Angiotensin-Converting Enzyme 2 Decreases Formation and Severity of Angiotensin II–Induced Abdominal Aortic Aneurysms

Sean E. Thatcher, Xuan Zhang, Deborah A. Howatt, Frederique Yiannikouris, Susan B. Gurley, Terri Ennis, John A. Curci, Alan Daugherty, Lisa A. Cassis

Objective—Angiotensin-converting enzyme 2 (ACE2) cleaves angiotensin II (AngII) to form angiotensin-(1–7) (Ang-(1–7)), which generally opposes effects of AngII. AngII infusion into hypercholesterolemic male mice induces formation of abdominal aortic aneurysms (AAAs). This study tests the hypothesis that deficiency of ACE2 promotes AngII-induced AAAs, whereas ACE2 activation suppresses aneurysm formation.

Approach and Results—ACE2 protein was detectable by immunostaining in mice and human AAAs. Whole-body deficiency of ACE2 significantly increased aortic lumen diameters and external diameters of suprarenal aortas from AngII-infused mice. Conversely, ACE2 deficiency in bone marrow–derived cells had no effect on AngII-induced AAAs. In contrast to AngII-induced AAAs, ACE2 deficiency had no significant effect on external aortic diameters of elastase-induced AAAs. Because ACE2 deficiency promoted AAA formation in AngII-infused mice, we determined whether ACE2 activation suppressed AAAs. ACE2 activation by administration of diminazene aceturate (30 mg/kg per day) to Ldlr−/− mice increased kidney ACE2 mRNA abundance and activity and elevated plasma Ang-(1–7) concentrations. Unexpectedly, administration of diminazene aceturate significantly reduced total sera cholesterol and very low-density lipoprotein–cholesterol concentrations. Notably, diminazene aceturate significantly decreased aortic lumen diameters and aortic external diameters of AngII-infused mice resulting in a marked reduction in AAA incidence (from 73% to 29%). None of these effects of diminazene aceturate were observed in the Ace2−/− mice.

Conclusions—These results demonstrate that ACE2 exerts a modulatory role in AngII-induced AAA formation, and that therapeutic stimulation of ACE2 could be a benefit to reduce AAA expansion and rupture in patients with an activated renin–angiotensin system. (Arterioscler Thromb Vasc Biol. 2014;34:2617-2623.)

Key Words: angiotensin II • angiotensin converting enzyme 2 • aortic aneurysms, abdominal • hypercholesterolemia

Abdominal aortic aneurysms (AAAs) affect >1 million people in the United States.1,2 There are no validated medical therapies that favorably affect aneurysm growth and rupture, making surgery the only available treatment option for AAAs. As AAA size increases, the risk of rupture increases, and ruptured aneurysms contribute to mortality rates of 60% to 80%.3–5 Therefore, there is a need for research to define mechanisms of AAA formation so that potential drug targets for this devastating disease can be identified.

Infusion of angiotensin II (AngII) into male mice is a commonly used animal model to gain insight into mechanisms of human AAAs.6,7 Similar to the human disease, AAAs that develop in response to AngII exhibit progressive leukocyte accumulation, extracellular matrix degradation, luminal expansion and thrombus.8 Manipulation of the renin–angiotensin system (RAS) pharmacologically through use of angiotensin type 1 receptor (AT1R) antagonists,9 or genetically through angiotensin type 1a receptor deficiency,10 substantially reduces AngII-induced AAA formation. Recent studies demonstrate that inhibition of the RAS also suppressed experimental aneurysms in an elastase-induced model.11 Currently, it is unclear whether manipulation of the RAS is an effective mode for AAA therapy in humans.12–16 However, several ongoing clinical trials (NCT01118520, NCT001904981) are either actively recruiting or in the process of evaluating efficacy of angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor antagonists on either the size or expansion rate of human AAAs. Moreover, a recent study demonstrated that long-term blockade of the RAS in hypertensive patients attenuated the expansion of nonaneurysmal abdominal aorta, suggesting that RAS blockade given before advancement of aortic remodeling may slow the development of AAAs.17

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ACE2 is a homolog of ACE that converts AngII to angiotensin-(1–7) (Ang-(1–7)). The ability of ACE2 to degrade a vasoconstrictor (AngII) and produce a vasodilator (Ang-(1–7)) provides rationale for this enzyme as a therapeutic target. Previous studies in our laboratory demonstrated that deficiency of ACE2 promoted hypercholesterolemia-induced atherosclerosis. Effects of ACE2 deficiency to promote atherosclerosis seem to result from elevations in AngII and from reductions in Ang-(1–7) concentrations because coinfusion of Ang-(1–7) with AngII in Ldlr−/− mice reduced atherosclerosis. Although these results suggest that manipulation of ACE2 influences atherosclerotic lesion formation, the role of ACE2 as a modulator of AAA formation and severity has not been defined.

Macrophages play an important role in the formation and progression of AngII-induced AAAs. Previous studies demonstrated that bone marrow deficiency of ACE2 promoted diet-induced atherosclerosis, suggesting that leukocyte ACE2 suppresses atherosclerotic lesion formation. Several studies have shown that deletion of specific proteins in bone marrow–derived cells can enhance or attenuate the formation of AAAs. It is unclear whether leukocyte ACE2 also modulates the susceptibility to AngII-induced AAAs.

