Genetic Ace2 Deficiency Accentuates Vascular Inflammation and Atherosclerosis in the ApoE Knockout Mouse

Merlin C. Thomas, Raelene J. Pickering, Despina Tsrorotes, Audrey Koitka, Karen Sheehy, Stella Bernardi, Barbara Toffoli, Thu-Phuc Nguyen-Huu, Geoffrey A. Head, Yi Fu, Jaye Chin-Dusting, Mark E. Cooper, Chris Tikellis

Rationale: Angiotensin-converting enzyme (ACE)2 opposes the actions of angiotensin (Ang) II by degrading it to Ang 1-7.

Objective: Given the important role of Ang II/Ang 1-7 in atherogenesis, we investigated the impact of ACE2 deficiency on the development of atherosclerosis.

Methods and Results: C57Bl6, Ace2 knockout (KO), apolipoprotein E (ApoE) KO and ApoE/Ace2 double KO mice were followed until 30 weeks of age. Plaque accumulation was increased in ApoE/Ace2 double KO mice when compared to ApoE KO mice. This was associated with increased expression of adhesion molecules and inflammatory cytokines, including interleukin-6, monocyte chemoattractant protein-1, and vascular cell adhesion molecule-1, and an early increase in white cell adhesion across the whole aortae on dynamic flow assay. In the absence of a proatherosclerotic (ApoE KO) genotype, ACE2 deficiency was also associated with increased expression of these markers, suggesting that these differences were not an epiphenomenon. ACE inhibition prevented increases of these markers and atherogenesis in ApoE/Ace2 double KO mice. Bone marrow macrophages isolated from Ace2 KO mice showed increased proinflammatory responsiveness to lipopolysaccharide and Ang II when compared to macrophages isolated from C57Bl6 mice. Endothelial cells isolated from Ace2 KO mice also showed increased basal activation and elevated inflammatory responsiveness to TNF-α. Similarly, selective inhibition of ACE2 with MLN-4760 also resulted in a proinflammatory phenotype with a physiological response similar to that observed with exogenous Ang II (10⁻⁷ mol/L).

Conclusions: Genetic Ace2 deficiency is associated with upregulation of putative mediators of atherogenesis and enhances responsiveness to proinflammatory stimuli. In atherosclerosis-prone ApoE KO mice, these changes potentially contribute to increased plaque accumulation. These findings emphasize the potential utility of ACE2 replent as a strategy to reduce atherosclerosis. (Circ Res. 2010;107:888-897.)

Key Words: angiotensin ■ angiotensin-converting enzyme 2 ■ atherosclerosis ■ inflammation

Atherosclerosis is a complex process in which a combination of pathogenic factors (dyslipidemia, hyperglycemia, shear stress, etc) activate common pathways that lead to the development of atherosclerotic plaques. One of the most important is activation of the renin–angiotensin system (RAS). Angiotensin (Ang) II has a number of direct proatherosclerotic effects, whereas blockade of the RAS has antiatherosclerotic actions. Additional and independent of systemic blood pressure. Such blockade has traditionally focused on inhibiting the synthesis of Ang II and/or preventing activation of the Ang II type 1 (AT₁) receptor. However, pathways that regulate the degradation of Ang II may also be important for regulating levels of Ang II, particularly at a tissue level. In the vasculature, angiotensin-converting enzyme (ACE)2 is the major enzyme that metabolizes Ang II. Deficiency of ACE2 results in increased tissue and circulating levels of Ang II. In this study, we explore the impact of genetic Ace2 deficiency on early plaque development in atherosclerosis-prone apolipoprotein E knockout (ApoE KO) mice.

It is now recognized that inflammation plays a pivotal role in early atherogenesis. In particular, leukocyte recruitment and adhesion to the nascent atherosclerotic lesion is regarded as one of the first steps toward plaque formation. Although a number of factors can promote leukocyte adhesion, there is strong evidence that Ang II is able to promote monocyte and endothelial cell activation. In addition, Ang 1-7, the major product of ACE2, has a range of antinflammatory effects.
which balance or oppose those of Ang II. In-so-far-as deficiency or inhibition of ACE2 increases Ang II levels by reducing the formation of Ang 1-7, we hypothesize that its net effects will be to promote vascular adhesion, and with it, early plaque development.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Animal Models

C57B16 and ApoE KO mice (on a C57B16 background) were sourced in house. Ace2 KO mice were provided by Prof Josef Penninger. To generate apoE/Ace2 KO mice, single knockout strains were backcrossed 10 generations, with genetic identity confirmed by tail genotyping at each generation. In these studies, male mice aged 10 weeks and weighing between 20 to 25 g were used: (1) C57B16; (2) Ace2 KO; (3) ApoE KO; and (4) ApoE/Ace2 double KO (n=20/group). ApoE and ApoE/Ace2 double KO mice were further randomized to receive the ACE inhibitor perindopril (Servier, Neuilly, France) at a dose of 2 mg/kg per day in drinking water. All groups were followed for 20 weeks. All experiments conform to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

At 30 weeks of age, all mice were culled using an intraperitoneal injection of Euthal (10 mg/kg) (Delvet Limited, Seven Hills, Australia) followed by exsanguination via cardiac puncture. Aortae were collected and placed in 10% neutral buffered formalin and quantitated for lesion area before being processed for subsequent immunohistochemical analysis or snap frozen and stored at -70°C for subsequent RNA extraction.

