Angiotensin-Converting Enzyme 2 Deficiency in Whole Body or Bone Marrow–Derived Cells Increases Atherosclerosis in Low-Density Lipoprotein Receptor−/− Mice

Sean E. Thatcher, Xuan Zhang, Deborah A. Howatt, Hong Lu, Susan B. Gurley, Alan Daugherty, Lisa A. Cassis

Objective—The renin-angiotensin system contributes to atherosclerotic lesion formation. Angiotensin-converting enzyme 2 (ACE2) catabolizes angiotensin II (Ang II) to angiotensin 1–7 (Ang-(1–7)) to limit effects of the renin-angiotensin system. The purpose of this study was to define the role of ACE2 in atherosclerosis.

Methods and Results—Male Ace2−/− mice in an low-density lipoprotein receptor–deficient background were fed a high-fat diet for 3 months. ACE2 deficiency increased atherosclerotic area (Ace2+/−, 17±1; Ace2−/−, 23±2 mm², P<0.002). This increase was blunted by losartan. To determine whether leukocytic ACE2 influenced atherosclerosis, irradiated low-density lipoprotein receptor–deficient male mice were repopulated with bone marrow–derived cells from Ace2−/− or Ace2−/− mice and fed a high-fat diet for 3 months. ACE2 deficiency in bone marrow–derived cells increased atherosclerotic area (Ace2+/−, 1.6±0.3; Ace2−/−, 2.8±0.3 mm²; P<0.05). Macrophages from Ace2−/− mice exhibited increased Ang II secretion and elevated expression of inflammatory cytokines. Conditioned media from mouse peritoneal macrophages of Ace2−/− mice increased monocyte adhesion to human umbilical vein endothelial cells. Incubation of human umbilical vein endothelial cells with Ang II promoted monocyte adhesion, which was blocked by Ang-(1–7). Coinfusion of Ang-(1–7) with Ang II reduced atherosclerosis.

Conclusion—These results demonstrate that ACE2 deficiency in bone marrow–derived cells promotes atherosclerosis through regulation of Ang II/Ang-(1–7) peptides. (Arterioscler Thromb Vasc Biol. 2011;31:758-765.)

Key Words: angiotensin II ■ atherosclerosis ■ leukocytes ■ macrophages ■ ACE2

Hypercholesterolemia stimulates components of the renin-angiotensin system (RAS), including angiotensin type 1 (AT₁) receptors1,2 and systemic concentrations of angiotensin peptides.3 Moreover, pharmacological interference with the RAS through inhibition of angiotensin-converting enzyme (ACE), renin, or AT₁ receptors has been demonstrated to reduce both experimental and human atherosclerosis.4–6 Similarly, genetic manipulation of the RAS, including AT₁a receptors3,7 or ACE deficiency,8,9 caused a striking reduction in experimental atherosclerosis, further supporting a role for the RAS in atherosclerosis. Recent studies in our laboratories demonstrated that deficiency of renin in bone marrow–derived stem cells reduced hypercholesterolemia-induced atherosclerosis in low-density lipoprotein receptor–deficient (Ldlr−/−) mice, suggesting that leukocyte production of angiotensin peptides influenced developing atherosclerotic lesions.6 Collectively, results support a prominent role of the RAS in developing atherosclerotic lesions; however, the relative importance of systemic versus local production of angiotensin peptides in lesion formation has not been fully resolved.

ACE2, a more recently discovered member of the RAS that has 40% homology to ACE,10–12 is a monocarboxypeptidase that cleaves the vasoconstrictor angiotensin II (Ang II) to a vasodilator peptide, angiotensin 1–7 (Ang-(1–7)). ACE2 is located on the sex-linked X chromosome and exhibits widespread tissue distribution.10,11 Although ACE2 can catabolize angiotensin I, it has greater catalytic efficiency in the catabolism of Ang II (≈400-fold higher).12 On the basis of its ability to degrade the vasoconstrictor Ang II and produce the vasodilator Ang-(1–7), ACE2 has been suggested to limit pathophysiologic activation of the RAS.

Previous studies localized ACE2 to atherosclerotic lesions of rabbit aorta and human carotid arteries.14,15 Interestingly, ACE2 localized to smooth muscle, endothelial cells, and macrophages in lesions of hypercholesterolemic rabbits.15 Functional studies have demonstrated that overexpression of ACE2 using adenoviral transfection in apolipoprotein E−/− mice and bone marrow–derived cells increases atherosclerotic plaque formation.