Because ACE2 catabolizes AngII to Ang-(1–7), activators of ACE2 are potential therapeutics in treatment of AngII-induced diseases. Diminazene aceturate (DIZE) has been described as an activator of ACE2 that lowered blood pressure and endothelin-1–induced ischemic stroke when administered centrally to rats and that attenuated pulmonary hypertension in rats. This compound has not been examined in AngII-induced AAAs. In this study, we first determined whether ACE2 localizes to human and murine AAAs. Then, we determined effects of whole-body ACE2 deficiency on AngII-induced AAAs in Ldlr−/− mice. As a potential therapeutic modality to suppress the RAS and thereby blunt AAA formation, we administered DIZE to ACE2 wild-type (Ace2+/+) or deficient mice (Ace2−/−) to determine whether ACE2 activation reduced AngII-induced AAAs. We also examined effects of whole-body ACE2 deficiency on elastase-induced AAAs in Ldlr−/− mice.

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

Results
ACE2 Localized to Murine and Human AAAs
In serial sections of abdominal aortas from saline or AngII-infused Ldlr−/− mice, ACE2 immunostaining localized predominately to the intima and adventitia (Figure 1). In AAA regions exhibiting a break in medial elastin, ACE2 immunostaining was pronounced in adventitia. ACE2 immunostaining was performed on tissue sections from human abdominal aortas and in sections from patients with AAAs (Figure 2; Figure 1 in the online-only Data Supplement). ACE2 localized to the intima and media of both non- and aneurysmal abdominal aorta and was also present in vaso vasorum (Figure IA in the online-only Data Supplement). In human AAA tissue sections, ACE2 was present in intimal plaque (Figure 2, arrow) with abundant immunostaining in cells of inflammatory foci (Figure 2, dotted arrow; Figure IB in the online-only Data Supplement). CD68-positive cells also stained positive for ACE2 in inflammatory foci of human AAAs (Figure I, lower panels, in the online-only Data Supplement).

Whole-Body ACE2 Deficiency Increased AngII-Induced AAAs
To determine the contribution of ACE2 to AngII-induced AAAs, male Ldlr−/− mice that were either Ace2+/+ or Ace2−/− were infused with AngII. ACE2 deficiency had no significant effect on body weight, plasma renin concentrations, serum ACE activity, or sera lipid concentrations in AngII-infused mice (Table I in the online-only Data Supplement). However, plasma AngII concentrations were significantly increased in AngII-infused Ace2−/− when compared with

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Figure 1. Localization of angiotensin-converting enzyme 2 (ACE2) to abdominal aortas of Ldlr−/− mice infused with either saline or angiotensin II (AngII). Left, Nonimmune IgG. Right, ACE2 immunostaining in abdominal aortic sections from saline (top 2 panels) vs AngII-infused mice (bottom 2 panels). ACE2 staining was present in all layers of the vascular wall in aortic sections from mice in each group. Black box represents area that magnified in bottom for saline-infused mice. Bottom panels for AngII-infused mice at medial break display ACE2 immunostaining. Scale bars, 50 μm.
Leukocyte ACE2 Deficiency Had No Effect on AngII-Induced AAAs

Previous studies demonstrated that ACE2 deficiency in whole bone marrow cells augmented diet-induced atherosclerosis.\(^\text{20}\) Because macrophages are a prominent cell type in AngII-induced AAAs,\(^\text{8}\) we transplanted whole bone marrow cells into lethally irradiated \(Ldlr^{−/−}\) recipients before infusing with AngII. Bone marrow from recipient mice exhibited donor mouse genotypes at study end point (Figure V in the online-only Data Supplement). Deficiency of ACE2 in bone marrow–derived cells had no significant effect on body weight, systolic blood pressure, white blood cell count, plasma renin concentrations, or serum cholesterol concentrations in AngII-infused \(Ldlr^{−/−}\) mice (data not shown on white blood cell; Table I in the online-only Data Supplement). In addition, deficiency of ACE2 in whole bone marrow cells had no effect on suprarenal aortic lumen diameters of AngII-infused \(Ldlr^{−/−}\) mice (donor genotype: \(Ace2^{+/−}\), \(1.31±0.06\); \(Ace2^{−/−}\), \(1.32±0.07\) mm; \(P>0.05\)). Similarly, external diameters of suprarenal aortas were not significantly different between donor genotypes (Figure VI in the online-only Data Supplement; \(P>0.05\)).

DIZE Reduced AngII-Induced AAAs in \(Ace2^{−/−}\), but Not in \(Ace2^{+/−}\) Mice

Because ACE2 deficiency promoted AngII-induced AAAs, this suggests that activation of ACE2 may reduce AAA formation and severity. We used DIZE as a previously described ACE2 activator\(^\text{27,29,30}\) to define whether ACE2 activation blunts AAA formation and severity. Moreover, to address specificity of DIZE, we determined effects of the compound on AngII-induced AAAs in \(Ace2^{+/−}\) and \(Ace2^{−/−}\) \(Ldlr^{−/−}\) mice. Pilot studies determined concentration-dependent effects of DIZE to activate ACE2 when infused subcutaneously by osmotic minipumps to \(Ldlr^{−/−}\) mice (Figure VII in the online-only Data Supplement). After 7 days of infusion of 30 mg/kg per day of DIZE, plasma Ang-(1–7) concentrations, kidney ACE2 mRNA abundance, and activity were increased significantly when compared with vehicle controls (Figure VII in the online-only Data Supplement; \(P>0.05\)). Unfortunately, when DIZE was placed in 28-day osmotic minipumps, the stability of the compound decreased markedly after 7 days (data not shown). Therefore, we administered vehicle or DIZE with a drug stabilizer (antipyrine) by daily intramuscular injections at a dose of 30 mg/kg per day.