Radiotelemetry Studies

Genetic Ace2 deficiency is associated with a mild increase in blood pressure when animals are bred onto a C57B16 background, as opposed to other mouse strains. To better characterize blood pressure levels in our animals, a subgroup of C57Bl6 mice and Ace2 KO mice were implanted with telemetry devices (TA11PA-C10; DataSciences International, St Paul, Minn) under halothane open circuit anesthesia. After allowing 10 days to recover, blood pressure recordings were taken in each group over a 72-hour period and then followed for 20 weeks. All experiments conform to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Plaque Area Quantitation

Plaque area was quantitated as described previously. In brief, aortae removed from mice were cleaned of excess fat and stained with Sudan IV–Herxheimer’s solution (0.5% wt/vol; Gurr, BDH Limited, Poole, UK). Aortae were dissected longitudinally, divided into arch, thoracic and abdominal segments, and pinned flat onto wax. Total and segmental (ie, arch, thoracic and abdominal) plaque area was quantitated as the percentage area of aorta stained red.

Immunohistochemistry

Immunohistochemical staining was performed for vascular cell adhesion molecule (VCAM)-1 using mouse anti-rat VCAM-1 immunoglobulin (IgG) at a dilution of 1:200 (BD Biosciences Pharmingen). To localize ACE2, a rabbit polyclonal anti-ACE2 antibody was used (Millennium Pharmaceuticals, Cambridge, Boston, Mass). Images were acquired and quantitated on an Olympus BX50 microscope using Optimis (Version 6.2) and digitized using a color video camera (3-charge coupled device; JVC, Wayne, NJ).

Quantitative Real-Time PCR

Gene expression of the adhesion molecules and proinflammatory cytokines were assessed in aortic homogenates by quantitative real-time RT-PCR. This was performed using the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700, Perkin-Elmer Inc, PE Biosystems, Foster City, Calif). Gene expression was normalized to 18S mRNA and reported as ratios compared to the level of expression in untreated control mice, which were given an arbitrary value of 1.

Ex Vivo Vessel Chamber Studies

Aortas taken from young preatherosclerotic ApoE KO mice and ApoE/Ace2 double KO mice (6 to 8 weeks of age, n=4 to 5/group) were isolated and mounted in a vessel chamber. Human whole blood was collected from healthy donors and labeled with DilC18 (1:100), and then perfused through the aorta at 0.12 mL/min. Images and videos of vessel wall-cell interactions were observed using a fluorescence microscope (Zeiss Discovery.V20). To examine responsiveness to proinflammatory mediators, vessels taken from ApoE KO mice and ApoE/Ace2 double KO mice (n=4 to 5/group) were pretreated with TNF-α (10 ng/mL; 4 hours at 37°C) before being perfused, as detailed above.

Studies in Isolated Macrophages

To examine the impact of ACE2 deficiency on inflammatory pathways, macrophages were isolated from the bone marrow as detailed in the Online Data Supplement. Their lineage was confirmed by the expression of ectodysplasin and staining for F4/80. After 48-hours of incubation in L-cell medium, cells were treated with LPS (10 ng/mL) for 1 hour. Proinflammatory responsiveness was assessed by the upregulated gene and protein expression of TNFα and IL-6, quantitated by RT-PCR and ELISA (R&D Systems, Minneapolis, Minn) respectively.

Studies in Endothelial Cells

To further examine the impact of ACE2 deficiency on proinflammatory pathways, primary endothelial cells were isolated and cultured from the aortae of C57B16 and Ace2 KO mice. Cells were then

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>ACE2</td>
<td>angiotensin-converting enzyme 2</td>
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<tr>
<td>Ang 1-7</td>
<td>angiotensin 1-7</td>
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<tr>
<td>Ang II</td>
<td>angiotensin II</td>
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<tr>
<td>ApoE</td>
<td>apolipoprotein E</td>
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<tr>
<td>F4/80</td>
<td>macrophage cell marker</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>JAM-A</td>
<td>junctional adhesion molecule-A</td>
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<td>KO</td>
<td>knockout</td>
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<tr>
<td>perindopril</td>
<td>angiotensin-converting enzyme inhibitor</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>MCP</td>
<td>monocyte chemotactic protein</td>
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<td>MLN-4760</td>
<td>angiotensin-converting enzyme 2 inhibitor</td>
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<tr>
<td>Svec4-10</td>
<td>saphenous vein endothelial cell</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<td>VCAM</td>
<td>vascular adhesion molecule</td>
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treated with TNFα (10 pg/mL) for 2 hours. Proinflammatory responsiveness was determined by changes in gene expression assessed by real-time RT-PCR and the secretion of soluble VCAM into the cell media, measured by ELISA (R&D Systems). Primary endothelial cells isolated and cultured from C57Bl6 mice were also treated with 1 μmol/L of the selective ACE2 inhibitor, MLN-4760 (Millennium Pharmaceuticals, Cambridge, Massachusetts, USA). This dose resulted in a reduction in ACE2 activity in cells by >90% (data not shown). After 30 minutes of incubation with MLN-4760, cells were treated with TNFα (10 pg/mL) for 2 hours and changes in gene expression determined as above.

In confirmatory experiments, immortalized mouse saphenous vein endothelial cells (Svec4-10) were transfected with Silencer Select small interfering (si)RNA specific for ACE2 (Applied Biosystems, CA, USA) that resulted in a >90% reduction in ACE2 gene expression (data not shown) or scrambled control RNA. After 24 hours of incubation in 2% FCS/OptiMEM media (Gibco), cells were treated with TNFα (1 ng/mL) for 1 hour and proinflammatory responsiveness determined as above. Finally, Svec4-10 cultured in 10% FBS/DMEM with 25 mmol/L glucose were pretreated with MLN-4760 (1 μmol/L) or Ang II (10 μmol/L) for 30 minutes, followed by treatment with TNFα (1 ng/mL) for 2 hours. Proinflammatory responsiveness was determined by changes in gene expression, as detailed above.

