Renin-Angiotensin System

Angiotensin-Converting Enzyme 2 Is a Critical Determinant of Angiotensin II–Induced Loss of Vascular Smooth Muscle Cells and Adverse Vascular Remodeling


Abstract—Angiotensin-converting enzyme (ACE) 2 is a key negative regulator of the renin–angiotensin system and metabolizes angiotensin II (Ang II) into Ang 1 to 7. Ang II is a vasoactive peptide, which plays an important role in vascular disease. The objective of the present study was to define the role of ACE2 in pathological vascular remodeling. We found upregulation of ACE2 in dilated human aorta with bicuspid aortic valve and in murine aorta in response to Ang II. Ex vivo pressure myography showed increased vascular stiffness in ACE2 knockout (KO) mesenteric arteries with loss of vascular smooth muscle cells. Aortic vascular smooth muscle cells from ACE2KO mice showed markedly increased reactive oxygen species and apoptosis in response to Ang II along with increased cleaved caspase-3 and cleaved caspase-8 levels in the ACE2KO aorta. Ang II type 1 receptor blockade and Ang 1 to 7 supplementation prevented the increase in Ang II–induced reactive oxygen species and apoptotic cell death. In the aorta, Ang II resulted in thoracic and abdominal aortic dilation with loss of vascular smooth muscle cell density in ACE2KO aorta as revealed by α-smooth muscle actin, calponin staining, and electron microscopy with increased promatrix metalloproteinase 2, matrix metalloproteinase 2, and matrix metalloproteinase 9 levels. ACE2 is upregulated in vascular diseases, and ACE2 deficiency exacerbates Ang II–mediated vascular remodeling driven by increased reactive oxygen species and vascular smooth muscle cell apoptosis. In conclusion, the key counter-regulatory role of ACE2 against an activated renin–angiotensin system provides novel insights into the role of ACE2 in vascular diseases. (Hypertension. 2014;64:157-164.) • Online Data Supplement

Key Words: angiotensin II ■ angiotensin converting enzyme 2 ■ irbesartan

The renin–angiotensin system (RAS) regulates vascular tone and plays a key role in adaptive and maladaptive vascular remodeling.1–4 Angiotensin II (Ang II), an effector peptide of RAS, plays an important role in normal vascular physiology, as well as disease progression, mostly through the Ang II type 1 receptor (AT1R) and partly through the Ang II type 2 receptor.5 The effects of Ang II on blood vessels include vascular smooth muscle cell (VSMC) contraction, increased growth and proliferation, modulation of reactive oxygen species (ROS) production, as well as cytoskeletal structural changes.6,7 Angiotensin-converting enzyme 2 (ACE2), a homolog of ACE, is a monocarboxypeptidase that metabolizes Ang II to yield Ang 1 to 7.8–13 Ang II receptor blockers that selectively antagonize the AT1R also upregulate ACE2, resulting in generation of Ang 1 to 7,14 and loss of ACE2 promotes cardiovascular disease in preclinical models of diabetes mellitus15 and atherosclerosis.16–18

Vascular remodeling is the result of a complex interplay of changes in vascular tone and structure. VSMCs maintain vascular tone mainly through coordinated contraction/relaxation, and they also play a key role in arterial wall remodeling through proliferation, hypertrophy, and apoptosis.19 The modulation of VSMC phenotypic changes is an early event in adverse vascular remodeling, leading to the formation of aortic aneurysm. We found increased ACE2 protein in human aortas with bicuspid aortic valve (BAV) and murine aortas in response to Ang II. We hypothesize that the upregulation of ACE2 levels is a compensatory response to minimize vascular injury. We assessed the role of ACE2 in vascular disease and showed a novel and critical role of ACE2 in the prevention of adverse vascular remodeling in resistance and conductance arteries. Loss of ACE2 potentiated the effects of Ang II on VSMC oxidative stress and apoptosis, whereas both AT1R blockade and...
Loss of ACE2 Potentiates Vascular Oxidative Stress and VSMC Apoptosis: Role of the AT1R and Ang 1 to 7

