measurements were statistically different between the 2 groups. ACE indicates angiotensin-converting enzyme; RBC, red blood cell; and WBC, white blood cell.
mRNA and secreted protein was diminished in macrophages from ACE-deficient mice. These results are consistent with an atherogenic role of ACE through promoting leukocyte infiltration into atherosclerotic lesions.

Although there are many studies demonstrating a role of pharmacological inhibition of ACE in experimental atherosclerosis, studies using ACE-deficient mice are sparse and have yielded conflicting results in atherosclerosis. One study reported that heterozygous deficiency of ACE did not change atherosclerosis in apolipoprotein E−/− mice.29 Another mouse model that has been studied is ACE−2 model, which lacks membrane-bound ACE but contains a truncated form of ACE in the circulating blood.30 Both heterozygous and homozygous ACE−2 mice (apolipoprotein E−/− background) had reduced atherosclerosis.10,32 To our knowledge, the present study is the first to define the role of leukocyte ACE in atherosclerosis. Bone marrow transplantation approach has been used extensively to determine effects of many genes of interest in leukocytes on the development of atherosclerosis in mouse models.16,33–36 The successful repopulation of bone marrow–derived cells did not reduce lesion area in aortic roots and presence versus absence of ACE mRNA in peritoneal macrophages in recipient LDL receptor−/− mice. Leukocyte ACE deficiency in chimeric LDL receptor−/− mice led to a reduction of percent atherosclerotic lesion area in the aortic arch region as measured by an en face method. The magnitude of lesion size reduction is comparable with what was observed previously in ACE−2 mice.32 However, ACE deficiency in bone marrow–derived cells did not reduce lesion area in aortic roots of chimeric mice as measured using serial cross sections. One potential interpretation of these differences is the diverse origins of the resident cell types in different regions of the aortic wall during embryonic development that may influence leukocyte infiltration.5 In addition, ACE is ubiquitously present in resident cell types of the aortic arch, such as endothelial cells, smooth muscle cells, and fibroblasts,10,34–41 inferring that ACE from these resident cell types has potentials to contribute to the development of atherosclerosis. As demonstrated in our previous studies and the present study, leukocyte renin16 and ACE contribute to atherosclerosis, whereas AT1α receptor in a single cell type (leukocytes, endothelial cells, or smooth muscle cells)16,36,42 is not sufficient to promote atherosclerosis. Lack of effect of AT1α receptor deletion in a single cell type may be as a result of the need for AT1α receptor stimulation exerting a coordinated response among several cell types. We will explore this assumption in a future study.

In summary, this study demonstrated that ACE inhibition reduced AngI but not AngII-induced atherosclerosis. Although systemic ACE inhibition markedly reduces atherosclerotic lesion formation in mice in the 2 vascular beds studied, leukocyte-specific ACE only modestly reduced atherosclerosis in the aortic arch but had no significant effect in the aortic root. In addition to leukocytes, ACE is present in many other cell types of atherosclerotic lesions, including endothelial and smooth muscle cells. Future studies are needed to determine the relative contributions of ACE in these cell types to atherosclerotic lesion formation.

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Disclosures
None.

References
Significance

Despite the convincing evidence that pharmacological inhibition of whole-body angiotensin-converting enzyme (ACE) reduces atherosclerosis in animal models, the mechanistic basis for this beneficial effect has not been elucidated. In addition, it has been wel recognized that ACE plays a critical role in converting angiotensin I into angiotensin II. This article provides insights into 2 aspects. First, our in vivo studies provide direct evidence that ACE is the enzyme responsible for conversion of angiotensin I into angiotensin II that contributes to the consequent induction of atherosclerosis. Second, deficiency of ACE in leukocytes modestly reduces hypercholesterolemia-induced atherosclerosis in the aortic arch region but not in the aortic root. Because ACE is present in resident vascular cell types in both experimental and human atherosclerotic lesions, subsequent studies will determine whether deficiency of ACE in resident cell types influences atherosclerosis and the potential cross-talk between and among different cell types.
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MATERIALS AND METHODS

Mice and Diets

LDL receptor -/- (B6.129S7-Ldlr<sup>tm1Her</sup>/J; Stock# 002207), ACE -/- mice (B6.129P2-Ace<sup>tm1Unc</sup>/J; Stock# 002679), and C57BL/6J (Stock# 000664) were obtained from The Jackson Laboratory (Bar Harbor, ME). LDL receptor -/- and ACE -/- mice have been backcrossed more than 10 times into a C57BL/6 background. Littermates were used for the experiments reported in this manuscript.

All mice were maintained in a barrier facility in individually vented cages with negative pressure containing Teklad Sani-Chip bedding (Cat# 7090A, Harlan Teklad, Madison, WI) on a light : dark cycle of 14 : 10 hours. Mice were fed a standard rodent diet (Diet# 2918, Harlan Teklad, Madison, WI) and provided drinking water from a reverse osmosis system. To induce hypercholesterolemia, mice were fed a diet supplemented with saturated fat (milk fat 21% wt/wt; Diet# TD.88137, Harlan Teklad). All studies were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

Infusion of AngI, AngII, and Enalapril

Alzet osmotic minipumps (Model 2004 or 1004, Durect Corporation, Cupertino, CA) were implanted into male LDL receptor -/- mice at the age of 8 weeks. Two infusion experiments were performed. In the first experiment, 3 groups of mice were studied: saline, AngI (1,000 ng/kg/min), and co-infusion of AngI (1,000 ng/kg/min) and enalapril (4 mg/kg/d). There were also 3 groups studied in the second experiment: AngI (1,000 ng/kg/min), AngII (1,000 ng/kg/min), and co-infusion of AngII (1,000 ng/kg/min) and enalapril (4 mg/kg/d). Co-infusion was performed with two separate osmotic minipumps (Model 1004 for AngI or AngII and Model 2004 for enalapril). AngI (Cat# H-1680) and AngII (Cat# H-1705) were purchased from Bachem (Torrance, CA), and enalapril (Cat# E6888) was purchased from Sigma-Aldrich (St. Louis, MO). All study mice were fed the saturated fat-enriched diet 1 week prior to pump implantation, and this diet was maintained during drug infusion.

Bone Marrow Transplantation

Recipient mice were provided drinking water containing sulfatrim (4 µg/ml) for 1 week prior to irradiation, which was maintained for another 4 weeks after bone marrow transplantation. For bone marrow transplantation, recipient male LDL receptor -/- mice (8 weeks of age) were lethally irradiated with 450 rads twice (total 900 Rads), 3 hours apart from a cesium source. Bone marrow-derived cells were isolated from pooled donor mice (male ACE +/- or -/- littermates at 6-8 weeks of age) as described previously. Bone marrow-derived cells were resuspended and injected (5 x 10<sup>6</sup> cells/mouse) into tail veins of irradiated recipient mice. Four weeks after bone marrow transplantation, mice were fed the saturated fat-enriched diet for 12 weeks. Body weights were monitored weekly. Reconstitution of the transplanted bone marrow cells was determined by polymerase chain reaction (PCR) of ACE on genomic DNA from bone marrow of recipient mice. ACE genotyping was performed using the following
primers: 5'-AGTGGAGGGTATTTGTCAGGGC, 5'-TAATTCCTTGAGGCAGCACT, and 5'-TAAAGCGCATGCTCCAGACTGC. PCR reaction was 1 run of 94 °C for 5 min; 35 cycles of 94 °C for 45 sec, 60 °C for 1 min, and 72 °C for 1 min; and 1 run of 72 °C for 6 min. The resultant bands for wild type and deficient alleles were 286 bp and 340 bp, respectively.

**Systolic Blood Pressure Measurements**

Systolic blood pressure (SBP) was measured on conscious mice using a non-invasive tail-cuff system (Code 8, Kent Scientific Corporation, Torrington, CT) as described previously. For osmotic minipump infusion studies, measurements were performed prior to pump implantation and on week 4 during drug infusion. For the bone marrow transplantation study, SBP was measured 1 week prior to (week 0), and every 4 weeks during the fat-enriched diet feeding.

**Peripheral Blood Profile**

Plasma cholesterol concentration was measured enzymatically using a commercial kit (Cat# 439-17501; Wako Chemicals USA, Richmond, VA). Plasma lipoprotein fractions were resolved with size exclusion chromatography using samples from individual mice and analyzed as described previously. Serum ACE activity was measured with a fluorimetric assay using hippuryl-His-Leu as a synthetic substrate as described previously. ACE activity was quantified using a standard curve prepared with different concentrations of His-Leu (Cat# H2504; Sigma-Aldrich). Assay specificity was determined using an ACE inhibitor, captopril. Plasma renin concentration was measured as described previously. Briefly, plasma samples (8 μl or each) harvested with EDTA (1.8 mg/ml) were incubated with an excess of rat angiotensinogen at 37 °C for 30 minutes. AngI generated in samples was quantified by radioimmunoassay using a commercially available kit (Cat# 1553; DiaSorin, Stillwater, MN).

Cell numbers in peripheral blood were counted using a Hemavet 950 (Drew Scientific Inc, Dallas, TX).

**Immunostaining**

To determine the cellular localization of ACE protein in atherosclerotic lesions, immunostaining of ACE was performed on serial sections from aortic roots of male LDL receptor -/- mice fed the saturated fat-enriched diet. A goat anti-ACE antibody (Clone N-20, Cat# sc-12184; Santa Cruz Biotechnology, Santa Cruz, CA) was used for ACE immunostaining. Macrophages were immunostained using a rat anti-mouse CD68 antibody (Clone FA-11, Cat# MCA 1957; AbD Serotec, Raleigh, NC). Smooth muscle cells and endothelial cells were immunostained using a rabbit anti-alpha smooth muscle actin (Cat# ab5694; Abcam, Cambridge, MA) and a biotin-conjugated rat anti-mouse CD31 monoclonal antibody (PECAM-1, Clone # MEC13.3, Cat# 553371; BD Biosciences, San Jose, CA), respectively. Secondary antibodies used were biotinylated rabbit anti-goat IgG (Cat# BA-5000; Vector Laboratories, Burlingame, CA) for ACE, biotinylated rabbit anti-rat IgG for CD68 (Cat# BA-4001, Vector Laboratories), and
biotinylated goat anti-rabbit IgG (Cat# BA-1000, Vector Laboratories) for alpha smooth muscle actin. Positive immunoreactivity was visualized as red color by oxidation of amino ethyl carbazole. Negative controls included non-immune IgG, no primary antibody control, and no primary and secondary antibody control as described previously.  

**Atherosclerosis Quantification**

Atherosclerosis was quantified both on the intimal surface of aortic arches using an en face method and throughout the aortic root on serial cross-sections, as described previously.  

**In vitro Macrophage Studies**

To determine whether cultured peritoneal macrophages secrete angiotensin peptides into culture media via an ACE-dependent pathway, mouse peritoneal macrophages were harvested from peritoneum of male C57BL/6 mice as described previously.  

Cells were incubated with DMEM supplemented with fetal bovine serum (FBS; 10% vol/vol). After 3 hours, cells were washed with serum-free DMEM. Adherent macrophages were incubated in DMEM with FBS (10% vol/vol) overnight. Subsequently, cells were washed thoroughly using serum-free DMEM and subsequently incubated in serum-free DMEM with or without an ACE inhibitor, captopril (0.1 μM) for 24 hours. AngII was measured in culture media from these cells using a high-performance liquid chromatographic assay followed by radioimmunoassay, as described previously.

Bone marrow-derived macrophages were cultured as described previously. Briefly, bone marrow cells were flushed from femurs and tibias of ACE +/+ or -/- mice and cultured for 7 days in Roswell Park Memorial Institute (RPMI) medium containing HEPES, L-glutamine, 10% FBS, and 100 IU/ml penicillin/streptomycin with addition of 15% L929 cell-conditioned medium to induce differentiation into macrophages. Adherent cells were subsequently plated in 6-well plates in serum free medium for 24 hours before cells and supernatant were collected for either mRNA or ELISA analysis.

To determine the influence of ACE deficiency in macrophages on the secretion of monocyte chemoattractant protein-1 (MCP-1), peritoneal or bone marrow-derived macrophages from ACE +/+ or -/- mice were harvested and incubated as described above. Adherent macrophages were incubated in serum-free medium for 24 hours. MCP-1 concentration in culture media from macrophages was measured using an ELISA kit (Cat# SMJE00; R&D system, Minneapolis, MN).

**Quantitative Real Time PCR**

Peritoneal or bone marrow-derived macrophages were extracted for total RNA using a SV total RNA Isolation System (Cat# 23100; Promega, Madison, WI). Reverse transcription was performed using an iScript™ cDNA Synthesis Kit (Cat#170-8891; Bio-Rad, Hercules, CA). Quantitative PCR was performed using a SsoFast™ EvaGreen® Supermix kit (Cat# 172-5203; Bio-Rad) on a Bio-Rad CFX96 cycler. Data were analyzed using the ΔΔCt method as described previously. Primers used for quantitative PCR are shown in Table I in the online-only Data Supplement.
Statistical Analyses

Data are represented as means ± standard error of means (SEM). SigmaPlot version 12.0 (SYSTAT Software Inc., Chicago, IL) was used for statistical analyses. To compare more than two groups of each study on a continuous response variable, we used one-way ANOVA for normally distributed variables and Kruskal-Wallis one way ANOVA on Ranks for non-normally distributed variables, and post-hoc analysis used Holm-Sidak method and Dunn’s method, respectively. For two groups, comparisons were performed using an unpaired two-tailed Student’s t-test for normally distributed continuous variables and a Mann-Whitney U test for non-normally distributed variables. A P<0.05 was considered statistically significant.
REFERENCES


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Supplemental Table I. Primer Sequences Used in Quantitative PCR Analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tr>
<td>MCP-1</td>
<td>Forward 5’-CCT GCT GCT ACT CAT TCA CC</td>
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<td>Reverse 5’-TGT CTG GAC CCA TTC CTT CT</td>
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<td>ACE</td>
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<td>β-actin</td>
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<td>Reverse 5’-GCA CTG TGT TGG CAT AGA GG</td>
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**Supplemental Table II. Characteristics of Hypercholesterolemic Mice Infused with Saline, AngI, or AngI and Enalapril**

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<td>n</td>
<td>19</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>135 ± 2</td>
</tr>
<tr>
<td>Plasma cholesterol (mg/dl)</td>
<td>1,313 ± 54</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SEM. Comparisons were performed using one way ANOVA. * P<0.001 compared to saline infusion and co-infusion of AngI and enalapril.

**Supplemental Table III. Characteristics of Hypercholesterolemic Mice Infused with AngI, AngII, or AngII and Enalapril.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Subcutaneous Infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AngI</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>164 ± 5</td>
</tr>
<tr>
<td>Plasma cholesterol (mg/dl)</td>
<td>1,328 ± 67</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM. Comparisons were performed using one way ANOVA.
Online Figure I. Atherosclerotic lesion area throughout the aortic root. Mean lesion areas throughout the aortic root were quantified on serial sections using ImagePro Plus software. The transition between the aortic sinus and ascending aorta is depicted as “0”. Symbols and error bars represent mean lesion areas ± SEM of each serial section in each group. Open triangles represent Saline group (n=15), black triangles are AngI group (n=12), and grey triangles are co-infusion of AngI and enalapril (n=9).
Online Figure II. Presence of ACE protein in atherosclerotic lesions. Immunostaining of ACE (upper panel) and CD68 (lower panel) was performed on fresh frozen sections from aortic roots. These atherosclerotic lesions on sections of aortic roots were from LDL receptor -/- male mice fed a saturated fat-enriched diet (Diet# TD.88137, Harlan Teklad).
Online Figure III. ACE deficiency in bone marrow-derived macrophages reduced mRNA and protein secretion of MCP-1. (A) MCP-1 mRNA abundance in cultured bone marrow-derived macrophages from ACE +/+ or -/- mice was determined using quantitative PCR. (B) MCP-1 protein released from cultured bone marrow-derived macrophages of ACE +/+ or -/- mice were measured using an ELISA kit. * P<0.05.
Online Figure IV. ACE deficiency in bone marrow-derived cells led to ablation of ACE mRNA in peritoneal macrophages from LDL receptor -/- recipient mice. Peritoneal macrophages were harvested from lethally irradiated LDL receptor -/- male mice that were subsequently repopulated with bone marrow-derived cells from ACE +/- or ACE +/- male donor mice (n=4 per group). mRNA abundance of ACE was determined using quantitative PCR. *P= 0.023.
Online Figure V. ACE deficiency in bone marrow-derived cells did not influence plasma lipoprotein distribution. Plasma lipoproteins of LDL receptor -/- recipient mice repopulated with bone marrow-derived cells from either ACE +/+ (n=4) or -/- (n=4) donor mice were resolved using a size exclusion chromatography and absorbance at 600 nm of separated fractions was measured using a cholesterol enzymatic kit. Circles and error bars denote mean absorbance ± SEM.
Online Figure VI. Representative images of the aortic arch region for en face measurements in LDL receptor -/- recipient mice. Male LDL receptor -/- mice were repopulated with bone marrow-derived cells from ACE +/+ (upper panel) or ACE -/- (lower panel) male donor mice.
Online Figure VII. Representative images of Oil Red O staining on serial sections of aortic roots for atherosclerotic lesion measurements. Male LDL receptor -/- mice were repopulated with bone marrow-derived cells from ACE +/+ (upper panel) or +/- (lower panel) male donor mice.
Online Figure VIII. ACE deficiency in bone marrow-derived cells did not affect atherosclerotic lesion area in aortic roots. Mean lesion areas of each group are represented throughout the aorta root, with the transition between the aortic sinus and ascending aorta depicted as “0”. Inverted triangles represent mean lesion areas ± SEM. Data presented are from male chimeric LDL receptor -/- mice repopulated with bone marrow-derived cells from either ACE +/+ (n=11) or -/- (n=10) mice.