Results

Circulating Lipid and Angiotensin Levels

Serum total cholesterol and triacylglycerol concentrations were elevated in ApoE KO mice (10.1±2.1 and 1.1±0.2) when compared to C57Bl6 mice (0.8±0.2 and 0.2±0.1, both P<0.01). Deficiency of ACE2 and treatment with perindopril decreased serum total cholesterol and triacylglycerol concentrations.

![Figure 1. Mean systolic (A) and diastolic blood pressure (B) levels over a 24-hour light-dark cycle as measured by radiotelemetry in conscious mice. White squares denote C57Bl6 mice. White diamonds denote C57Bl6 mice treated with the ACE inhibitor perindopril (2 mg/kg per day). Black squares, dotted line, denotes Ace2 KO mice. Black diamonds, dotted line, denote Ace2 KO mice treated with perindopril (2 mg/kg per day). Data show mean±SEM.](image)

![Figure 2. Flat mounts of aortic vessels stained red with Sudan IV to denote the presence of atherosclerotic plaque. From left to right denote ApoE KO (A), ApoE KO+perindopril (2 mg/kg per day) (B), ApoE/Ace2 double KO (C), ApoE/Ace2 double KO+perindopril (2 mg/kg per day) (D), and Ace2 KO (E). Within each panel, the 3 segments from left to right denote arch, thoracic, and abdominal segments, respectively. Bottom, Percentage of each segment of the aorta stained red with Sudan IV in each group. Data show mean±SEM. Data from Ace2 KO is not shown, as there was no plaque present in any segment. *P<0.05 compared to ApoE KO mice, #P<0.05 compared to ApoE+perindopril mice and $P<0.05 compared to ApoE/Ace2 double KO mice.](image)
had no effect on circulating lipid levels (data not shown). Ace2 KO mice had increased circulating levels of Ang II when compared to wild-type mice (control 115±H11006 17 pg/mL; Ace2 KO 270±H11006 35 pg/mL, P<0.01). Aortic tissue levels of Ang II were also elevated in Ace2 KO mice (26±H11006 5 pg/mg protein) when compared to aortae from C57Bl6 mice (12±2 pg/mg protein, P<0.01). Treatment with perindopril reduced circulating Ang II levels to a similar extent in wild-type and Ace2 KO mice (125±H11006 22 pg/mL and 122±H11006 32 pg/mL respectively, P=NS).

Blood Pressure Levels
ACE2 deficiency was associated with a modest elevation in systolic blood pressure as measured by tail cuff plethysmography and radiotelemetry (Ace2 KO 112±2 mm Hg, C57Bl6 103±2 mm Hg, P<0.01, Figure 1A). Diastolic blood pressures were also higher in Ace2 KO mice (Figure 1B). Treatment with perindopril reduced systolic blood pressure levels in both C57Bl6 (~19 mm Hg) and Ace2 KO (~16 mm Hg), although the decline was greatest in the former (P<0.01, Figure 1A). These data were confirmed in tail cuff plethysmography, in which treatment with perindopril reduced systolic blood pressure levels in ApoE KO and ApoE/Ace2 double KO mice (93±3 and 99±3 respectively, versus untreated P<0.01). There were no differences in the achieved diastolic or mean arterial blood pressure with perindopril between C57Bl6 and Ace2 KO.

Aortic Plaque Area
The total plaque area, quantitated as a percentage area of aorta stained red with Sudan IV, was significantly increased in ApoE/Ace2 double KO mice when compared to ApoE KO mice (P<0.001, Figure 2). This increase in plaque accumulation was observed in all aortic segments (arch, thoracic and abdominal). Treatment with the ACE inhibitor, perindopril, significantly reduced plaque area in ApoE/Ace2 double KO mice, to levels not significantly different from control ApoE KO mice (Figure 2). In the absence of genetic susceptibility conferred by the ApoE gene deletion, no plaque was detected in Ace2 KO mice (Figure 2E) or in C57Bl6 mice (data not shown).

Aortic Expression of Proinflammatory Mediators
Deficiency of ACE2 in apoE KO mice was associated with increased gene expression of a number of adhesion molecules inflammatory cytokines and matrix metalloproteinases (MMPs) when compared to ApoE KO mice, including TNFα, IL-6, monocyte chemoattractant protein (MCP)-1, VCAM-1, JAM-A, proMMP-2 and proMMP-9 (Figure 3B). Levels of IL-6 protein were also elevated in the aorta of ApoE/Ace2 double KO mice (134±26 pg/mg protein) when compared to
ApoE KO mice (34±9 pg/mg protein, P<0.05). In addition, immunohistochemical staining of the aortae also showed increased expression of VCAM-1 protein in the endothelium in ApoE/Ace2 double KO mice when compared to apoE KO mice (Figure 4). Treatment with perindopril completely prevented increases in aortic VCAM expression at a gene level (ApoE/Ace2 double KO mice + perindopril, 0.8±0.2; P<0.05 versus ApoE/Ace2 double KO mice) and at a protein level (Figure 4C).