Ang II is a well-known stimulant of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system and induces production of ROS in the vascular cells. Ang II resulted in greater activation of the NADPH oxidase system (Figure 2A), as well as greater immunoreactivity for nitrotyrosine (Figure 2B), a marker for peroxynitrite formation, in the ACE2KO MA compared with WT MA. Dihydroethidium staining showed markedly increased ROS production in ACE2KO aortic VSMCs (Figure 2C; Figure S3A and S3B) and an increased NADPH oxidase activity in response to Ang II stimulation (Figure 2D). AT1R blockade using irbesartan and Ang 1 to 7 supplementation was equally effective in suppressing the elevated oxidative stress in ACE2KO VSMCs in response to Ang II (Figure 2C–2E; Figure S3A and S3B). Apoptosis represents a dominant mode of VSMC loss, leading to adverse vascular remodeling.21–25 Terminal deoxynucleotidyltransferase-mediated 2′-deoxyuridine-5′-triphosphate nick-end labeling (TUNEL) staining in the MA shows increased apoptosis of VSMCs in ACE2KO MA in response to Ang II (Figure 2E; Figure S4), which correlated with reduced VSMC density as determined by calponin that is a specific marker of VSMCs and wheat germ agglutinin double staining (Figure 2F–2H), as well as immunofluorescence staining for α-smooth muscle actin (Figure 2I).

To provide definitive evidence for this, we isolated aortic VSMCs from WT and ACE2KO mice and treated them with equal concentration of Ang II (100 nmol/L). Calponin and vimentin double immunostaining confirmed that our preparation was composed mostly of VSMCs (Figure S5). The annexin V-fluorescein isothiocyanate/propidium iodide staining followed by flow cytometric analysis revealed markedly increased apoptosis in ACE2KO aortic VSMCs in response to Ang II (Figure 3A and 3B; Figure S6A). We next performed TUNEL assay along with calponin staining in aortas exposed to Ang II to assess VSMC-specific apoptosis. TUNEL assay revealed a marked increase in VSMC apoptosis in Ang II–treated ACE2KO mice (Figure 3C; Figure S6B). TUNEL-positive cells were also detected in the adventitia in the Ang II–treated ACE2KO mice (Figure 3C). Ang II resulted in a greater increase in cleaved caspase-3, as well as cleaved caspase-8 levels, with no change in the caspase-3 levels in the ACE2KO aorta compared with WT aortas (Figure 3D; Figure S6C), consistent with increased apoptosis in the ACE2KO-Ang II aortas. Matrix metalloproteases (MMPs) are known to play a crucial role in adverse vascular remodeling.26–28 Ang II resulted in a greater increase in pro-MMP2, as well as MMP2 and MMP9 protein levels, in ACE2KO compared with the WT aortas (Figure 3E). Importantly, in situ gelatin zymography showed increased MMP activity within the ACE2KO aorta in response to Ang II, confirming that the increased MMP2 and MMP9 proteins are concordant with increased MMP activity (Figure 3F). Clearly, loss of ACE2 increased the production of ROS and apoptotic loss of VSMCs, leading to progression of adverse vascular remodeling, a process suppressible by antagonizing the AT1R or enhancing Ang 1 to 7 action.

Loss of ACE2 Potentiates Adverse Aortic Remodeling in Response to Ang II

The role of ACE2 in the progression of adverse vascular remodeling in resistance arteries implies a critical role of ACE2 in disease progression in the major conductance artery, the aorta. Ang II administration resulted in greater activation of NADPH oxidase (Figure 4A) along with greater immunoreactivity for nitrotyrosine (Figure 4B) in ACE2KO aorta compared with WT aortas. Ultrastructural analysis with transmission electron
microscopy exhibited worsened structural damage in the aorta from ACE2KO mice in response to Ang II infusion compared with the WT mice characterized by disruption of the inner elastic membrane and disarranged smooth muscle layer with swollen mitochondria (Figure 4C; Figure S7). Next, we examined the long-term outcome of these effects. Four weeks of Ang II infusion resulted in greater aortic dilation in ACE2KO mice based on gross morphometry (Figure 4D) and ultrasonic imaging (Figure 4E). The aortic systolic expansion index, a measure of aortic elasticity and recoil properties during systole and diastole, was significantly suppressed in the Ang II–infused ACE2KO mice in the thoracic (Figure 4F–4H) and abdominal
Pathological aortic dilation and aortic aneurysms are associated with decreased medial VSMC density. Gomori trichrome staining and calponin immunofluorescence images (along with elastin autofluorescence) revealed a marked increase in aortic medial thickness in WT compared with ACE2KO mice (Figure 4I–4K). Next, we performed calponin and wheat germ agglutinin double staining to assess the VSMC density, which revealed that WT thoracic aorta showed a marked increase in medial VSMCs which was lacking in the ACE2KO thoracic aorta in response to 4 weeks of Ang II infusion (Figure 4J and 4K; Figure S9). Thus, loss of ACE2 increased adverse vascular remodeling in the aorta in response to Ang II resulting in loss of VSMCs and aortic dilation, a prelude to the formation of aortic aneurysms.