Administration of DIZE did not significantly influence systolic blood pressure, plasma renin, AngII concentrations or sera triglyceride concentrations in AngII-infused \(Ace2^{+/−}\) or \(Ace2^{−/−}\) mice (Table I in the online-only Data Supplement; \(P>0.05\)). Although DIZE administration had no significant effect on plasma Ang-(1–7) concentrations (data not shown), kidney ACE2 mRNA abundance, and activity were increased significantly in \(Ace2^{−/−}\) mice (Figure VIII in the online-only Data Supplement). Body weight was decreased significantly by DIZE administration in \(Ace2^{+/−}\) and \(Ace2^{−/−}\) mice (VEH \(Ace2^{+/−}\), 27±1 g; DIZE 30 mg/kg per day \(Ace2^{+/−}\), 23±0.4 g; DIZE \(Ace2^{−/−}\), 23±1 g; \(P<0.05\) compared with VEH \(Ace2^{−/−}\)). Unexpectedly, sera cholesterol concentrations were decreased significantly in \(Ace2^{+/−}\), but not in \(Ace2^{−/−}\) mice administered DIZE when compared with vehicle controls (Figure 4A; \(P<0.05\) compared with VEH \(Ace2^{+/−}\) mice). The decrease in

\[\text{Ace2}^{+/−}\] mice (Table I in the online-only Data Supplement; \(P<0.05\)). Systolic blood pressure was increased significantly by AngII infusion in both genotypes, with significantly increased pressures in \(Ace2^{−/−}\) when compared with \(Ace2^{+/−}\) mice (Table I in the online-only Data Supplement). AngII infusion significantly increased suprarenal aortic lumen diameters at day 14 and 28 when compared with baseline mice (Table I in the online-only Data Supplement). AngII size is illustrated in representative aortas from \(Ace2^{−/−}\) mice (Figure III in the online-only Data Supplement). At sites of medial degeneration, CD68 immunostaining was more pronounced in AAA sections from \(Ace2^{−/−}\) mice (Figure III in the online-only Data Supplement). We performed CD68 immunostaining in AAA sections from \(Ace2^{+/−}\) and \(Ace2^{−/−}\) mice (Figure III in the online-only Data Supplement). We also examined effects of ACE2 deficiency on dilations of the infrarenal aorta in response to intra-aortic elastase perfusion.\(^\text{21}\) The percentage increase in aortic diameter, quantified 14 days after elastase perfusion, was not significantly different between \(Ace2^{+/−}\) and \(Ace2^{−/−}\) \(Ldlr^{−/−}\) mice (Figure IV in the online-only Data Supplement).

\[\text{Ace2}^{−/−}\] mice administered DIZE when compared with vehicle controls (Figure 4A; \(P<0.05\) compared with VEH \(Ace2^{+/−}\) mice).
serum total cholesterol concentrations was attributable to decreased very low-density lipoprotein-cholesterol concentrations by DIZE administration in \textit{Ace2\textsuperscript{+/y}}, but not in \textit{Ace2\textsuperscript{--/y}} mice (Figure 4B and 4C; \(P<0.05\)).

DIZE administration decreased significantly lumen diameters (Figure 5A; \(P<0.05\)) and external diameters of suprarenal aortas (Figure 5B; \(P<0.05\)) in \textit{Ace2\textsuperscript{+/y}} and \textit{Ace2\textsuperscript{--/y}} mice. Representative abdominal aortas from \textit{Ace2\textsuperscript{+/y}} and \textit{Ldlr\textsuperscript{--/y}} mice are shown in Figure 5C. \(\ast P<0.05\) compared with day 0 ultrasound. \(\text{**} P<0.05\) compared with \textit{Ace2\textsuperscript{+/y}}.

**Discussion**

We first demonstrated that ACE2 localized to murine AngII-induced AAAs and human AAAs. To determine whether ACE2 modulates AAA formation, we examined the effects of ACE2 deficiency on formation and severity of AngII-induced AAAs. Whole-body ACE2 deficiency promoted both formation and the severity of AngII-induced AAAs. In contrast, whole-body ACE2 deficiency had no effect on elastase-induced AAAs. Leukocyte ACE2 seemed to play no role because ACE2 deficiency in whole bone marrow cells had no effect on AngII-induced AAAs. Because ACE2 deficiency promoted AAA formation, we turned...
to therapeutic activation of ACE2 to suppress AAA formation and severity. Administration of the ACE2 activator, DIZE, reduced the size, severity, and incidence of AngII-induced AAs in wild-type, but not in ACE2-deficient mice. These results suggest that ACE2 activation may serve as a novel therapeutic target in the treatment of AAAs in patients with an activated RAS.