In the absence of a proatherosclerotic (apoE KO) genotype, ACE2 deficiency was still associated with increased expression of adhesion molecules, inflammatory cytokines and MMPs when compared to C57Bl6 control mice (Figure 3A), suggesting that these differences were not simply an epiphenomenon associated with plaque accumulation. Levels of IL-6 in the aorta were also elevated in Ace2 KO mice (41±12 pg/mg protein, P<0.05) when compared to wild-type mice (20±6 pg/mg protein, P<0.05).

**Ex Vivo Vessel Chamber Studies**

Consistent with the increased expression of adhesion molecules and plaque accumulation in ApoE/Ace2 double KO mice, whole aortas taken from young preatherosclerotic ApoE/Ace2 double KO mice showed increased adhesion of labeled human white cells in a dynamic flow adhesion assay (Figure 5A). The number of cells adhering to aortic endothelium was also increased in aortas that had been pretreated with TNF-α (10 ng/mL for 4 hours). However, the response to TNF-α was substantially enhanced in aortas taken from ApoE/Ace2 double KO mice (Figure 5B).

**Isolated Macrophage Experiments**

Macrophages play a key role in the development and progression of atherosclerosis and are potentially responsible for the production of many proinflammatory cytokines and up-regulation of adhesion molecules within atherosclerotic plaques. Because we found the expression of proinflammatory cytokines and adhesion molecules to be upregulated in
Ace2 KO mice, we also investigated the role of ACE2 in circulating macrophage/monocytes in determining this proinflammatory proatherogenic phenotype. In these experiments, macrophages were isolated from the bone marrow of C57Bl6 and Ace2 KO mice. ACE2 expression in wild-type macrophages was demonstrated by real time RT-PCR (data not shown). Bone marrow macrophages isolated from Ace2 KO mice showed increased gene expression of the adhesion molecule VCAM-1 when compared to macrophages isolated from wild-type mice (Ace2 KO 3.1/0.6, C57Bl6 1.0/0.2, P<0.01), as well as increased basal production of TNFα (Figure 6A).

Because the half-life of Ang II is prolonged in the absence of ACE2 expression, the effect of Ang II (10^{-7}M) on the expression of proinflammatory markers was first assessed. As expected, ACE2 deficient macrophages showed an increased response to Ang II, as indicated by the release of TNFα into the cellular media (Figure 6A). However, to assess overall proinflammatory responsiveness, isolated macrophages from C57Bl6 and Ace2 KO mice were also treated with a proinflammatory stimulus (LPS). In the absence of ACE2 expression, isolated macrophages from Ace2 KO mice released higher levels of TNFα protein into the culture medium in response to LPS when compared to macrophages isolated from C57Bl6 mice (Figure 6B). Similar changes were observed with respect to the production of IL-6 (Figure 6C), with ACE2 deficiency augmenting its synthesis and secretion into the media in response to LPS. The gene expression of IL-6, TNFα, MCP-1, IL-6, MMP-2, and MMP-9 mRNA in cultured primary bone marrow macrophages isolated from C57Bl6 mice (white bars) and Ace2 KO mice (black bars) as measured by real time RT-PCR in the presence and absence of prestimulation with 1 μmol/L LPS. Data are standardized to the gene expression levels observed in macrophages from C57Bl6 mice, which are given an arbitrary value of 100%. Data show mean±SEM. *P<0.05 vs macrophages from C57Bl6 mice; #P<0.05 vs stimulated cells from C57Bl6 mice.

Studies in Endothelial Cells

Activation of endothelial cells is an early mediator of atherosclerotic plaque development.1–3 To examine whether ACE2 deficiency directly results in a proinflammatory phenotype in the endothelium, primary endothelial cells were isolated and cultured from C57Bl6 and Ace2 KO mice. Endothelial cells from Ace2 KO mice demonstrated increased gene expression of VCAM, MCP-1 and IL-6 (Figure 7A), and increased secretion of soluble VCAM into the cell media when compared to cells cultured from wild-type controls (Figure 7C). In response to treatment with TNFα (10 pg/mL), wild-type cells showed a marked upregulation in the expression of genes associated with inflammation and secretion of soluble VCAM.
into the cell media. This increase was substantially augmented in endothelial cells from Ace2 KO mice. A similar proinflammatory effect was observed when wild-type cells were treated with the selective ACE2 inhibitor, MLN-4760 (Figure 7B).

In parallel experiments, immortalized murine endothelial cells (Svec4-10) treated with the selective ACE2 inhibitor, MLN-4760 (Figure 8A) or an siRNA to ACE2 (Figure 8B) also demonstrated increased gene expression of proinflammatory markers, as well as augmented proinflammatory responsiveness to TNFα (1 ng/mL). A similar proinflammatory effect was observed when Svec-4 to 10 cells were pretreated with Ang II (Figure 8C).

**Discussion**

Activation of the RAS significantly contributes to the development and progression of atherosclerosis in susceptible individuals/animals. Our group and others have previously shown that both ACE inhibition and blockade of the angiotensin II receptor are able to reduce atherosclerosis in ApoE KO mice.9,10,24 In this study, we now show that genetic deletion of ACE2, the major enzyme that degrades Ang II to form Ang 1-7 in the vasculature, significantly increases plaque accumulation in ApoE KO mice. This is consistent with increased atherogenesis following an infusion of Ang II6 and the atheroprotective effects of Ang1–7 in ApoE KO mice.25 These data point to ACE2 as an important and novel target for vascular-protective therapies. Indeed, recent studies suggest that ACE2 delivered as an adenovirus26 may, in part, attenuate atherosclerosis in ApoE KO mice.