Discussion

The RAS plays a central role in the pathophysiology of several vascular diseases, and the suppression of the ACE/Ang II/AT1R axis has important clinical benefits. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity (A) and immunofluorescence (IF) of nitrotyrosine formation (B) showing greater increase in reactive oxygen species (ROS) production in ACE2 knockout (KO) MA in response to Ang II. Representative dihydroethidium (DHE) staining (C) showing increased ROS production in vascular smooth muscle cells (VSMCs) isolated from ACE2KO compared with wild-type (WT) thoracic aorta in response to Ang II (100 nmol/L) and equivalent suppression by AT1 receptor blockade and Ang 1 to 7 stimulation. Greater increase in Ang II–mediated NADPH oxidase activity in ACE2KO compared with WT aortic VSMCs is also suppressed by AT1 receptor blockade and Ang 1 to 7 stimulation (D). Representative terminal deoxynucleotidyltransferase-mediated 2′-deoxyuridine-5′-triphosphate nick-end labeling (TUNEL) and calponin IF staining (E) images showing increased VSMC apoptosis in the ACE2KO mesenteric arteries in response to Ang II. Representative calponin IF staining (F), calponin and wheat germ agglutinin double staining images (G), quantification of VSMC density in the mesenteric arteries (H), and representative α-smooth muscle actin IF (I) images showing decreased medial VSMC density in ACE2KO MA in response to Ang II. In the IF images, red color represents nitrotyrosine (B), calponin (E and F; G), or α-smooth muscle actin (I), green color represents autofluorescence (B; F; I), TUNEL-positive nuclei (E) or wheat germ agglutinin staining (G), and the blue color represents 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei. IRB=irbesartan, 1 μmol/L; Ang 1 to 7, 100 nmol/L. *P<0.05 for the main effects and #P<0.05 for the interaction using 2-way ANOVA (A). §P<0.05 compared with the corresponding vehicle group; ‡P<0.05 compared with WT-Ang II group; $P<0.05 compared with ACE2KO-Ang II group (D and E).
remodeling, which is an accepted preclinical model of aortic aneurysm in dyslipidemic mice.\textsuperscript{3,34,35} We found greater vascular stiffness with the ex vivo passive characteristics of MA vessels in ACE2KO mice in response to Ang II, which is likely because of the loss of VSMCs. Importantly, these mice showed senile changes in vascular function, resulting in increased vascular stiffness. Adverse vascular remodeling in conductance arteries from ACE2KO mice was exacerbated in response to Ang II. These changes in the ACE2KO vessels were associated with decreased smooth muscle cell density in the media and correlate with increased sensitivity to age\textsuperscript{39} and Ang II–dependent hypertension.\textsuperscript{9} In the absence of ACE2, blocking the AT1 receptor or enhancing Ang 1 to 7 action had therapeutic effects, illustrating an active contribution from both arms of the RAS.

Histopathologic changes in human aortic aneurysms predominantly affect the medial layer that is normally dominated by elastic fibers and VSMCs. VSMCs maintain vascular tone mainly through coordinated contraction/relaxation and also play a key role in arterial wall remodeling through proliferation, hypertrophy, and apoptosis.\textsuperscript{19,24} The modulation of VSMC phenotypic changes is an early event of adverse vascular remodeling, often leading to aortic dilation and aneurysm.\textsuperscript{31} We showed greater increase in NADPH oxidase–dependent ROS production and secondary nitrotyrosine formation and increased VSMC apoptosis in Ang II–infused ACE2KO mice aorta associated with increased cleaved caspase-3/caspase-8 levels. Importantly, ROS mediates protein kinase C–mediated VSMC apoptosis,\textsuperscript{37} and the role of ROS has been implicated in the formation of aortic aneurysms because inhibition of ROS

Figure 3. Loss of angiotensin-converting enzyme (ACE) 2 potentiates apoptosis of vascular smooth muscle cells and matrix metalloproteinase (MMP) activation in response to angiotensin II (Ang II) with a critical role of the angiotensin II type 1 (AT1) receptor and Ang 1 to 7. Flow cytometric analysis of annexin V/propidium iodide–stained vascular smooth muscle cells (VSMCs; A) and quantification of % apoptotic cells (B) showing increased apoptosis in the ACE2 knockout (KO) VSMCs in response to Ang II (100 nmol/L) and suppressed by AT1 receptor blockade and Ang 1 to 7 stimulation. Representative terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) and calponin immunofluorescence staining (C) showing increased smooth muscle cell apoptosis in the ACE2KO aorta in response to Ang II. In these images, green color represents TUNEL-positive nuclei, red color represents calponin, and blue color represents 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei. Western blot analysis showing greater cleaved caspase 3 and cleaved caspase 8 (D) in ACE2KO compared with wild-type (WT) aortas in response to Ang II. Ang II produced a greater increase in pro-MMP2, active MMP2, and MMP9 (E) in the ACE2KO compared with WT aortas. Representative in situ gelatin zymography images (F) showing increased gelatinase activity in the ACE2KO aorta in response to Ang II. IRB=irbesartan, 1 μmol/L; Ang 1 to 7, 100 nmol/L. *P<0.05 compared with the corresponding vehicle group; #P<0.05 compared with the WT-Ang II group; $P<0.05 compared with ACE2KO-Ang II group (B). P<0.05 for the main effects and P<0.05 for the interaction using 2-way ANOVA (D and E). R.R. indicates relative ratio.
Our results are consistent with previous findings linking ACE2 deficiency to increased Ang II–mediated ROS production, NADPH oxidase activity and nitrotyrosine levels in the aorta, and increased Ang II levels in the vasculature. Ang II–induced VSMC proliferation is seen with short-term exposure to Ang II in WT VSMCs. In our study, Ang II infusion to WT mice leads to increased media-to-lumen ratio in both the MA and aorta, implying increased media thickness (possibly because of increased VSMC proliferation or generation attenuated aneurysm formation. Our results are consistent with previous findings linking ACE2 deficiency to increased Ang II–mediated ROS production, NADPH oxidase activity and nitrotyrosine levels in the aorta, and increased Ang II levels in the vasculature. Ang II–induced VSMC proliferation is seen with short-term exposure to Ang II in WT VSMCs. In our study, Ang II infusion to WT mice leads to increased media-to-lumen ratio in both the MA and aorta, implying increased media thickness (possibly because of increased VSMC proliferation or...
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Hypertrophy). However, Ang II exposure in the ACE2KO background increased VSMC apoptosis, highlighting a critical role of ACE2 in the inhibition of VSMC apoptosis. This correlates with the phenotype-dependent effects of Ang II on the VSMCs. A phenotype-dependent effect of Ang II on VSMCs is characterized by a lack of Ang II effect on cell survival of the spindle VSMCs, whereas delayed Ang II exposure is proapoptotic in the epitheloid VSMCs. Importantly, despite the increased apoptosis in the ACE2KO VSMCs, the media thickness and the VSMC density did not decrease compared with the saline infused aorta. This suggests that, unlike in the WT vessels, Ang II–induced cellular proliferation is counterbalanced by increased apoptosis in the ACE2KO vessels. TUNEL staining in the Ang II–infused ACE2KO aorta showed increased apoptosis of not only the VSMCs but also the non-VSMC cell types present in the adventitia. However, further work is needed to determine which adventitial cell types are undergoing apoptosis and their possible role in vascular disease in the ACE2KO mice. Loss of ACE2 resulted in increased MMP2 and MMP9 levels and increased MMP activity within the aortic wall in response to Ang II. MMPs contribute to the extracellular matrix turnover by degrading existing proteins and increased activated MMP2 and MMP9 levels in patients with aortic aneurysms degrade the vascular extracellular matrix, leading to aortic dilation. Importantly, increased MMP2 activity coexists with VSMC apoptosis in the aorta from patients with Marfan syndrome, as well as BAV. In summary, our results provide a link between Ang II–induced ROS production, MMP activation, and VSMC apoptosis with the potential counter-regulatory role of ACE2 in adverse vascular remodeling. The pathophysiological significance of this constellation of molecular, biochemical, and structural changes in ACE2-deficient vasculature is further strengthened by the well-established link between ACE2 deficiency and the promotion of an atherosclerotic phenotype. Collectively, these results strongly implicate the ACE2 pathway as a critical mediator of vascular disease. Enhancing ACE2 action may have therapeutic benefits in vascular diseases.

Perspectives

BAV is the most common congenital cardiac abnormality, occurring in 1% of the population. Patients with BAV have increased risk (and at a younger age) of ascending aortic dilatation than it does in patients with normal trileaflet aortic valves. Aortic dilatation has a propensity for dissemination and rupture, making it a potentially lethal disease. RAS regulates vascular tone and plays a key role in adaptive and maladaptive vascular remodeling. ACE2 is a newly discovered member of RAS family, which converts Ang II into a vasculoprotective peptide Ang 1 to 7). We found upregulation of ACE2 in the aorta from patients with BAV. We also found ACE2 upregulation in the Ang II–infused mice aorta. Ang II infusion is a widely accepted clinically relevant preclinical model of RAS activation. Ang II infusion in ACE2KO mice resulted in increased adverse vascular remodeling, showing marked aortic dilation that was associated with increased VSMC apoptosis. These data are in agreement with the histological features of aortic aneurysm. Histopathological changes in human aortic aneurysms predominantly affect the medial layer that is normally dominated by elastic fibers and VSMCs. The modulation of VSMC phenotypic changes including VSMC apoptosis is an early event of adverse vascular remodeling, often leading to aortic dilation and aneurysm. Our data suggest that ACE2 is a key negative regulator of activated RAS–driven adverse vascular remodeling, and enhancing ACE2 could have beneficial effects in vascular diseases.

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Disclosures

None.

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Angiotensin converting enzyme 2 is a critical determinant of angiotensin II-induced loss of vascular smooth muscle cells and adverse vascular remodeling

by

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Supplemental Methods

Experimental animals and protocols. Ace2⁻¹/²Y mutant mice that were backcrossed into the C57BL/6 background for at least 8 generations were used in the present study.¹⁻⁴ As Ace2 is an X-linked gene, Ace2⁻¹/²Y represents ACE2 null of ACE2 knockout mice. Mice were housed in pathogen-free conditions and had access to sterilized food and water ad libitum. All experiments were performed in accordance to institutional guidelines, Canadian Council on Animal Care and 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). Alzet micro-osmotic pumps (Model 1002, Durect Corp.) were implanted subcutaneously in male mice of the indicated genotypes to deliver 1.5 mg/kg/day Ang II or saline (control) for 2 or 4 wk.¹,⁵

Human aortic specimen. Ascending aorta from patients with bicuspid aortic valve (BAV) and aortopathy undergoing aortic valve replacement and ascending repair at the Mazankowski Alberta Heart Institute. Non-diseased controls were collected as part of the Human Organ Procurement and Exchange (HOPE) program for research. All experiments were performed in accordance with the institutional guidelines and were approved by Institutional Ethics Committee.

Ex vivo vascular passive characteristics. Second-order mesenteric arteries were used from the mice to study the ex vivo vascular passive characteristics as previously described.⁶,⁷ Second-
order mesenteric arteries were used from the mice to study the ex vivo vascular passive characteristics as previously described. Briefly, tissue dissections were performed in ice-cold physiological saline solution (PSS), composition (in mmol/L): 10 HEPES, 5.5 glucose, 1.56 CaCl2, 4.7 KCl, 142 NaCl, 1.17 MgSO4, 1.18 KH2PO4, pH 7.5. Arteries were cleaned of all surrounding adipose and connective tissues and mounted on two glass cannulae in a two-bath pressure myograph (Living Systems, Burlington, VT). Vessels were given a 40-minute equilibration period during which they were exposed to a stepwise increase in pressure from 60 to 80 mmHg with regular changes of the PSS bathing solution. Vessels were equilibrated in Ca2+-free PSS in the presence of papaverine (0.1 μmol/L) to initiate complete dilation. Passive characteristics were then assessed using pressures from 0 to 140 mmHg. Ex vivo analysis of circumferential wall stress and wall strain (change in diameter/original diameter) mesenteric arteries were calculated as previously described.6

Ultrasonic imaging of aorta. Ultrasonic images of the aorta were obtained in mice anesthetized with isoflurane using a Vevo 770 high resolution imaging system equipped with a real time microvisualization scan head (RMV 704, Visual Sonics, Toronto, Canada). The aortic diameters were measured by M-mode at thoracic aorta and abdominal aorta The maximum aortic lumen diameter (aortic systolic diameter corresponding to cardiac systole) and the minimum aortic lumen diameter (aortic diastolic diameter corresponding to cardiac diastole) monitored by simultaneous ECG recordings were measured and used to calculate the aortic expansion index [(Systolic aortic diameter - Diastolic aortic diameter)/Systolic aortic diameter X 100].

Isolation and culture of Vascular Smooth Muscle Cells. Vascular smooth muscle cells (VSMCs) were isolated from 8- to 10-wk old WT and ACE2KO mice aortas and cultured as described previously. Briefly, thoracic and abdominal aortas were obtained from 8- to 10- weeks-old mice. The adventitial and inner layers of aorta were dissected off. The aortic media were cut into small pieces and attached to the surface of 35 mm dishes by drying for 15 minutes. The explants were cultured in DMEM (Gibco) containing 10% fetal bovine serum (FBS, Sigma) supplemented with 50 μg/mL gentamicin (Gibco) at 5% CO2 incubator. Cells migrated from the explants and were passaged when they reached 100% confluency. The purity of the VSMCs was examined with smooth muscle-specific calponin and mesenchymal cell marker, vimentin, double immunostaining. VSMCs were serum-deprived for 24 hours by incubation in DMEM with 0.1% BSA and gentamicin and then treated with or without 1 mM hydrogen peroxide in DMEM+0.1% BSA as a positive control for apoptotic analysis. Vascular smooth muscle cells were treated with 100 nM Ang II in DMEM+0.1% BSA for 24 hours and were used between passages 4 and 5. Irbesartan (1 μM) and Ang 1-7 (Bachem, Germany; 100 nM) were added to the VSMCs for 30 min prior to exposure of Ang II.

Histological analyses, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, Wheat germ agglutinin staining and immunofluorescence. Mice were perfuse-fixed at 80 mmHg with paraformaldehyde after 2 wk of Ang II or saline infusion allowing the blood vessels to be fixed in its native state as previously described. Subsequently, mesenteric arteries were dissected out, carefully not to alter the structure of the vessels, further fixed in 10%
buffered formalin for 48 hr and paraffin-embedded. Aorta were collected and fixed in 10% buffered formalin for 48 hr from the mice infused with Ang II or saline for 2 and 4 wk. In separate experiments, aortas were collected and fixed in OCT for cryosections. Five µm thick formalin fixed paraffin embedded (FFPE) sections were used for the histological staining including Movat’s pentachrome and Gomori Trichrome as previously described. Collagen-positive area in the MA was quantified by the morphometric analysis using the Metamorph Basic (version 7.7.0.0) software. It was represented as the normalized ratio to the vessel cross-sectional area. In situ DNA fragmentation was detected in 5 µm thick FFPE sections of mesenteric arteries and aorta using the commercially available terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay kit according to manufacturer’s instructions (Invitrogen) as previously described. Five µm thick FFPE sections were used for the immunofluorescence staining for ACE2, alpha-smooth muscle actin, calponin, and nitrotyrosine immunofluorescence as well as wheat germ agglutinin staining as previously described. Elastin autofluorescence was used to assess the media thickness in the aorta. Briefly, media thickness was assessed in the calibrated images by measuring the mean distance between the external elastic lamina and the internal elastic lamina from 8 different regions in the cross-section of aorta using the Metamorph Basic (version 7.7.0.0). Calponin positive cells in the aorta delineated with WGA cell membrane staining were imaged using Confocal microscope (SP5, Leica) and counted to assess the VSMC density at the brightest focal plane using Fiji/ImageJ v1.48q. Paraformaldehyde fixed VSMCs were double stained for calponin and vimentin to assess the purity of the VSMC culture. Briefly, the VSMCs were stained with rabbit anti-calponin (Abcam) and rabbit anti-vimentin (Abcam) primary antibodies and Alexa Fluor 488 conjugated anti-rabbit and Alexa Fluor 594 conjugated anti-mouse secondary antibodies. The cells were imaged using the fluorescence microscope (Olympus IX81) and analyzed using the Fiji/ImageJ v1.48q.

**In situ gelatin zymography.** In situ gelatin zymography was performed on 14 µm thick aorta cryosections as described previously. Briefly, aorta cryosections were incubated with 0.1% agarose containing DQ-gelatin (Invitrogen) at 37°C for 1 hour and then imaged using fluorescence microscope (Olympus IX81) using GFP filter. Autofluorescence was captured using Texas red filter to show the elastin fibers.

**Transmission Electron Microscope analysis.** For transmission electron microscope analysis, samples of mice aorta tissues were fixed in 2.5% glutaraldehyde and then washed three times with phosphate buffered saline, followed by postfixation with 1% osmium tetroxide for 1 h, stained with 2% aqueous uranyl acetate, and dehydrated in a graded series of ethanol. After infiltration and polymerization, ultrathin sections were cut, flattened with xylene vapor, collected on nickel grids, and observed on a HITACHI-600 electron microscope (Hitachi, Japan) with magnification 2500x and 7400x.

**Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity assay and dihydroethidium staining.** Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity in mesenteric arteries, aorta as well as aortic VSMCs was quantified by lucigenin-enhanced chemiluminescence using a single-tube luminometer (Berthold FB12, Berthold
Technologies, Germany) modified to maintain the sample temperature at 37°C as we have previously described. Briefly, tissue homogenates or VSMCs were suspended in phosphate buffered saline (PBS) with protease and phosphatase inhibitors, and the amount of protein in each sample was quantified after lysis with RIPA. NADPH (1 mM) and Lucigenin (50 µM) were added to the suspended cell sample equivalent to containing 100 µg of protein in the presence or absence of diphenylene iodonium (DPI; 10 µM), a selective inhibitor of flavin-containing enzymes including NADPH Oxidase. Light emission was measured every 1 second during a 5-minute period using a single-tube luminometer (Berthold FB12, Berthold Technologies, Germany) at 37°C. The emission over a 3-minute period was averaged for each sample. Dihydroethidium (DHE) staining was performed on VSMCs as previously described and visualized under fluorescence microscope (Olympus IX81). Briefly, control or Ang II treated cells, pretreated with or without IRB, Ang 1-7, were incubated with DHE (20µM DHE in culture media; Sigma Aldrich) at 37°C for 30 minutes in dark. Fluorescence images were subsequently taken after washing with PBS with a fluorescence microscope (IX81, Olympus). Quantitative measurements of DHE fluorescence intensity were carried out using Metamorph Basic (version 7.7.0.0). Briefly, regions congruent to the cell nuclei boundaries were drawn and the average pixel intensities were calculated. The average pixel intensity was then corrected by subtracting the average pixel intensity of the background and represented as DHE fluorescence.

**Annexin V staining for detection of apoptosis.** Annexin V staining was carried out in VSMCs to detect the apoptosis. Control as well Ang II treated cells were trypsinized, and both monolayers and any cells in the culture supernatant were centrifuged at 1000 rpm for 5 minutes. The cell pellets were washed three times in PBS and then resuspended in annexin binding buffer at 1 x 10^6 cells/mL. The cells were then incubated with FITC conjugated Annexin V antibody (Invitrogen, Burlington, Canada) and propidium iodide (1 µg/mL) at room temperature for 15 minutes. After the incubation annexin-binding buffer was added and samples were analysed as soon as possible with a LSR Fortessa flow cytometer (BD Pharmingen, Franklin Lakes, NJ) and FACS Diva software (version 6.1.3, BD Pharmingen, Franklin Lakes, NJ) measuring the fluorescence emission at 530 nm and 575 nm (or equivalent) using 488 nm excitation.

**Western Blot Analysis.** The proteins from aorta tissues were measured by Western blot analysis as described previously. Total protein was extracted using the BCA Protein Array Kit (Pierce, Rockford, IL). The primary antibody against cleaved caspase 3 (18 kD), caspase 3 (35 kD), cleaved caspase 8 (18 kD), MMP2 (72 kD; 63kD), MMP9 (90 kD) and GAPDH (37 kD) were obtained from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The primary antibody for ACE2 was obtained from Abcam Inc, (Toronto, ON, Canada). For the loading control using MemCode staining, the nitrocellulose membrane containing the transferred proteins was first rinsed with ultrapure water and the MemCode™ Reversible Protein Stain (Thermo Scientific, Ottawa, Ontario, Canada) was added. After agitating the membrane at room temperature for 30 secs, the turquoise-blue bands appeared. To remove background, MemCode™ Destain Reagent was added twice to the membrane for 5 min. Images were taken whole lanes were used as the loading control. Representative blots were shown as the MemCode loading control images.
**Statistical Analysis.** All data are shown as mean ± SEM. All statistical analyses were performed using SPSS software (Chicago, Illinois; Version 19). Comparison between two groups was made using a nonpaired Student t-test. Two-way ANOVA using ACE2 status and Ang II-infusion status as the two independent variables (factors) was performed to compare the data between the four experimental groups (WT, WT-Ang II, ACE2KO, and ACE2KO-Ang II). In experiments with multiple treatments, one-way ANOVA was followed by multiple comparison using the Student Neuman-Keuls test. Statistical significance is recognized at p<0.05.
References:


Figure S1. Upregulation of ACE2 in human aorta with bicuspid aortic valve. Representative ACE2 immunofluorescence (IF) staining images showing increased ACE2 protein levels in the human aorta with bicuspid aortic valve compared to non-diseased control (NDC). In the IF images, red color represents ACE2, green color represents autofluorescence and blue color represents DAPI stained nuclei.
Figure S2. Ang II administration results in equivalent fibrosis, though loss of ACE2 or Ang II administration does not affect the vascular stiffness in the WT mesenteric resistance arteries. Quantification of collagen-positive area/vessel cross-sectional area (A) showing equivalent fibrosis in the WT and ACE2KO mesenteric arteries in response to Ang II-infusion. Ex vivo passive pressure myography showing pressure-dependent changes in lumen diameter of mesenteric arteries in young WT and ACE2KO mice (B), as well as in WT mesenteric arteries after 2 wk of Ang II-infusion (C) showing no change in the vascular stiffness compared with the WT.
Figure S3. Loss of ACE2 leads to increased vascular oxidative stress in response to Ang II with a critical role of the AT1 receptor and Ang 1-7. Representative dihydroethidium (DHE) staining images (A) and the DHE fluorescence (B) showing increased ROS production in VSMCs isolated from ACE2KO compared to WT thoracic aorta in response to Ang II and equivalent suppression by AT1 receptor blockade and Ang 1-7 stimulation. BF: bright field, DHE: dihydroethidium. $p<0.05$ compared with the corresponding vehicle group; $^\ddagger p<0.05$ compared with the WT-Ang II group; $^§ p<0.05$ compared with ACE2KO-Ang II group.
Figure S4. Loss of ACE2 potentiates apoptosis of mesenteric artery vascular smooth muscle cells in vivo in response to Ang II. Smooth muscle cell specific % apoptotic cells quantified from TUNEL assay images showing increased smooth muscle cell apoptosis in the ACE2KO mesenteric arteries in response to Ang II-infusion. #p<0.05 for the interaction using two-way ANOVA.
Figure S5. Characterization of isolated aortic vascular smooth muscle cells. Representative calponin and vimentin double immunofluorescence staining confirming the purity of the isolated and cultured vascular smooth muscle cells to be more than 95%. In the immunofluorescence images, green color represents calponin, red color represents vimentin and blue color represents DAPI stained nuclei.
Figure S6. Loss of ACE2 potentiates apoptosis of vascular smooth muscle cells in response to Ang II with a critical role of the AT1R and Ang 1-7 with no effect on the caspase 3 protein levels. Overlays of the flow cytometric analysis of Annexin V/propidium iodide stained VSMCs showing increased apoptosis, as shown by increased Annexin V positive cells, in the ACE2KO VSMCs in response to Ang II and suppressed by AT1 receptor blockade and Ang 1-7 stimulation (A). Smooth muscle cell specific % apoptotic cells quantified from TUNEL assay images showing increased smooth muscle cell apoptosis in the ACE2KO aorta in response to Ang II infusion (B). Quantification of immunoblotting showing no change in the caspase 3 levels in ACE2KO aorta in response to Ang II compared with WT (C). *p<0.05 for the interaction using two-way ANOVA.
Figure S7. Loss of ACE2 leads to enhanced adverse aortic remodeling and ultrastructural disruptions and diarrangements. Ultrastructural analysis with transmission electron microscopy showing disruption of the inner elastic membrane (black arrow), swollen mitochondria (white asterik) and disarranged smooth muscle layer (white arrow) in the ACE2KO aorta in response to Ang II for 2 wk (x7400 magnification).
Figure S8. Loss of ACE2 leads to enhanced adverse remodeling of the abdominal aorta in response to Ang II. Quantification of abdominal aortic diastolic (A) and systolic (B) diameters showing equivalent effect of Ang II-infusion these diameters in ACE2KO aorta compared with WT aorta with decreased expansion index (C) in the ACE2KO aorta in response to Ang II compared with WT. *p<0.05 for the main effects and #p<0.05 for the interaction using two-way ANOVA.
Figure S9. Loss of ACE2 leads to decreased aortic vascular smooth cell density in response to Ang II infusion. Representative calponin and wheat germ double stained images (A) and cell count (B) showing decreased vascular smooth cell density in the ACE2KO aorta in response to Ang II infusion. In the fluorescence images, green color represents wheat germ agglutinin stained cell membranes, red color represents calponin and blue color represents DAPI stained nuclei. #p<0.05 for the interaction using two-way ANOVA.