An interesting feature of ACE2 is that it catabolizes AngII to form Ang-(1–7), 2 angiotensin peptides that counterbalance each other in vaso-contractile versus vaso-relaxation, respectively.32–40 This makes activation of ACE2 an attractive target to reduce the relative balance of AngII/Ang-(1–7) and thereby inhibit vascular disease.41 In this study, although whole-body deficiency of ACE2 promoted AngII-induced AAs, ACE2 activation inhibited AngII-induced AAs. Beneficial effects of ACE2 manipulation against AAA formation could result from the regulation of AngII concentrations (eg, plasma concentrations increased in ACE2-deficient mice) or from regulation of Ang-(1–7). These results suggest that effects of ACE2 may be attributed to regulation of Ang-(1–7).

In contrast to previous findings demonstrating a role for leukocyte ACE2 in diet-induced atherosclerosis,39 results from this study demonstrate that leukocyte ACE2 deficiency had no effect on AngII-induced AAs. However, AAs from ACE2-deficient mice exhibited pronounced macrophage immunostaining at sites of medial degeneration. Because leukocyte ACE2 deficiency had no effect on AngII-induced AAs, it is likely that ACE2 deficiency in other cell types (eg, vascular wall cells) promoted macrophage recruitment to developing AAs. It is clear that angiotensin type 1a receptors are required for AngII-induced AAs.10 Similar to the lack of effect of leukocyte ACE2 deficiency on AngII-induced AAs, deficiency of angiotensin type 1a receptors on leukocytes did not significantly influence AngII-induced AAs in Ldlr−/− mice.10 In addition, recent studies demonstrated that neither endothelial nor smooth muscle cell angiotensin type 1a receptor deficiency influenced AngII-induced AAs in male Ldlr−/− mice.10 Thus, the primary cell type(s) responding to AngII to induce AA formation have not been identified. Additional studies should address cell type(s) expressing ACE2 that modulate local angiotensin peptide concentrations to promote AA formation and severity.

Whole-body deficiency of ACE2 in the present study resulted in an increased blood pressure response to AngII infusion. In mice, infusion of norepinephrine to hypercholesterolemic male mice at a rate that increased blood pressure to a similar extent as observed in AngII-infused mice did not result in AAA formation.43 Moreover, administration of the vasodilator hydralazine to AngII-infused mice had no significant effect on AAA formation. Given these findings, it is unlikely that blood pressure increases in ACE2-deficient mice infused with AngII contributed to increases in AA severity.

Because there are no proven medical therapies that modify AAA growth and rupture, an important finding of the present study was the ability of ACE2 activation to reduce the formation and severity of AngII-induced AAs. We used DIZE, a compound previously shown to activate ACE2 and lower blood pressure and endothelin-1–induced ischemic stroke when administered centrally to rats.30,44 Recent studies demonstrated that administration of DIZE not only prevents the development of pulmonary hypertension in rats but also arrests the progression of established pulmonary hypertension.29 However, recent studies indicate that DIZE may also exhibit ACE2-independent effects.45 We included the drug stabilizer, antipyrine, to improve the stability of DIZE because the drug has a short half-life.46 Our results also demonstrate that DIZE increased ACE2 mRNA abundance and elevated plasma Ang-(1–7) concentrations.

Figure 5. Activation of angiotensin-converting enzyme 2 (ACE2) by diminazene aceturate (DIZE) administration reduced angiotensin II (AngII)–induced abdominal aortic aneurysms. A, Aortic lumen diameter was quantified by ultrasound on day 0, 7, 14, and 28 of AngII infusion in mice administered vehicle (VEH) or DIZE (15 or 30 mg/kg per day). Data are mean±SEM of n=7 to 14 mice per group. *P<0.05 compared with day 0. **P<0.05 compared with VEH. B, External diameters of suprarenal aortas from mice administered VEH or DIZE. Circles are individual mice, whereas triangles represent mean±SEM. C, AAA incidence in mice from each group. *P<0.05 compared with VEH.
These findings are in agreement with results from studies using pulmonary hypertensive rats, where DIZE administration significantly increased the ACE2:ACE ratio. Importantly, administration of DIZE to ACE2-deficient mice had no effect on AngII-induced AAAs, supporting an ACE2-dependent mechanism of the compound. In addition to efficacy to decrease AAA formation and severity, DIZE administration resulted in other ACE2-dependent (sera cholesterol) and ACE2-independent (body weight) effects, suggesting ancillary properties of the compound. These results support the need to include ACE2-deficient mice in studies examining novel ACE2 activators to define the specificity of drug action on the parameter of interest. In addition, these results support further studies examining efficacy of ACE2 activation to retard AAA progression.