Leukocyte recruitment and adhesion to an activated endothelium is an important early step in plaque formation. We demonstrate that preatherosclerotic ApoE/Ace2 double KO mice show augmented adhesion of white cells to their aortic wall, both in the presence and absence of a proinflammatory stimulus (TNF-α), together with an upregulation of inflammatory and adhesion markers in vivo in response to Ace2 deletion. Consistent with these in vivo and functional findings, we also show increased inflammatory responsiveness of primary macrophages and endothelial cells taken from Ace2 KO mice. Although our studies haven chiefly focused on leukocyte adhesion, other components of the inflammatory cascade that influence lesion development and progression may also be adversely affected by an imbalance in the RAS, including innate immunity,27 platelet adhesion28 and the accumulation and activation of CD4+ T-lymphocytes.29,30

The breakdown of extracellular matrix by MMPs in inflamed atherosclerotic plaques is an important determinant of
plaque stability. In addition, some MMPs can degrade Ang I to locally generate Ang II. The potential importance of this non-ACE pathway is illustrated by the finding that MMP-8 KO mice have reduced Ang II levels alongside reduced plaque accumulation when crossed onto an ApoE KO background. Angiotensin II is also a potent stimulant of MMP expression and activity. In our study, genetic Ace2 deficiency was associated with increased aortic expression of gelatinases, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B), and augmented production of MMP-9 in macrophages. This is consistent with increased gelatinase activity observed in mice infused with subpressor doses of Ang II that contributes to aneurysm formation in this model, but could reflect the dose and concentration of Ang II delivered by minipump. Another potential contributor to augmented atherosclerosis in our model was the modestly increased systolic blood pressure observed in ACE2 deficient mice. Previous studies have documented a positive relationship between BP and size of atherosclerotic lesions in proatherogenic ApoE KO. However, we were able to clearly demonstrate proinflammatory effects of ACE2 deficiency in vitro. Moreover, increased plasma concentrations of Ang II following a continuous infusion also increases severity of aortic atherosclerotic lesions and their content of macrophages in the absence of changes in blood pressure.

Because of very efficient lipoprotein metabolism in mice, a proatherogenic phenotype is required for the development of an atherosclerotic plaque. In this study we used the ApoE KO mouse, the model most widely used to investigate experimental atherosclerosis, with plaque accumulation and morphology in this strain resembling human disease. However, in studying the mechanisms whereby ACE2 deficiency might lead to atherosclerosis we also examined changes in C57Bl6 and Ace2 KO animals. Although in the absence of dyslipidemia these animals cannot develop plaques, this strategy has allowed us to examine the proatherogenic mediators specifically associated with ACE2 deficiency, without the confounding effects of atherosclerosis, where changes associated with vascular disease could be interpreted as being epiphenomena rather than causative in the disease process. In this light we observed that ACE2 deficiency was associated with significant upregulation of proinflammatory pathways, even in the absence of atherosclerosis, albeit to a lesser extent, suggesting that the increased inflammation in ApoE/Ace2 double KO mice was not simply attributable to enhanced plaque accumulation. Consistent with this hypothesis, we also observed increased inflammatory responsiveness in macrophages and endothelial cells derived from Ace2 KO mice and murine endothelial cells treated with a selective inhibitor of ACE2.

It is possible to speculate that the imbalance of increased tissue and circulating levels of Ang II alongside reduced synthesis of Ang 1-7, contributes to the proinflammatory and

Figure 8. The expression of IL-6, MCP-1, and VCAM mRNA in saphenous vein endothelial cells (SVEC-40) in the presence or absence of prestimulation with TNFα (1 ng/mL), as measured by real time RT-PCR. Results are shown in the presence or absence of a selective inhibitor of ACE2, MLN-4760 (1 μmol/L) (A), in the presence or absence of a selective siRNA to ACE2 (B) and in the presence or absence of Ang II (10 μmol/L) (C). Data are standardized to the gene expression levels observed in untreated cells, which are given an arbitrary value of 100%. Data are expressed as mean±SEM. *P<0.05 vs untreated cells; #P<0.05 vs cells treated with TNFα alone.
atherogenic phenotype associated with ACE2 deficiency. However, the substrate affinity of ACE2 is not confined to the angiotensin peptides. For example, ACE2 is able to cleave the C-terminal amino acid from vasoactive bradykinin (1-8) (also known as des-Arg9-bradykinin) to form the inactive peptide, bradykinin (1-7) and metabolize des-Arg-kallidin (also known as des-Arg10 Lys-bradykinin).53 Other peptide compounds including dynorphin A, costamorphin, neurotensin 1 to 13, apelin 13, apelin-36, and kinetensin may also be hydrolyzed by ACE2 in vitro.54 Whether changes in the regulation of any of these other peptides contribute to the proinflammatory effects of ACE2 deficiency is unclear. However, the antiatherogenic effect of ACE inhibition in ApoE KO mice is not mediated by bradykinin35 and atherogenesis is specifically enhanced following an infusion of Ang II.6

In our study, treatment with the ACE inhibitor, perindopril was able to reduce plaque accumulation in ApoEL/Alce2 double KO mice. This is consistent with the antiatherosclerotic effects of RAS blockade observed in diabetic models.9,10 However, the degree of protection was significantly less than that observed following treatment in ApoE KO mice, despite a comparable reduction in blood pressure and circulating Ang II levels. This difference may partly reflect the lead time exposure of ace2 KO mice to Ang II before the initiation of perindopril at 10 weeks of age. It is also possible that increased levels of Ang 1-7 may play a role in the antiatherosclerotic actions of ACE inhibition, because ACE is the major enzyme that degrades Ang 1-7. However, in the setting of ACE2 deficiency, where Ang 1-7 synthesis is impaired, changes in Ang 1-7 induced by ACE inhibition may also be attenuated. We have described this phenomenon previously with respect to the renoprotective effects of RAS blockade.18 Furthermore, we have recently reported that perindopril reduces ACE2 expression in the vasculature,36 possibly as part of negative feedback or escape phenomenon to restore Ang II levels, potentially underlining the effects of chronic blockade. For both reasons, augmentation of ACE2 in the setting of ACE inhibition potentially offers a useful means to improve the both the reno- and vasculoprotective actions of conventional RAS blockade.