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Atherosclerosis in ApoE−/− mice or in hypercholesterolemic rabbits decreased atherosclerosis.6,17 Recent studies demonstrated that whole body ACE2 deficiency increased atherosclerosis in ApoE−/− mice.18 In this study, we examined effects of whole body or leukocytic ACE2 deficiency on high-fat (HF) diet–induced atherosclerosis in Ldlr−/− mice. To define mechanisms of ACE2 to regulate lesion formation, we focused on interactions between Ang II and Ang-(1–7) as the substrate and product of ACE2, respectively, in the regulation of monocyte adhesion to endothelial cells and on lesion development.

Methods
A detailed description of the methods section is provided in the Supplemental Materials, available online at http://atvb.ahajournals.org.

To examine effects of whole body ACE2 deficiency on atherosclerosis, male Ace2+/− and Ace2−/− mice on an Ldlr−/− background (no. 002207, obtained from the Jackson Laboratory, Bar Harbor, ME) were fed a HF diet (42% caloric intake from fat, TD88137, Harlan Teklad, Indianapolis, IN) for 3 months. In separate studies, losartan was administered by osmotic minipump (25 mg/kg per day; no. 002207, obtained from the Jackson Laboratory, Bar Harbor, ME) were fed a HF diet (42% caloric intake from fat, TD88137, Harlan Teklad, Indianapolis, IN) for 3 months. In separate studies, losartan was administered by osmotic minipump (25 mg/kg per day) to Ace2−/− male mice during month 3 of HF feeding. For bone marrow transplantation, male Ldlr−/− mice (2 months of age) were irradiated with a total of 900 rads from a cesium source delivered in 2 doses.3 Mice were maintained on antibiotic water (sulfadimethoxine, 4 μg/mL) for 1 week before and 6 weeks after irradiation before feeding the HF diet for an additional 3 months. To define interactions between Ang-(1–7) and Ang II in the regulation of lesion formation, Ang-(1–7) (400 ng/kg per minute) was infused to fat-fed Ldlr−/− male mice for 7 days before coinfusion of Ang II (1000 ng/kg per minute) for 28 days. Macrophages were harvested from the peritoneum of Ace2−/− or Ace2−/− Ldlr−/− mice. All studies were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Results
Whole Body ACE2 Deficiency Increased Atherosclerosis in Ldlr−/− Mice
Deficiency of ACE2 (insertion of a neomycin cassette in exon 9 of the ACE2 gene19) was confirmed by ablation of mRNA of ACE2 in kidneys of Ace2+/− and Ace2−/− female mice (Figure 1A). Deficiency of ACE2 had no effect on body weight, systolic blood pressure, serum ACE activity, plasma renin concentrations, serum cholesterol concentrations, or lipoprotein cholesterol distributions in Ldlr−/− mice fed an HF diet (Table, Supplemental Figure IIA). Plasma concentrations of Ang II were modestly but not significantly increased in Ace2−/− mice fed a HF diet (1 month of HF feeding: Ace2+/−, 29±4; Ace2−/−, 39±6 pg/mL; P>0.05). Whole body deficiency of ACE2 increased lesion area in aortic arches (Figure 1B and 1D, P<0.002) and aortic sinuses (Figure 1E, P<0.05) compared with Ace2+/− wild-type controls. Quantification of CD68 immunostaining revealed that macrophage accumulation was greater in aortic sinus lesions from Ace2−/− mice compared with Ace2+/− Ldlr−/− controls (Ace2+/−, 0.25±0.02; Ace2−/−, 0.44±0.04 mm²; P<0.007) (Figure 1F). Administration of the AT1 receptor antagonist losartan to Ace2−/− mice decreased lesion formation in aortic arches (Figure 1C and 1D) and sinuses (Supplemental Figure IA).

To define cell types implicated in effects of ACE2 deficiency, aortic sinus sections from Ace2−/− Ldlr−/− mice were immunostained for ACE2 or CD68 (Figure 2). Lesions with pronounced CD68 immunostaining also exhibited ACE2 immunoreactivity. In addition, ACE2 immunoreactivity was also evident in media and adventitia of aortic sinuses.
Ace2 Parameters

Because ACE2 immunoreactivity localized to macrophages of lesions, we assessed ACE2 enzymatic activity in macrophages harvested from the peritoneal cavity (mouse peritoneal macrophages). ACE2 enzymatic activity in mouse peritoneal macrophages was similar to that in heart and liver (Supplemental Figure IB).