It is noteworthy that DIZE administration reduced sera cholesterol concentrations, specifically very low-density lipoprotein-cholesterol, in wild-type but not in ACE2-deficient mice. Mechanisms responsible for ACE2-mediated regulation of sera cholesterol concentrations are unclear. Recent studies identified novel effects of ACE2 to regulate dietary amino acid homeostasis in the gut; effects that were independent of ACE2-mediated regulation of the RAS. In addition to the gut, ACE2 is also expressed in liver where it has been suggested to modulate cirrhosis. It is conceivable that ACE2 exerts unidentified effects, potentially related to the gut and liver, to influence cholesterol absorption and metabolism. Effects of DIZE to reduce sera cholesterol concentrations may result from increased levels of systemic Ang-(1–7) because recent studies demonstrate that administration of an oral formulation of Ang-(1–7) to high-fat–fed mice significantly reduced sera cholesterol concentrations. Although beneficial in the treatment of atherosclerosis, reductions (32%) in sera cholesterol are unlikely to be the mediator of DIZE’s ability to decrease AAA formation in response to AngII because sera cholesterol concentrations in mice administered DIZE (571 mg/dL) remained considerably higher than those typically present in C57BL/6 mice (<80 mg/dL). Even at low levels of sera cholesterol concentration, male C57BL/6 mice continue to exhibit some susceptibility (20%–30%) to AngII-induced AAAs.

In conclusion, results from these studies support a role for ACE2 as a modulator of the formation and severity of AngII-induced AAAs. Whole-body, but not leukocyte deficiency of ACE2, promoted AngII-induced AAAs, suggesting that ACE2 in other cell types regulates AAA formation and severity. Finally, the activation of ACE2 suppressed both the formation and the severity of AngII-induced AAAs, identifying ACE2 as a potential novel target in the medical therapy of AAAs in patients with an activated RAS.

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Disclosures
None.

References
ACE2 Regulates AAA Formation and Severity


Significance

Abdominal aortic aneurysms (AAAs) and ruptures are associated with significant mortality. Currently, there are no drug therapies that are effective to slow the progression of AAAs. Infusion of angiotensin II induces AAA formation in experimental mice. Angiotensin-converting enzyme 2 (ACE2) catalyzes angiotensin II to form its functional antagonist, angiotensin-(1–7). Our results demonstrate that ACE2 localizes to murine and human AAAs. To determine the functional role of ACE2 in AAA formation and severity, we examined effects of ACE2 deficiency versus ACE2 activation on angiotensin II–induced AAAs. Deficiency of ACE2 augmented AAA formation and severity, whereas increasing ACE2 activity therapeutically suppressed AAA formation and severity. However, ACE2 deficiency had no effect on elastase-induced AAAs. These results suggest that ACE2 activators may serve as a novel therapeutic modality to reduce the formation and severity of AAAs in patients with an activated renin–angiotensin system.
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SUPPLEMENTAL MATERIAL

ACE2 Decreases the Formation and Severity of Angiotensin II-induced Abdominal Aortic Aneurysms

Sean E. Thatcher¹, Xuan Zhang², Deborah A. Howatt³, Frederique Yiannikouris¹, Susan B. Gurley⁴, Terri Ennis⁵, John A. Curci⁵, Alan Daugherty³, and Lisa A. Cassis¹
Table I. Systolic blood pressures, plasma renin concentrations, serum ACE and triglyceride concentrations in AngII-infused mice from each study

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BMT – Bone marrow transplantation
VEH, Vehicle (antipyrine in 0.9% saline); DIZE, Diminazine aceturate (30 mg/kg/day)
PRC = plasma renin concentration
ND = not determined
*P<0.05 compared to baseline blood pressure
†P<0.05 compared to Ace2⁺/⁺ mice
Figure I. Top, Non-immune IgG and ACE2 immunostaining in human abdominal aortas (upper panels), and CD68 immunostaining in inflammatory foci of human AAAs (lower panels). (A), Represents ACE2 staining in the vaso vasorum of a human non-AAA (arrows). (B), Represents ACE2 staining in an inflammatory foci of a human AAA (arrow). Lower panels: Non-immune IgG (Neg Control), CD68, and ACE2 immunostaining in inflammatory foci of human AAAs. Scale bars represent 50 µm.
Figure II. Gomori trichrome stain of medial break (arrows) in $\text{Ace2}^{+/y}$ and $\text{Ace2}^{-/-}$ mice. Scale bar represents 500 μm.
Figure III. CD68 staining in Ace2\(^{+/y}\) and \(^{-/y}\) AAA sections. (A) represents CD68 staining of sections at the medial break. Scale bar is 500 µm (B) represents increased magnification (200X) and images used for quantification of macrophages. Scale bar is 50 µm. (C) represents the average macrophage area (mm\(^2\)) in sections taken from Ace2\(^{+/y}\) (N=7) and Ace2\(^{-/y}\) (N=8) Ldlr\(^{-/-}\) mice (*P<0.05)
Figure IV. Percent increase from baseline for elastase-induced AAA model. *Ace2+/y* and *-y* mice were infused with elastase and infrarenal aortas were examined 14 days later (N=4-5).
Figure V. PCR analysis of Ldlr−/− recipients that were transplanted with bone marrow-derived stem cells either from Ace2+/*/* or −/*/* mice. DNA bands of bone marrow from mice transplanted with cells from Ace2+/*/* (380 base pairs (bp)) or −/*/* mice (580 bp).
Figure VI. External aortic diameters of Ace2<sup>+/y</sup> mice that were transplanted with either Ace2<sup>+/y</sup> or -/y bone marrow and infused with AngII for 28 days (1,000 ng/kg/min). Closed or open circles represent individual mice and triangles represent the average of each group.
Figure VII. (A) Plasma Ang-(1-7) concentrations in mice infused with selected doses of DIZE for 7 days by osmotic minipump (N = 5/dose; P=0.04 for 30 mg/kg/day DIZE-treated mice compared to saline). ACE2 mRNA abundance (B) and activity (C) in kidneys from mice administered either saline or DIZE (30 mg/kg/day; N = 5; *P<0.05 compared to saline).
Figure VIII. ACE2 mRNA abundance (A) and activity (B) in kidneys from AngII-infused Ace2+/y or Ace2−/y mice; N = 5-8 mice/group) administered either vehicle (VEH) or DIZE (30 mg/kg/day by intramuscular injection). ND = not detected. *, P<0.05 compared to vehicle Ace2+/y; **, P<0.05 compared to DIZE Ace2+/y.
Figure IX. Kaplan-Meier survival curves for death due to AAA ruptures in AngII-infused Ace2+/y or Ace2−/y mice administered either vehicle (VEH) or DIZE (30 mg/kg/day). DIZE treatment prevented AAA ruptures in Ace2+/y male mice (*P<0.05 compared to vehicle and DIZE-treated Ace2−/y mice).
SUPPLEMENTAL MATERIAL