In summary, genetic ACE2 deficiency is associated with upregulation of putative mediators of atherogenesis, such as cytokines and adhesion molecules, and also appears to enhance the responsiveness to proinflammatory stimuli. In atherosclerosis prone apoE KO mice, these changes resulting from ACE2 deficiency significantly increase accumulation of atherosclerotic plaque. These findings emphasize the potential utility of ACE2 repletion as a strategy to reduce atherosclerosis.

Sources of Funding
This work was supported by research funding from the National Health Foundation of Australia, Kidney Health Australia (Bootle bequest), Juvenile Diabetes Research Foundation, and the National Health and Medical Research Council of Australia.

Disclosures
None.

References
ACE2 Deficiency Accentuates Atherosclerosis

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Novelty and Significance

What Is Known?

- Angiotensin (AngII) contributes to the development and progression of atherosclerosis, partly by augmenting vascular inflammation.
- Ang 1-7 antagonizes the vascular effects of Ang II.
- Angiotensin-converting enzyme (ACE2) plays a key role in the local regulation of the renin–angiotensin system by converting Ang II to form Ang 1-7.

What New Information Does This Article Contribute?

- ACE2 deficiency accelerates atherogenesis.
- ACE2 deficiency results in increased vascular inflammation and an augmented responsiveness to inflammatory signals in macrophages and endothelial cells.

Deficiency of ACE2 results in increased levels of the proatherogenic and proinflammatory peptide Ang II, while reducing the synthesis of Ang 1-7. In this study, we found that genetic Ace2 deficiency was associated with enhanced early plaque development in atherosclerosis-prone Apoe-null mice. ACE2 deficiency was also associated with increased expression of adhesion molecules and inflammatory cytokines, in the presence and absence of atherosclerosis, suggesting that these differences were not an epiphenomenon. Primary macrophages and endothelial cells from Ace2-null mice showed increased basal activation and augmented responsiveness to proinflammatory stimuli when compared with cells from wild-type animals. These data confirm the key role of the rennin-angiotensin system in the development and progression of atherosclerosis and highlight the importance of ACE2 in its regulation. Although blockade of the rennin-angiotensin system has traditionally focused on reducing the synthesis of Ang II, the findings of our study emphasize the potential utility of ACE2 repletion as a strategy for balancing the rennin-angiotensin system and reducing atherosclerosis.
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Circ Res. 2010;107:888-897; originally published online July 29, 2010; doi: 10.1161/CIRCRESAHA.110.219279

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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MATERIALS AND METHODS

Animal models

C57Bl6 and ApoE KO mice (on a C57Bl6 background) were sourced from the Precinct Animal Centre of the Baker IDI Heart and Diabetes Institute. ApoE KO mice (also on a C57Bl6 background) were kindly provided by Professor Josef Penninger, and generated as previously described by his group. To generate apoE/Ace2 KO mice, single knockout strains were backcrossed 10 generations, with genetic identity confirmed by tail genotyping at each generation.

In these studies, male mice aged 10-weeks and weighing between 20-25 g were used from four groups (1) C57Bl6 (2) Ace2 KO (3) ApoE KO and (4) ApoE/Ace2 double KO (n=20/group), with all animals on the same C57Bl6 background. ApoE and ApoE/Ace2 double KO mice were further randomised to receive the ACE inhibitor, perindopril (Servier, Neuilly, France) at a dose of 2 mg/kg/day in drinking water. All groups were then followed for 20 weeks (until 30 weeks of age). Throughout the study animals were given access to standard mouse chow (Animal Resources Centre, Perth, Australia) and water ad libitum. All experiments were approved by the animal ethics committee of the Alfred Medical Research Precinct and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Systolic blood pressure levels were measured by tail cuff plethysmography. At 30 weeks of age, all mice were culled using an intraperitoneal injection of Euthal (10mg/kg) (Delvet Limited, Seven Hills, Australia) followed by exsanguination via cardiac puncture. In half of the animals in each group, aortae were collected and placed in 10% neutral buffered formalin and quantitated for lesion area before being processed for subsequent immunohistochemical analysis. In the other half, aortae were snap frozen in liquid nitrogen and stored at –70°C for subsequent RNA extraction.

Radiotelemetry studies

Genetic Ace2 deficiency is associated with a mild increase in blood pressure when animals are bred onto a C57Bl6 background, as opposed to other mouse strains. To better characterise blood pressure levels in our animals, a subgroup of C57Bl6 mice and Ace2 KO mice were implanted with telemetry devices (TA11PA-C10; DataSciences International, St. Paul, MN) under halothane open circuit anaesthesia to monitor mean arterial pressure. The catheter tip was placed in the aortic arch via the carotid artery, and the transmitter was placed under the skin at the flank. The radiotelemetry signals were collected by the receiver (model RPC-1; Data Sciences International) and were passed to an analogue converter (model RP11A; Data Sciences International). After allowing 10 days to recover, blood pressure recordings were taken in each group over a 72-hour period and then repeated after one week of treatment with perindopril (2mg/kg/day in drinking water).