**ACE2 Deficiency in Bone Marrow–Derived Cells Increased Atherosclerosis in Ldlr−/− Mice**

Because lesional macrophages stained positive for ACE2, we repopulated irradiated Ldlr−/− mice with bone marrow–derived cells harvested from either Ace2+/y or Ace2−/− mice to define the role of leukocyte ACE2. After 6 weeks of repopulation, mice in each group were fed a HF diet for 3 months. Genomic analyses of irradiated mice demonstrated effective repopulation with Ace2+/y or Ace2−/− donor cells (Supplemental Figure III). Deficiency of ACE2 in bone marrow–derived cells had no effect on body weight, systolic blood pressure, serum ACE activity, plasma renin concentration, or lipoprotein cholesterol distribution (Table and Supplemental Figure IIB). ACE2 deficiency in bone marrow–derived cells increased lesion areas in both aortic arches (Ace2+/y, 1.6±0.3; Ace2−/−, 2.8±0.3 mm²; *P*<0.05, Figure 3A) and aortic sinuses (Figure 3B; *P*<0.05). In addition, CD68-positive immunostaining was increased in sections from aortic sinuses of mice transplanted with Ace2−/− compared with Ace2+/y marrow (Figure 3C and 3D).

**Peritoneal Macrophages From ACE2-Deficient Mice Exhibited Increased Expression of Inflammatory Cytokines and Promoted Monocyte Adhesion to Endothelial Cells**

Concentrations of Ang II released from MPMs of Ace2−/− mice were increased compared with Ace2+/− cells (Ace2+/−, 70±8; Ace2−/−, 115±13 pg/mL; *P*<0.05, Figure 4A). To determine whether elevated Ang II concentrations influenced inflammatory cytokines, we examined mRNA abundance and culture media protein levels of various inflammatory cytokines and their receptors using MPMs harvested from Ace2+/−

### Table. Systemic Parameters Between Ace2+/y and Ace2−/− Ldlr−/− Male Mice

<table>
<thead>
<tr>
<th>Parameters</th>
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<th>1 Month Coinfusion Study</th>
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<td>Plasma renin (ng/mL)</td>
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*Compared to basal blood pressure, *P* < 0.05. BMT indicates bone marrow transplantation; ND, not determined.

**Figure 2.** ACE2 localized to lesional macrophages in Ldlr−/− male mice. CD68 and ACE2 immunostaining in aortic sinus sections from Ldlr−/− male mice. IgG staining (anti-rat, control for CD68) is illustrated on left. Boxed areas are shown at a higher magnification in lower panels. Scale bars represent 200 μm.
In MPMs from Ace2/H11002/y mice, mRNA abundance of C-C motif chemokine receptor 2 was increased (Figure 4B), and protein concentrations of interleukin 6 and plasminogen activator inhibitor 1 released into culture media were elevated (Figure 4C). In addition, deficiency of ACE2 resulted in modest elevations in mRNA abundance or protein release of CCL2, tumor necrosis factor-α and its receptor, and nuclear factor-κB (Supplemental Tables II and III). To determine whether MPMs from Ace2/H11002/y mice promoted monocyte adhesion to endothelial cells, we cocultured human umbilical vein endothelial cells (HUVECs) and MPMs from Ace2/H11001/y and Ace2/H11002/y mice by placing cells in the upper and lower chamber of Transwells, respectively. Human monocytic leukemia cell line 1 monocytes were placed in the upper chamber to assay for adhesion to HUVECs. Coculture of MPMs from Ace2/H11002/y mice with HUVECs resulted in increased THP-1 monocyte adhesion compared with coculture with MPMs from Ace2/H11001/y controls (Ace2/H11001/y, 100 ± 7; Ace2/H11002/y, 142 ± 13% control; *P<0.05; Figure 4D). Losartan reduced THP-1 monocyte adhesion when HUVECs were cocultured with MPMs from Ace2/H11002/y or Ace2/H11001/y mice (Figure 4D).

**The ACE2 Product Ang-(1–7) Functionally Antagonized Ang II-Induced Monocyte Adhesion to HUVECs and Reduced Ang II-Induced Atherosclerosis in Ldlr−/− Mice**

Recent studies demonstrated that Ang-(1–7) antagonized effects of Ang II to promote vascular smooth muscle cell proliferation and migration. Therefore, we investigated the relative contribution of the ACE2 substrate, Ang II, to the ACE2 product, Ang-(1–7), in the regulation of monocyte adhesion to HUVECs. Incubation of HUVECs with Ang II increased monocyte adhesion, and this effect was abolished by losartan (Figure 5A). By itself, Ang-(1–7) had no effect on monocyte adhesion. However, Ang-(1–7) functionally antagonized Ang II-induced stimulation of monocyte adhesion (Figure 5A), and this effect was blocked by D-Ala. To determine whether these peptides interacted in vivo in the regulation of atherosclerosis, we infused Ldlr−/− mice with Ang II in the absence or presence of coinfused Ang-(1–7). Coinfusion of Ang-(1–7) with Ang II reduced (2-fold) atherosclerosis in the aortic arch (Figure 5B and 5E) and sinus (Figure 5D) and decreased CD68 immunostaining in aortic sinus sections (Figure 5C).
Discussion
Results from this study demonstrate that whole body deficiency of ACE2 increased the development of atherosclerosis in fat-fed Ldlr/−/− mice. ACE2 localized to macrophage-rich regions of atherosclerotic lesions, and ACE2 enzymatic activity was evident in mouse peritoneal macrophages. Interestingly, deficiency of ACE2 in bone marrow–derived cells also promoted the development of atherosclerosis. Although plasma concentrations of Ang II were not markedly elevated in whole body ACE2-deficient mice, macrophages from ACE-2 deficient mice released greater concentrations of Ang II. Moreover, macrophages from ACE2-deficient mice exhibited increased expression and release of inflammatory cytokines and promoted monocyte adhesion to endothelial cells. Although Ang-(1–7) had no effect on monocyte adhesion by itself, this angiotensin peptide functionally antagonized Ang II-induced stimulation of monocyte adhesion and lesion formation. These results demonstrate that ACE2 deficiency promotes atherosclerosis and suggest that endogenous ACE2 protects against atherosclerosis by controlling the relative balance between Ang II and Ang-(1–7) in pivotal cell types.

Previous investigators demonstrated that whole body ACE2 deficiency had strain-dependent effects on blood pressure. In C57BL/6 mice, ACE2 deficiency resulted in a modest increase in blood pressure (~7 mm Hg). We did not observe an effect of ACE2 deficiency on systolic blood pressures in Ldlr−/− mice. It is possible that ACE2 deficiency on an Ldlr−/− background blunted blood pressure increases in C57BL/6 mice. Previous studies demonstrated that although AT1a receptor deficiency had divergent effects on systolic blood pressure in Ldlr−/− and ApoE−/− mice, the loss of AT1a receptor signaling resulted in pronounced reductions in atherosclerosis. Therefore, it is unlikely that blood pressure concentrations of renin or Ang II. Our results are in agreement with several studies that document no change in plasma or kidney angiotensin peptide concentrations in ACE2-deficient mice. However, it should be noted that a previous study reported that ACE2 deficiency increased systemic concentrations of Ang II in mice experiencing heart failure. An interesting aspect of the present study is that whole body and bone marrow cell deficiency of ACE2 promoted atherosclerosis but had no effect on the systemic RAS. These results suggest that ACE2 primarily influences concentrations of Ang II in pivotal cell types involved in developing lesions.

Figure 4. ACE2 deficiency promoted Ang II release, chemokine receptor expression, and inflammatory cytokine release from cultured peritoneal macrophages and increased monocyte adhesion to endothelial cells. A, Ang II release from MPMs of Ace2+/y or Ace2−/− mice. B, C-C motif chemokine receptor 2 mRNA abundance in MPMs from Ace2+/y or Ace2−/− mice (n=6 mice per genotype). C, Interleukin 6 (IL-6) and plasminogen activator inhibitor 1 (PAI-1) protein levels released from MPMs of Ace2+/y or Ace2−/− mice (n=6 mice per genotype). D, Coculture of MPMs from Ace2−/− mice with HUVECs increased THP-1 monocyte adhesion compared with control (expressed as percentage of THP-1 adhesion in macrophage media from Ace2+/y mice). Coincubation of MPMs from each genotype with losartan (1 μmol/L; n=3 to 6 mice per genotype) reduced monocyte adhesion, whereas coincubation with D-Ala (5 μmol/L; n=3 to 6 mice per genotype) had no effect. *P<0.05 compared with Ace2+/y (A to D), †P<0.05 compared with vehicle within genotype.
effects from ACE2 deficiency contributed to the augmented atherosclerosis observed in the present study.

Our results are in agreement with previous studies demonstrating that ACE2 localized to macrophage-rich areas of atherosclerotic lesions from hypercholesterolemic rabbits. However, these results extend previous findings by demonstrating that murine macrophages exhibit ACE2 enzymatic activity and that macrophages from ACE2-deficient mice release greater concentrations of Ang II. Similar to previous findings demonstrating reductions in thoracic aortic lesion areas following bone marrow transplantation in HF-fed Ldlr−/− mice,6,23 in this study, bone marrow transplantation reduced lesion areas in the aortic arches of HF-fed Ldlr−/− mice of both genotypes. Even though background levels of atherosclerosis were reduced in bone marrow–transplanted mice, chimeric Ldlr−/− mice with ACE2 deficiency in bone marrow–derived cells exhibited increased atherosclerosis of a magnitude (≈2-fold) similar to that seen from whole body ACE2 deficiency. Moreover, chimeric mice with ACE2 deficiency in bone marrow–derived cells exhibited increased atherosclerosis in the absence of changes in the systemic RAS. These results suggest that local effects of ACE2 on bone marrow–derived cells, potentially macrophages, mediate effects of ACE2 deficiency to promote atherosclerosis.

Previous investigators demonstrated that adenoviral overexpression of murine ACE2 in rabbits subjected to endothelial injury and fed an atherogenic diet attenuated lesion progression.16 Similarly, recent studies demonstrated that adenoviral overexpression of ACE2 in atherosclerotic lesions of rabbits attenuated fatty streak formation.20 In ApoE−/− mice, adenoviral overexpression of ACE2 reduced atherosclerosis.17 Moreover, recent studies demonstrated that whole body deficiency of ACE2 in ApoE−/− mice increased atherosclerosis.18 In agreement with this, results from the present...
study demonstrate that whole body deficiency of ACE2 in Ldlr<sup>−/−</sup> mice increased atherosclerosis. Collectively, these results support a pivotal role for ACE2 in developing atherosclerotic lesions.

Our studies extend previous findings by contrasting the effects of whole body versus bone marrow deficiency of ACE2 on atherosclerosis. Our results demonstrate an increase of similar magnitude in lesion formation in whole body ACE2-deficient mice compared with chimeric mice lacking ACE2 in bone marrow–derived cells. Although these findings do not indicate a specific cell type responsible for augmented lesion formation in mice lacking ACE2 in bone marrow–derived cells, macrophage-rich regions of lesions from Ldlr<sup>−/−</sup> mice stained positive for ACE2. In addition, MPMs exhibited ACE2 enzymatic activity, and macrophages from ACE2-deficient mice released greater concentrations of Ang II. Recent studies demonstrated that aortas from ACE2-deficient ApoE<sup>−/−</sup> mice exhibited increased cell adhesion when perfused with whole blood. Moreover, bone marrow–derived macrophages from ACE2-deficient mice exhibited increased expression of inflammatory cytokines in response to lipopolysaccharide. Results from the present study demonstrate increased monocyte adhesion when HUVECs were cocultured with MPMs from ACE2-deficient mice, and extend previous findings by demonstrating that these effects are Ang II/AT<sub>1</sub> receptor mediated. Moreover, in this study, in the absence of external stimuli (eg, lipopolysaccharide), ACE2-deficient MPMs exhibited increased expression and release of inflammatory cytokines. These results demonstrate that deficiency of ACE2 in pivotal cell types promotes inflammation and monocyte adhesion, 2 mechanisms invoked in lesion formation.

In the setting of ACE2 deficiency, where Ang II is formed but not catabolized to Ang-(1–7), our results using losartan support a prominent role for Ang II effects at AT<sub>1</sub> receptors to augment atherosclerosis. In contrast, in wild-type mice, both Ang II and Ang-(1–7) are present to regulate lesion formation. Recent studies demonstrated that long-term infusion of Ang-(1–7) to ApoE<sup>−/−</sup> mice reduced atherosclerosis. Moreover, recent studies demonstrated functional antagonism of Ang II-mediated regulation of smooth muscle cell proliferation and migration by Ang-(1–7), supporting functional interactions between these angiotensin peptides that are regulated by ACE2. Similar to findings in smooth muscle cells, in the present study, although Ang-(1–7) had no effect on monocyte adhesion when incubated alone with HUVECs, this peptide functionally antagonized effects of Ang II to promote monocyte adhesion. Because the effects of Ang-(1–7) to blunt Ang II-induced monocyte adhesion were abolished by d-Ala, these findings implicate the mas receptor as the mediator of Ang-(1–7)-induced antagonism of Ang II. Infusion of Ang-(1–7) with Ang II markedly lowered atherosclerosis in Ldlr<sup>−/−</sup> mice, supporting an in vivo interplay between these ACE2-regulated angiotensin peptides in the development of atherosclerosis.