ACE2 Decreases the Formation and Severity of Angiotensin II-induced Abdominal Aortic Aneurysms

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Supplemental Materials and Methods

Experimental Animal Models. Male Ace2<sup>+/y</sup> (ACE2 is an X-linked gene) or Ace2<sup>-/y</sup> on an Ldlr<sup>/−</sup> background (10 times backcrossed on a C57BL/6 background; 8-12 weeks of age) were bred in-house and littermate controls were used for all studies. For each study, mice were fed a high-fat diet (42% kcal from fat, Teklad Diets, TD.88137) beginning 1 week prior to minipump implantations until study endpoint. AngII (1,000 ng/kg/min; Bachem) was infused (Alzet, Model 1004, Durect Corporation) into mice for 28 days. Bone marrow transplantation was performed as described previously,<sup>1</sup> and as described in detail in Supplemental Materials. Vehicle (antipyrin, Sigma, A5882, drug stabilizer in 0.9% saline) or DIZE (30 mg/kg/day, Sigma, D7770):antipyrin (1:1 ratio) were administered daily by intramuscular injection to Ace2<sup>+/y</sup> and -/y mice for 7 days prior to implantation of minipumps containing AngII (1,000 ng/kg/min) and throughout the 28-day AngII-infusion protocol. All experiments involving mice conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Elastase AAA model. Ace2<sup>+/y</sup> and -/y Ldlr<sup>/−</sup> male mice, aged 6-12 months, are anesthetized with 2% isoflurane. After determination of surgical anesthesia, the abdomen and inguinal area are clipped with a #40 blade and then the mouse is placed on its back and secured to the operating tray. The mouse is then scrubbed and prepped with Betadine and alcohol. A midline abdominal incision is made using sterile instruments. The wound is retracted with two clips to expose the abdominal cavity. The mouse is draped with sterile gauze. The intestines will be retracted and placed to the mouse’s left, placed in sterile gauze and kept moist. The infrarenal aorta will be exposed from the left renal vein to the aortic bifurcation. Posterior lumbar artery branches and the inferior mesenteric artery will be ligated with 9-0 suture. The aorta will be circumferentially dissected at the proximal and distal ends to allow placement of 6-0 silk sutures to be used as clamps. The aortic diameter will be measured using the micrometer within the microscope eyepiece. The proximal clamp then will be tightened, a microclip placed at the iliac junction, and a small aortotomy will be made using the tip of a 30g needle. The tip of a custom made microrenathane catheter .010 diameter (Braintree) will be placed into the aortotomy and the distal silk tie will be tightened to hold the intraluminal catheter in place. The aorta will be perfused for 5 ½ minutes with 0.49 ml of elastase solution via infusion pump. Following the 5 ½ minute perfusion, the distal tie will be released, the catheter tubing removed, and the aortotomy closed with 10-0 suture. Then the distal clamp will be removed, followed by removal of the proximal tie. After assessment of a patent, non-leaking aorta, and collecting post perfusion measurements of the aorta, the intestines will be replaced and the wound closed in two layers with 4-0 Vicryl and 4-0 nylon suture. Following surgery, prior to closure, the mouse may be given sterile warmed fluids, 0.25-1 ml, IP to replace minimal blood loss and to remoisten the abdominal cavity. The mouse will be placed on a circulating heated water blanket and allowed to recover. As described above, aseptic technique and postoperative care will be given.
Measurements of Vascular Pathologies. Ultrasound measurements (Vevo 660 or 2100) were performed at baseline (day 0 of infusion), and during AngII infusions on day 14 and 27. Mice exhibiting a 50% increase in aortic lumen diameter compared to baseline were defined as exhibiting an AAA. At the end of the study, mice were euthanized with a lethal dose of ketamine/xylazine mix (100/10 mg/kg, ip) and tissues (spleen, kidney, liver) were snap-frozen in liquid nitrogen. Aortas were placed in 10% formalin and cleaned of adherent tissue to quantify maximal external diameters of suprarenal aortas of mice exhibiting an AAA as described previously.²

Measurements of Systolic Blood Pressure. Systolic blood pressure was quantified by tail cuff as described previously.¹ Mice were acclimated to the system prior to and during week 3 of AngII-infusion and recordings were obtained for 5 consecutive days.