Angiotensin peptide studies

The effects of genetic Ace2 deficiency on circulating and aortic Ang II and its response to ACE inhibition was examined by measuring Ang II concentrations in the presence and absence of treatment in wild type and Ace2 KO mice by a commercial radioimmunoassay (ProSearch International, Malvern, Australia) utilising an Ang II selective polyclonal antibody and 125I-AngII. Blood samples were collected on ice into a cocktail of protease inhibitors [50 mmol/L ethylenediaminetetraacetic acid, 0.5-mmol/L o-phenanthroline, 1 mmol/L N-ethylmaleimide, and 0.1 mmol/L pepstatin A], spun and then snap frozen for subsequent analysis.
Plaque Area Quantitation

Plaque area was quantitated by en face whole analyses to examine the extent of atherosclerosis in the aorta. The entire aorta was cleaned of peripheral fat under a dissecting microscope, opened longitudinally, and cut into 3 different segments: arch, descending thoracic aorta, and abdominal aorta. The aortas were stained with Sudan IV–Herxheimer’s solution (0.5% w/vol) (Gurr, BDH Limited, Poole, UK) and pinned out flat onto a wax pad. Photographs of the stained aortas were digitized by use of a dissecting microscope equipped with a high-resolution camera Axiocam camera (Zeiss, Heidelberg, Germany). Digitized images were then evaluated with an image analysis system (Adobe Photoshop v7.0). Total and segmental (i.e. arch, thoracic and abdominal) plaque area was quantitated as the percentage area of aorta stained red. Tissue was subsequently embedded in paraffin and sections cut for immunohistochemical analysis.

Immunohistochemistry

For immunohistochemistry 4 micron paraffin serial sections were prepared from 4% paraformaldehyde fixed, paraffin embedded mouse aorta. Sections were de-waxed and hydrated, then endogenous peroxidase was quenched for 20 minutes using 3% (v/v) hydrogen peroxide in phosphate-buffered saline (PBS). To identify adhesion molecules, immunohistochemical staining was performed for VCAM-1 using mouse anti-rat VCAM-1 immunoglobulin (IgG) at a dilution of 1:50 (BD Biosciences Pharmingen, CA, USA). To localise ACE2, a rabbit polyclonal anti-ACE2 antibody at a dilution of 1:250 raised against ACE2 residues 489-508 was used (antibody kindly donated by Millennium Pharmaceuticals, Cambridge, Boston, M.A., USA). Specific staining was detected using the standard ABC (avidin-biotin complex) method. Sections were lightly counter-stained with haematoxylin. Non-specific staining was tested with 1% non-immunized goat serum. Images were acquired and quantitated on an Olympus BX50 microscope using Optimis (Version 6.2) and digitised using a colour video camera (3-charge coupled device; JVC, Wayne, NJ).

Quantitative Real-Time PCR

Gene expression of the adhesion molecules and pro-inflammatory cytokines were assessed in aortic homogenates and cell preparations by real-time quantitative RT-PCR. Samples were homogenised using the Ultra-Turrax (Janke & Kunkel IKA, Labortechnik, Germany) in TRIZOL (Life Technologies Inc, Gaithersburg M.D., USA). Total RNA was isolated and genomic DNA removed using DNase I (Applied Biosystems, Foster City, CA, USA). cDNA was synthesised with a reverse transcriptase reaction carried out using standard techniques (Superscript First Strand Synthesis System for RT-PCR, Life Technologies Inc, Gaithersburg, M.D., USA) with random hexamers, dNTPs and total RNA extracted from control and diabetic rat kidneys. An aliquot of the resulting single-stranded cDNA was used in the real time PCR experiments as described below. To assess genomic DNA contamination, controls without reverse transcriptase were included. Gene expression was normalised to 18S mRNA and reported as ratios compared to the level of expression in untreated control mice/cells, which were given an arbitrary value of 1.

Real time (RT)-PCR is a fully quantitative method for the determination of amounts of mRNA performed using the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700, Perkin-Elmer Inc, Applied Biosystems, Foster City, CA, USA). Gene specific 5’-oligonucleotide corresponding to genes listed in the table below were designed using the software program, ‘Primer Express’ (Applied Biosystems, Foster City, C.A., USA). The generation of amplicons was defined by the point during cycling when amplification of the PCR product is first detected.

The RT-PCR reaction took place with 500nmol/L of forward and reverse primer and 50nmol/L of FAM/MGB ACE/ACE2 probe and VIC™/MGB 18S ribosomal probe, in 1x
Taqman universal PCR master mix (Applied Biosystems, Foster City, C.A., USA). Each sample was run and analyzed in triplicate. The samples from control aorta/cell preparations were then used as the calibrator with a given value of 1 and all other groups were compared with this calibrator.