In conclusion, the present study demonstrated that whole body deficiency of endogenous ACE2, as well as deficiency in bone marrow–derived cells, increased atherosclerosis in Ldlr<sup>−/−</sup> mice. Elevated macrophage concentrations of Ang II in ACE2-deficient mice promoted expression of inflammatory cytokines and increased monocyte adhesion to endothelial cells. When both Ang II and Ang-(1–7) were present, Ang-(1–7) functionally antagonized effects of Ang II to promote monocyte adhesion and lesion development. These results demonstrate that regulation of local concentrations of Ang II versus Ang-(1–7) in pivotal cell types by enzymes such as ACE2 influences developing atherosclerotic lesions.

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Disclosures
None.

References


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Supplemental Methods

Mice. Ace2+/y or Ace2-/y mice (Dr. S. Gurley, Duke University) were bred onto an Ldlr-/- background (C57BL/6 ten times backcrossed). At 8 weeks of age, male mice of both genotypes were fed a high-fat diet (HF; TD.88137, 42% caloric intake from fat; Harlan Teklad, Madison WI) for 3 months. Mice were anesthetized using ketamine/xylazine (100/10 mg/kg, i.p.), aortas were harvested and post-fixed with 10% formalin and en face analysis was performed as previously described.1 All other tissues were either snap frozen in liquid nitrogen, placed in RNA lysis solution (Promega, Madison WI), or set in OCT (Tissue-Tek, Torrance CA) for sectioning. All studies using animals were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Bone Marrow Transplantation. Bone marrow transplantation procedures were performed as described previously.2 Mice were maintained on antibiotic water (sulfatrim, 4 ug/ml) for 1 week prior to and then 4 weeks after irradiation. Recipient mice were irradiated with a total of 900 Rads from a cesium source that was delivered in 2 divided doses (3 hours apart). Bone marrow-derived cells were obtained from the tibias and femurs of donor mice (Ldlr-/- mice that were either Ace2+/y or Ace2-/y) and injected into the tail vein of irradiated recipient mice (1 x 10^7 cells/mouse). Six weeks after irradiation, mice were fed the HF diet for a total of 3 months.

Ang-(1-7) with AngII Co-infusion Studies. 8-12 week old Ldlr-/- male mice were placed
on a high-fat diet (TD.88137) 1 week prior to the start of measurements. Body weights, baseline blood pressures, and ultrasounds were recorded before a 7-day mini-pump (Alzet, Model 2001) was placed subcutaneously into these mice (Groups, saline and 400 ng/kg/min Ang-(1-7)). During the 7-day infusion, blood pressure measurements and an ultrasound recording was performed on these two groups. No significant differences were seen in blood pressure or ultrasound recordings (data not shown). After removal of this pump, a 28-day pump (Alzet, Model 1004) was placed in these mice with either AngII (1,000 ng/kg/min) or Ang-(1-7)(400 ng/kg/min) plus AngII (1,000 ng/kg/min). Ultrasound recordings were performed at day 0, day 14, and day 28. Blood pressure measurements were done during the third week of post-infusion of the peptides. After 28 days, mice were sacrificed and tissues/plasma/serum were collected for analysis.

ACE2 Activity Measurements. ACE2 enzymatic activity was measured as described previously. Briefly, mouse peritoneal macrophages (MPMs) and other tissues were isolated and homogenized in a 100 mM Tris buffer containing 0.3 M NaCl, 10 µM ZnCl2, and 10 µM Z pro-prolinal containing protease inhibitors without EDTA (Roche, Indianapolis IN). Samples were centrifuged at 15,000 rpm and pellets were reconstituted in buffer containing 0.5% Triton-X. Samples were kept at 4°C overnight and centrifuged at 5,000 rpm to get rid of cellular debris. Supernatant was then measured for protein by BCA assay (ThermoFischer, Rockford IL) and placed into buffer (0.5 mg/mL) containing 1 µM pepstatin A, 10 µM captopril, 100 µM bestatin
hydrochloride, 10 µM phosphoramidon, and 10 µM thiorphan. Samples were then incubated with $^{125}$I-AngII for 30 minutes at 37°C and peptides were separated by HPLC (Beckman Coulter, Brea CA) followed by scintillation counting (Perkin Elmer, Waltham MA). ACE2 activity is expressed as femtomoles per milligram protein per minute, based on the specific activity of $^{125}$I-AngII (2,175 Ci/mmol).

**Immunohistochemistry.** Aortic tissue was taken from the start of the aortic semilunar valves and sectioned every 10 microns. Sections were placed on MicroProbe slides (Fischer, Chicago IL) and immunostained for CD68 (5 µg/mL; rat monoclonal ab53444, Abcam, Cambridge MA), ACE2 (5 µg/mL; rabbit polyclonal, ab15348 Abcam, Cambridge MA) or control IgG antibody (anti-rat for CD68, anti-rabbit for ACE2; 5 µg/mL; Vector Labs, Burlingame CA). Both non-immune rat IgG and rabbit IgG yielded similar results (data not shown). Sections were first placed in methanol at -20°C for 5 minutes followed by incubation with 0.05% redusol for 2 minutes with subsequent rinsing (1X automation buffer, GeneTex, Irvine CA). Sections were incubated with hydrogen peroxide in methanol for 2 minutes followed by blocking in rabbit or goat serum, respectively (Vector Labs, Burlingame, CA). Sections were then rinsed in 1X automation buffer and incubated with primary antibody for 30 minutes at 40°C. Sections were rinsed again and incubated with a biotinylated secondary antibody for 15 minutes at 40°C (Vector Labs). An AEC kit was used for detection of the biotin-avidin complex (Biomeda, Foster City CA). Sections were counterstained with aqueous hematoxylin followed by rinsing with automation buffer and distilled water. Sections were then placed in glycerol gelatin and kept at room temperature (Sigma, St. Louis MO). Oil Red
O staining was performed as previously described. Quantification of Oil Red O and CD68 staining was performed on 4-8 mice with 9 sections per mouse (approximately 240 μm above and below the transition point) at 40X magnification. Data are the mean of values across 9 sections/mouse/genotype or peptide treatment.

Monocyte Adhesion Assay. Human umbilical endothelial cells (HUVECs, Lonza, Walkersville MD) were plated in 12 well plates (Corning, Flushing NY) at a density of 40,000 cells/well and allowed to reach confluence. HUVECs were also co-incubated on human fibronectin coated inserts (lower chamber contained MPMs at a density of 1 X 10^6 cells/well for 24 hours)(BD Biosciences, San Jose CA) before performing monocyte adhesion assay. HUVECs were incubated with losartan (Merck, Whitehousestation NJ), Ang II (Bachem, Torrance, CA) or Ang-(1-7)(Bachem, Torrance, CA) at a 1 µM concentration for 30 minutes prior to AngII stimulation (losartan plus AngII) for 24 hours. D-Ala (Bachem, Torrance, CA) was used at a 5 µM concentration. Human acute monocytic leukemia cells (THP-1, ATCC, Manassas, VA) were counted (50,000 cells/well or 12,500 cells/insert) and activated with 10 ng/mL TNF-α for 10 minutes at 37°C. THP-1 cells were then incubated with calcein AM (Invitrogen, Carlsbad CA) for 15 minutes at 37°C. Fluorescently labeled cells were then incubated in either human fibronectin coated inserts or 12 well plates for 45 or 30 minutes, respectively. Wells were gently rinsed twice and fixed with 1% glutaraldehyde (Sigma, St. Louis, MO). After fixation, cells were gently rinsed in PBS containing Mg^{2+} and Ca^{2+} and taken to inverted Nikon Eclipse TE2000-U fluorescent microscope for cell counting. For co-culture of MPMs with HUVECs, data was normalized to the vehicle treatment of Ace2^{+/−} MPMs.
(Figure 4C). For other adhesion assay, data was normalized to control HUVECs (Figure 4D).

**Real-time PCR.** MPMs were cultured in DMEM for 48 hours prior to RNA extraction or isolation of culture media for cytokine analysis. MPMs were extracted for total RNA using a Promega SV Total RNA kit. RNA absorbance was measured at 260 and 280 nm and a reverse transcription reaction was performed on 0.1 micrograms of RNA using a Retroscript kit (Ambion, Austin, TX). Real time PCR was performed using an Applied Biosystems SYBR Green kit (Warrington, UK) and data were analyzed using the delta delta Ct method. Primers used in real-time PCR experiments are depicted below.

### Supplemental Table I. Primer sequences used in real-time PCR analysis.

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE2</td>
<td>Forward 5’-ACGAGATGGGACACATCCA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-GAAAATCGGATGGCAGAAGA-3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward 5’-CCCACTCTGACCCCTTTTACTC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-TCACTGTCCAGCATCTTTGT-3’</td>
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<tr>
<td>TNF-α receptor</td>
<td>Forward 5’-CAGTCTGAGGAGTGTAAGG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-CACGCAGAGTGCTTCT-3’</td>
</tr>
<tr>
<td>NF-kappa B (p105 subunit)</td>
<td>Forward 5’-CTGACCTGAGCCTTCTTGAC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-GCAGGCTATTGCTCATCACA-3’</td>
</tr>
<tr>
<td>CCL2</td>
<td>Forward 5’-CCTGCTGTACTCATTCCACC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-TGCTGGACCATTCTTCTT-3’</td>
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</table>
Total Serum Cholesterol Concentrations and Lipoprotein Cholesterol Distributions. Sera cholesterol was measured using a colorimetric kit (Wako Chemicals, Richmond, VA) as described previously. Sera lipoproteins were resolved by size exclusion chromatography (Bio-rad, Hercules, CA) as described previously.

Blood Pressure and Plasma RAS Characterization. Systolic blood pressure was measured as previously described using a tail cuff system (Visitech, Apex, NC) and plasma renin and AngII concentrations were performed as described previously. Serum ACE activity was measured according to the manufacturer’s instructions (Fujirebio Diagnostics, Malvern PA).

Measurement of Inflammatory Cytokines. Protein levels of IL-6 and PAI-1 were assessed using Millipore’s Luminex assay system (Billerica, MA). Ten microliters of standard or sample were used for each well and the quality controls fell within the recommended range.

Statistical Analysis. Data are reported as mean ± SEM. Data were tested for use of parametric or nonparametric post hoc analysis. For two groups, data were analyzed by
Student’s t-test, while ANOVA was used for analyses of more than two groups. P<0.05 values were considered to be statistically significant.

Supplemental Results

Supplemental Table II. mRNA abundance of various inflammatory factors in MPMs from \textit{Ace2}\textsuperscript{+/y} and \textit{Ace2}\textsuperscript{+/y} \textit{Ldlr}\textsuperscript{−/−} mice

<table>
<thead>
<tr>
<th></th>
<th>\textit{Ace2}\textsuperscript{+/y}</th>
<th>\textit{Ace2}\textsuperscript{+/y}</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2</td>
<td>1.7 ± 0.9</td>
<td>3.0 ± 1.1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.5 ± 0.7</td>
<td>3.1 ± 0.9</td>
</tr>
<tr>
<td>TNF-α receptor</td>
<td>1.0 ± 0.2</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>NF-kappa B</td>
<td>1.1 ± 0.3</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

Data are mean ± SEM from n = 3.

Supplemental Table III. Protein levels released from MPMs of \textit{Ace2}\textsuperscript{+/y} and \textit{Ace2}\textsuperscript{+/y} \textit{Ldlr}\textsuperscript{−/−} mice

<table>
<thead>
<tr>
<th></th>
<th>\textit{Ace2}\textsuperscript{+/y}</th>
<th>\textit{Ace2}\textsuperscript{+/y}</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2 (pg/mL)</td>
<td>715 ± 161</td>
<td>1216 ± 265</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>249 ± 68</td>
<td>324 ± 54</td>
</tr>
</tbody>
</table>

Data are mean ± SEM from n = 3.

References


2. Cassis LA, Rateri DL, Lu H, Daugherty A. Bone marrow transplantation reveals that recipient at1a receptors are required to initiate angiotensin ii-induced atherosclerosis and aneurysms. \textit{Arterioscler Thromb Vasc Biol}. 2007;27:380-386


Supplemental Figure I. Oil red O lesion quantification in aortic sinuses of saline and losartan-infused Ace2^-/- male mice (A). ACE2 activity of tissues from Ace2^-/- Ldlr^-/- male mice (B).
Supplemental Figure II. Lipoprotein cholesterol distributions in mice with whole body (A) or bone marrow transplanted ACE2 deficiency (B).
Supplemental Figure III. Genotyping of bone marrow from Ldlr⁻/⁻ recipients to demonstrate efficient repopulation with appropriate donor genotype (Ace2⁺/⁺ or -/-).

Supplemental Figure

A. Ace2⁺/⁺  Ace2⁻/⁻