Genotyping by polymerase chain reaction. C57BL/6J mice (ten times backcrossed) were provided by Drs. Thomas Coffman and Susan Gurley from Duke University.³ Ace2+/y or −/y males were crossed to Ldlr−/− female mice until breeding pairs were established. Tail DNA was obtained by using the Qiagen DNeasy Tissue and Blood Kit (Qiagen, cat#69506). Mice were screened for Ace2 and Ldlr deficiency using primers listed, (Ace2+/y, Forward primer, 5’-GGGCCAGAGTATCTGCCCAG-3’ Reverse primer, 5’-GCAGGATCTCCTCTGATCTCAACC-3’ 380 bp)(Ace2+/y, Forward primer, 5’-TCGCCCTCTATCGCCTTCTGCACC-3’ Reverse primer, 5’-GGCGGATAATGCGCTTCTAG-3’ 583 bp)(Ldlr, neo cassette primer, 5’-AAATCCATCTTGTTCAATGGCCGATC-3’, upstream of neo cassette, 5’-CCATATGCCATCCCAGTCTT-3’, exon 4, 5’-GCGATGGATACACTCACTGC-3’, Ldlr+/+ 167 bp; Ldlr+/− 350 bp).

Bone marrow transplantation. Male Ldlr−/− mice (8 weeks old) were irradiated lethally with a total of 900 rads divided into two doses (450 rads/dose; 3 hours apart) from a cesium γ source. Bone marrow-derived cells were harvested from femurs of Ace2+/y or −/y mice on an Ldlr−/− background and injected into irradiated recipient Ldlr−/− mice (1 x 10⁷ donor cells/mouse). Eight weeks after irradiation, recipient mice were fed a high fat diet (42% caloric intake from fat, TD88137, Harlan Teklad, Indianapolis, IN) beginning 1 week prior to infusions of AngII (1,000 ng/kg/min). After completion of AngII infusions, DNA was isolated from bone marrows of anesthetized (ketamine/xylazine, 100/10 mg/kg, ip) recipient mice and PCR was performed to verify the successful repopulation of donor cells.

ACE2 mRNA and activity measurements. Mouse kidneys were dissected in half and one portion was used for total RNA extraction and the other for ACE2 activity. RNA extraction was performed using a kit (Promega, Total RNA extraction, cat#Z3105). RNA was quantified by spectrophotometry using a Nanodrop 2000 (ThermoScientific), diluted to 0.4 μg per reaction and reverse transcribed using a cDNA synthesis kit (qScript cDNA Supermix, Quanta Biosciences, cat#95048-500). cDNA was diluted at a ratio of 1:50 (0.4 ng/μL) and 5 μLs were used per PCR reaction (PerfeCta SYBR Green FastMix for iQ, Quanta Biosciences, cat#95071-012). ACE2 and 18S mRNA abundances were analyzed using the 2⁻^ΔΔCt method. Primers sequences were
ACE2 enzymatic activity was quantified as described previously.\textsuperscript{4} Briefly, samples were placed in a 0.5 mL Tris-NaCl solution containing 10 μM of ZnCl\textsubscript{2} and Z-pro-proline (ACE2 buffer) and homogenized with metal beads using a GenoGrinder for 30 seconds at 1,250 RPMs. Samples were centrifuged at 14,000 RPMs for 20 minutes at 4°C. Supernatants were discarded and the pellets were diluted with 0.5 mL of 0.5% Triton-X in ACE2 buffer. Samples were vortexed and refrigerated (4°C) overnight. The next day, samples were centrifuged at 5,000 RPMs for 10 minutes (4°C). The supernatant was used for quantifying ACE2 activity and protein was determined using a BCA kit (Thermoscientific, cat#23225) with BSA used as the standard (BioRad, cat#500-0007). We quantified ACE2 activity using 0.01 mg of kidney protein in buffer (total volume of 250 μL; Tris-NaCl buffer containing 1 μM pepstatin A, 10 μM captopril, 100 μM bestatin hydrochloride, 10 μM phosphoramidon, and 10 μM thiorphan). Samples were incubated with \textsuperscript{125}I-AngII (specific radioactivity, 2,200 Ci/mmol) for 30 minutes at 37°C. Reactions were stopped with 50 μL of 1% phosphoric acid and frozen at -20°C until angiotensin peptides could be resolved by HPLC (AngII, Ang-(1-7) were resolved using HPLC as described previously).\textsuperscript{5} HPLC fractions (1 ml) were collected and analyzed by gamma spectrometry to quantify radioactivity within fractions containing AngII or Ang-(1-7). ACE2 activity is expressed as femtomoles per milligram protein per minute, based on the specific activity of [\textsuperscript{125}I]AngII (2,175 Ci/mmol).

**Plasma and Serum Component Analysis.** Plasma renin concentrations were quantified as described previously.\textsuperscript{1} Plasma concentrations of Ang-(1-7) were quantified using a commercial ELISA kit (Bachem, cat#S-1330) as described previously.\textsuperscript{6} Sera cholesterol and triglyceride concentrations were quantified using a commercial kit (Total Cholesterol E kit, Wako cat#439-17501; Total Serum Triglycerides kit, Wako cat#290-63701) as described previously.\textsuperscript{7} To quantify lipoprotein cholesterol concentrations, fast protein liquid chromatography (FPLC) was performed on individual serum samples (n = 4 mice/group) to resolve lipoproteins, and cholesterol was quantified in individual fractions as described previously.\textsuperscript{8} Measurements were imported into PeakFit (v.4.12) and chylomicrons (CM)/VLDL, LDL, and HDL cholesterol concentrations were determined from areas under the curve (AUC). AUCs were calculated and multiplied to total serum cholesterol concentrations to determine cholesterol content (mg/dl) for each lipoprotein fraction.\textsuperscript{8}

**Immunohistochemistry of murine AAAs.** A 3% low, melting-point agarose solution was made and a green marking tissue dye was added (Polysciences Inc., cat#24110). Mice were perfused with a 10% formalin solution for approximately 15 minutes at physiological pressure and peripheral organs were removed. A 1 mL syringe with needle (gauge 23) was filled with green agarose solution and perfused slowly via the left ventricle. Aortas were cleaned of adherent tissue. Abdominal aorta was placed in 30% sucrose solution until the tissue sank to the bottom of a 15 mL conical tube. Abdominal segments were then placed in OCT media and serially sectioned (10 μm). Sections were placed on positively charged slides (Probe On Plus, Fischer Scientific, cat#22-230-900) and stained using the Microprobe system (Fischer Scientific). Sections were stained with Gomori’s Trichrome stain as described previously.\textsuperscript{9} For ACE2 immunostaining, sections were cleared using 100% xylene before using a step-down
series of alcohol-water mixes (100%, 95%, 75%, 100% water). Sections were then incubated with 0.05% chromic acid for 2 minutes at 40°C for 2 minutes followed by a rinse in 1X automation buffer (10X automation buffer, GeneTex, cat#GTX30931). Antigen retrieval was then performed using a 1:100 dilution of warmed citrate buffer (low pH citrate buffer, Vector Labs, cat#H-3300) for 10 minutes followed by a 10 minute incubation at room temperature. Sections were then washed 4 times in automation buffer and then pre-incubated with 1% hydrogen peroxide in methanol for 3 minutes at 40°C. Sections were again washed with automation buffer and blocked with goat serum for 5 minutes at 40°C. Sections were then incubated with a monoclonal goat, anti-rabbit IgG for ACE2 for 30 minutes at 40°C (anti-rabbit, Abcam, cat#ab15348, 1mg/mL, stock)(final concentration 20 μg). A negative IgG control was used through a non-immune rabbit IgG at the same concentration as the ACE2 antibody (GeneTex, cat#GTX35035). Sections were rinsed with automation buffer and incubated with a biotinlated goat anti-rabbit IgG linked to horseradish peroxidase (HRP)(Vector Labs, cat#BA-1000, final concentration 7.5 μg/mL) for 30 minutes at 40°C. Sections were rinsed again and an ABC kit was utilized for avidin-biotin conjugation for 30 minutes at room temperature (Vector Lab, cat#PK-6100). Sections were rinsed and a peroxidase enhancer step was used as a subsequent wash (GeneTex, cat#GTX82979). Finally, an AEC kit (Vector Labs, cat#SK-4200) was used for detection of antibody-peroxidase complex and counterstained with hematoxylin. Glycerol-gelatin (Sigma, cat#GG-15ML) was heated and used as a mounting medium for slides. For macrophage staining, CD68 was utilized (anti-rat, Abcam, clone FA-11, cat#ab53444, 1 mg/mL, stock)(final concentration 20 μg). Staining was performed in the same manner as ACE2, however the antigen retrieval step and counter staining with hematoxylin was omitted. For macrophage quantification, a similar protocol was used for detection of macrophages at the medial break. All images were taken at the same exposure settings and images were threshold to include positive staining (red-brown) and areas were summed to get the total macrophage area in mm².

**Immunohistochemistry of human AAAs.** The same protocol used in ACE2 immunostaining of murine AAAs (see above) was utilized in staining human abdominal aortic sections. CD68 immunostaining was performed using a monoclonal mouse anti-human antibody (clone KP1, DakoCytomation, cat#M 0814) using a peroxidase-conjugated ImmPRESS anti-mouse IgG (Vector Labs, cat#MP-7402). The reactions were visualized using ImmPACT DAB peroxidase substrate (Vector Labs, cat#SK-4105). Negative control for CD68 was incubation with the secondary only. Human AAA sections were surgical samples procured at Washington University, MO.

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<th>Sample</th>
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Statistics. Data are represented as mean ± SEM. For data that passed normality and equal variance, a Student’s t-test was used to evaluate differences between genotypes or in mice administered vehicle versus DIZE. For two group comparisons where data did not pass normality and/or equal variances, a Mann-Whitney U test was performed. Ultrasound measurements were analyzed by repeated measures two-way ANOVA followed by a pairwise multiple comparison test (Holm-Sidak). A Fischer’s exact test was used to analyze AAA incidence between groups of mice. Statistical analysis was performed through SigmaPlot (v.12) with significance at P < 0.05.
References

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