<table>
<thead>
<tr>
<th>Gene</th>
<th>5'-oligonucleotide, 5'&gt;3'</th>
<th>probe (FAM-dye) 5'&gt;3'</th>
<th>3'-oligonucleotide, 5'&gt;3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>GGCTGCCCCCAGACTACGT</td>
<td>TCACCCACACCGTCAG</td>
<td>TTCTCTGGTATGAGATAGCAAATC</td>
</tr>
<tr>
<td>IL-6</td>
<td>GGGAAATCGTGGGAAATGAGAAA</td>
<td>ATTTGCCATAGCACAACCT</td>
<td>AAGTGCAATCATGTGGTTCATACA</td>
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<tr>
<td>MCP-1</td>
<td>GTCTGTGCTGCCCAAGAGAG</td>
<td>AATGGGTCAGCGACATAC</td>
<td>TGGTTCCGAAGAGTTTTTA</td>
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<tr>
<td>VCAM</td>
<td>CTGCTAAGTGTGAGTGGATACCA</td>
<td>CCAAAATCTGTGGAGCGAG</td>
<td>ATCTGCCTTTTTGTAGCAGAAG</td>
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<tr>
<td>JAM-A</td>
<td>GACCGGAAAGGACAATGGA</td>
<td>CAGCATCCACCTCAG</td>
<td>TCGTGGGTGGATGGA</td>
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<tr>
<td>MMP-2</td>
<td>TCACCTTCTGCGGCAACAAGT</td>
<td>TGCACGAGCAGCGG</td>
<td>GCCAGAGGAATAGGCTATATCC</td>
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<tr>
<td>MMP-9</td>
<td>TGAGTCCCGGCAACAATCCT</td>
<td>CATCAAAAACATCCACATGG</td>
<td>CGCCTGGATCTCAGCAATA</td>
</tr>
</tbody>
</table>

Ex Vivo Vessel Chamber Studies

Aortas taken from young pre-atherosclerotic ApoE KO mice and ApoE/Ace2 double KO mice (6-8 weeks of age, n=4-5/group) were isolated and mounted in a vessel chamber primed with Krebs buffer and maintained at physiological pH by infusing carbogen gas (95% O₂; 5% CO₂) through the buffer at 37°C. Human whole blood (25U/ml heparin) was collected from healthy donors and labelled with DilC18 (1:1000), and then perfused through the aorta at 0.12ml/min. Images and videos of vessel wall-cell interactions were observed using a fluorescence microscope (Zeiss Discovery.V20), coupled to a digital camera (HAMAMATSU ORCA-ER) and analysed with AxioVison software. 2-3 frames were taken at each time points to determine dynamic cell adhesion. To examine responsiveness to pro-inflammatory mediators, in additional experiments, vessels taken from ApoE KO mice and ApoE/Ace2 double KO mice (n=4-5/group) were pre-treated with TNF-α (10ng/ml; 4hrs at 37°C) before being perfused with labelled human whole blood, as detailed above.

Studies in isolated macrophages

To examine the impact of ACE2 deficiency on inflammatory pathways implicated in the development of atherosclerosis, macrophages were isolated from the bone marrow and their responsiveness was assessed. The femur and tibia were removed from mice, cleaned of excess fat, muscle and tendons and placed in ice cold PBS. A 3ml syringe with a 26g needle was used to flush the marrow from the bone. The resulting solution was spun at 700g for 15min at 4°C and the pellet resuspended in warm DMEM media containing L-cell medium. The solution was allowed to sit in T75ml flasks and fibroblast cells adhered whilst macrophages remained in solution. The solution was decanted into 20cm petri dishes on the following day and the macrophages were allowed to adhere over a week. Their lineage was confirmed by the expression of ED1 and staining for F4/80. The adhered macrophages were scrapped off the plastic and aliquoted into 6 well plates. After 48 hours of incubation in L-cell medium, cells were treated with LPS (10ng/ml) for 1 hour. Pro-inflammatory responsiveness was assessed by the up-regulated gene and protein expression of TNFα and IL-6, quantitated by RT-PCR and ELISA (RnD Systems, Minneapolis, MN) respectively.
To further examine the impact of ACE2 deficiency on pro-inflammatory pathways, primary endothelial cells were isolated and cultured from the aortae of C57Bl6 and Ace2 KO mice. Cells were then treated with TNFα (10 pg/mL) for 2 hours in serum free DMEM with 25mM glucose. Pro-inflammatory responsiveness was determined by changes in gene expression assessed by quantitative real time RT-PCR and the secretion of soluble VCAM into the cell media, measured by ELISA (RnD Systems, Minneapolis, MN).

Primary endothelial cells isolated and cultured from C57Bl6 mice were pre-treated with 1µM of the selective ACE2 inhibitor, MLN-4760 (Millennium Pharmaceuticals, Cambridge, Massachusetts, USA). This dose resulted in a reduction in ACE2 activity in cells by > 90% (data not shown). After 30 minutes of incubation with MLN-4760, cells were treated with TNFα (10 pg/mL) for 2 hours and changes in gene expression determined as above.

In confirmatory experiments, immortalised mouse saphenous vein endothelial cells (Svec4-10) were transfected with Silencer Select siRNA specific for ACE2 (Applied Biosystems, CA, USA) that resulted in a >90% reduction in ACE2 gene expression (data not shown) or scrambled control RNA. After 24 hours of incubation in 2% FCS/OptiMEM media (Gibco, CA, USA), cells were treated with TNFα (1ng/mL) for 2 hours and pro-inflammatory responsiveness determined as above. Finally, Svec4-10 cultured in 10% FBS/DMEM with 25mM glucose were pre-treated with MLN-4760 (1µM) or Ang II (10µM) for 30 minutes, followed by treatment with TNFα (1ng/mL) for 2 hours. Pro-inflammatory responsiveness was determined by changes in gene expression, as detailed above.

Statistics

Continuous data are expressed as mean ± SEM. Differences in the mean among groups were compared using 2-way ANOVA with ApoE/Ace2 double KO and Ace2 KO groups as the 2 variables. Pair-wise multiple comparisons were made with the Student-Newman-Keuls posthoc analysis to detect significant differences between groups. P<0.05 was considered statistically significant.
References:


