Cardioprotective Effects Mediated by Angiotensin II Type 1 Receptor Blockade and Enhancing Angiotensin 1-7 in Experimental Heart Failure in Angiotensin-Converting Enzyme 2–Null Mice


Abstract—Loss of angiotensin (Ang)-converting enzyme 2 (ACE2) and inability to metabolize Ang II to Ang 1-7 perpetuate the actions of Ang II after biomechanical stress and exacerbate early adverse myocardial remodeling. Ang receptor blockers are known to antagonize the effect of Ang II by blocking Ang II type 1 receptor (AT1R) and also by upregulating the ACE2 expression. We directly compare the benefits of AT1R blockade versus enhancing Ang 1-7 action in pressure-overload–induced heart failure in ACE2 knockout mice. AT1R blockade and Ang 1-7 both resulted in marked recovery of systolic dysfunction in pressure-overloaded ACE2-null mice. Similarly, both therapies attenuated the increase in NADPH oxidase activation by downregulating the expression of Nox2 and p47phox subunits and also by limiting the p47phox phosphorylation. Biomechanical stress-induced increase in protein kinase C-α expression and phosphorylation of extracellular signal–regulated kinase 1/2, signal transducer and activator of transcription 3, Akt, and glycogen synthase kinase 3β were normalized by irbesartan and Ang 1-7. Ang receptor blocker and Ang 1-7 effectively reduced matrix metalloproteinase 2 activation and matrix metalloproteinase 9 levels. Ang II–mediated cellular effects in cultured adult cardiomyocytes and cardiofibrolasts isolated from pressure-overloaded ACE2-null hearts were inhibited to similar degree by AT1R blockade and stimulation with Ang 1-7. Thus, treatment with the AT1R blocker irbesartan and Ang 1-7 prevented the cardiac hypertrophy and improved cardiac remodeling in pressure-overloaded ACE2-null mice by suppressing NADPH oxidase and normalizing pathological signaling pathways.

Key Words: renin-angiotensin system • angiotensin 1-7 • angiotensin-converting enzyme 2 • NADPH oxidase • AT1 receptor • heart failure • signaling

Several lines of experimental and clinical evidence implicate a key role for the renin-angiotensin system in the pathophysiology of a number of cardiovascular diseases, such as myocardial infarction, hypertension, and heart failure.1,2 Angiotensin II (Ang II), acting via the Ang II type 1 receptor (AT1R) and Ang II type 2 receptor, modulates production of reactive oxygen species (ROS), impairing myocardial contractility and extracellular matrix remodeling, thereby negatively impacting on heart function.3 Angiotensin-converting enzyme 2 (ACE2), a homologue of angiotensin-converting enzyme, is a monocarboxypeptidase that metabolizes Ang II to yield angiotensin 1-7 (Ang 1-7) and lowers the Ang II/Ang 1-7 ratio.4–9 Ang II receptor blockers that selectively antagonize the AT1R became a valid alternative approach to interfere with the renin-angiotensin system axis and also upregulates ACE2, resulting in the generation of Ang 1-7.10,11 Ang 1-7 acts on the Mas receptor and plays an important role in counteracting the actions of Ang II.12–18 Ang II–mediated oxidative stress, cardiac hypertrophy, contractile dysfunction, and fibrosis are exacerbated in ACE2-deficient mice,5,7 whereas recombinant human ACE2 is able to attenuate these responses and improve cardiac function, with a marked reversal of Ang II–mediated signaling.6 Many of the cardiac pathological effects of renin-angiotensin system activation and Ang II appear to be mediated through ROS, produced by a specific NADPH oxidase–dependent pathway in an AT1R-dependent manner.19,20 Also involved are a battery of prohypertrophic signaling pathways with downstream activation of matrix metalloproteinase (MMPs).3,6 We hypothesized that the car-
diabetic phenotype in ACE2-null mice can be rescued by switching off the Ang II-AT,R axis and/or switching on the Ang 1-7/Mas receptor axis as an integral dual counterregulatory system.

**Methods**

Detailed methods are available in the online-only Data Supplement.

**Experimental Animals and Protocols**

Ace2−/− mutant mice that were backcrossed into the C57BL/6 background for ≥8 generations were used in the present study.5,6,21 All of the experiments were performed in accordance with institutional guidelines, the Canadian Council on Animal Care, and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

**Pressure-Overload**

Young (8- to 9-week–old) male Ace2−/− (ACE2KO) mice were subjected to pressure-overload as described previously.7,22 ACE2KO mice were treated with the AT,R blocker irbesartan (50 mg/kg per day)5,6 or the antioxidant and NADPH oxidase inhibitor apocynin (240 mg/kg per day)7 in the drinking water, and in parallel experiments, ACE2KO mice were implanted with miniosmotic pumps to deliver Ang 1-7 (24 μg/kg per hour; Bachem).7,14

**Isolation and Culture of Adult Cardiomyocytes and Cardioblasts**

Adult murine left ventricular (LV) cardiomyocytes and cardioblasts from ACE2KO-2 Weeks AB mice were isolated and cultured as described previously.5,8 Isolated cardiomyocytes and cardioblasts for 20 minutes before exposure of Ang II (Sigma; 100 nmol/L).

**Echocardiographic and Hemodynamic Measurements**

Transthoracic echocardiography and hemodynamic measurements were performed in anesthetized mice as described previously.6

**Superoxide Assay and Dihydroethidium Fluorescence**

The chemiluminescence lucigenin assay to measure NADPH oxidase activity and dihydroethidium fluorescence were performed as described previously.6

**Taqman Real-Time PCR, Western Blot Analysis, and Gelatin Zymography**

Taqman real-time PCR, Western blot analysis, and gelatin zymography were carried out as described previously.6,7 Assessment of phosphorylated p47phox was measured by immunoprecipitation, followed by Western blot, as described previously.7

**Plasma and Myocardial Ang 1-7 Levels**

Myocardial and plasma Ang 1-7 levels were measured at the Hypertension Core Laboratory (Wake Forest University, Winston-Salem, NC), as described previously.6,7

**Histological Analysis and Immunofluorescence**

LV fibrosis and cardiomyocyte hypertrophy were measured by Masson trichrome and Picrosirius red staining, as described previously.6 Isolated cardioblasts were double-stained for α-smooth muscle actin (SMA) and vimentin, along with nuclear-staining with 4′,6-diamidino-2-phenylindole (Invitrogen) and visualized and imaged using fluorescence microscopy.

Statistical Analysis

All of the data are shown as mean±SEM. All of the statistical analyses were performed using SPSS software (Chicago, IL; version 10.1). The effects of genotype, irbesartan, and Ang 1-7 were evaluated using ANOVA followed by the Student-Neuman-Keuls test for multiple comparison testing, and comparison between 2 groups were made using the Student t test.

**Results**

**AT,R Blockade and Ang 1-7 Supplementation Reversed the Adverse Myocardial Remodeling in Pressure-Overload ACE2-Deficient Hearts**

Pressure-overload resulted in a hypertrophic response at 2 weeks, as assessed by morphometry, LV weight normalized to tibial length, and expression of hypertrophic markers compared with atrial natriuretic factor, brain natriuretic peptide, and β-myosin heavy chain in ACE2KO hearts (Figure 1). Pressure-overload resulted in increased LV wall thickness and LV end-diastolic dimension with lowered fractional shortening in ACE2KO hearts (Table S1, available in the online-only Data Supplement). The functional deterioration was confirmed by invasive hemodynamic measurement showing elevated LV end-diastolic pressure and reduced ±dP/dt max (Table S1). Myocardial Ang II level is elevated and Ang 1-7 level is reduced in ACE2KO hearts subjected to pressure-overload.7 We tested the hypothesis that blocking AT,R using irbesartan or supplementing Ang 1-7 would prevent the deleterious effects of Ang II actions in vivo and improve cardiac function. Both AT,R blockade and Ang 1-7 supplementation resulted in less cardiac hypertrophy with marked improvement in heart function in ACE2KO mice receiving irbesartan or Ang 1-7 in response to 2 weeks of pressure-overload (Figure 1 and Table S1). Although treatment with irbesartan and Ang 1-7 resulted in a significant lowering of systolic blood pressure, the proximal aortic pressure was equivalent in all of the experimental groups (Figure S1). We confirmed that systemic delivery of Ang 1-7 elevated plasma Ang 1-7 levels with a mild elevation seen in response to AT,R blockade without changes in myocardial Ang 1-7 levels (Figure S2). These results show that both AT,R blockade and Ang 1-7 supplementation have equivalent protective effects against adverse myocardial remodeling in an ACE2-null environment.

**Suppression of NADPH Oxidase, Pathological Signaling, and MMP Axis by AT,R Blockade and Enhanced Ang 1-7 Signaling**

NADPH oxidase activation and enhanced oxidative stress are common features of the pathological effects of Ang II.3,24,25 The antioxidant and NADPH oxidase inhibitor apocynin suppressed the increased myocardial NADPH oxidase activity and superoxide production in pressure-overloaded ACE2KO hearts (Figure 2A and 2B), resulting in suppression of brain natriuretic peptide expression and improvement in heart function (Figure 2C through 2E). AT,R blockade and Ang 1-7 supplementation effectively mitigated NADPH oxidase activation and ROS production measured by dihydroethidium fluorescence (Figure 2F and 2G), which correlated with reduction in the expression of NADPH oxidase subunits p47phox and NOX2 (gp91phox; Figure 2H and
Furthermore, immunoprecipitation followed by Western blotting indicated that there is a significant increase in phosphorylated-p47phox levels with increased gp91phox protein levels in pressure-overloaded ACE2KO hearts, which were effectively blocked by treatment with irbesartan and Ang 1-7 (Figure 2J and 2K).

Ang II–mediated activation of AT1R leads to a cascade of molecular events, including activation of the extracellular signal–regulated kinase (ERK) 1/2, signal transducer and activator of transcription 3 (STAT3), and phosphatidylinositol 3-kinase signaling pathways.3,6,7 Both irbesartan and Ang 1-7 completely blocked the activation of ERK1/2 (Figure 3A) and STAT3 (Figure 3B), suggesting that AT1R blockade or Mas receptor activation was capable of suppressing pathological signaling. We then examined the role of irbesartan and Ang 1-7 in regulating the activity of the phosphatidylinositol 3-kinase/Akt pathway. During cardiac hypertrophy, glycogen synthase kinase 3β (serine 9) is phosphorylated by Akt, leading to suppression of its kinase activity.26 We found that pressure-overloaded ACE2KO hearts exhibit increased phosphorylation of Akt and glycogen synthase kinase 3β, which was blocked by irbesartan and Ang 1-7 (Figure 3C and 3D).

We hypothesized that there might be a common upstream target that regulates the activity of these pathways. Indeed, protein kinase C (PKC)-α is capable of regulating the activity of ERK1/2 and Akt pathways in response to Ang II.27,28 Whereas Ang II–induced activation of NADPH oxidase involves PKC-α–induced phosphorylation of p47phox and its translocation to the membrane.3 Pressure-overload in ACE2KO hearts increased PKC-α expression by 2-fold compared with sham control, and this effect was prevented by irbesartan and Ang 1-7 (Figure 3E).

Enhanced formation of ROS is linked to the activation of MMPs and degradation of key components of the extracellular matrix.29,30 Gelatin zymography showed greater activation of the MMP2 system with higher levels of the active MMP2, in addition to increase MMP9 levels. Both pro- and active-MMP2 and -MMP9 levels returned to normal levels as in the sham control, on exposure to irbesartan and Ang 1-7 (Figure 4A through 4C). These results show that AT1R blockade and Mas receptor activation suppress MMP expression. Picrosirius red staining revealed marked hypertrophy and disorganization of the extracellular matrix, which was prevented by treatment with irbesartan and Ang 1-7 (Figure 4D and 4E), suggesting both cardiomyocyte and extracellular matrix–dependent effects.

### Cellular Effects of AT1R Blockade and Ang 1-7 Supplementation in Cardiomyocytes and Cardiofibroblasts

To gain further insight into the beneficial effects of AT1R blockade and Ang 1-7 effects, we isolated and cultured adult cardiomyocytes and cardiofibroblasts from pressure-overloaded ACE2KO hearts. Acute stimulation of cardiomyocytes with Ang II (100 nmol/L) resulted in a marked increase in NADPH oxidase activity and superoxide forma-
tion with increased phosphorylation of ERK1/2 (Figure 5A through 5C). In cardiofibroblasts, Ang II stimulation increased the expression of α-SMA (Figure 5D) with activation of the ERK1/2 signaling pathway (Figure 5E), resulting in increased accumulation of α-SMA, a well-accepted marker of activated fibroblasts (Figure 5F). In both cell types, irbesartan and Ang 1-7 were equally efficacious at preventing the Ang II–mediated changes (Figure 5). These results demonstrate that the adverse remodeling in pressure-overloaded ACE2-deficient hearts is mediated by a combination of pathological

Figure 2. Treatment with apocynin, irbesartan, and angiotensin 1-7 inhibits NADPH oxidase and superoxide generation and reverses the adverse remodeling in angiotensin-converting enzyme 2 knockout (ACE2KO) mice subjected to pressure-overload. Apocynin inhibited NADPH oxidase activity (A) and reactive oxygen species formation assessed by dihydroethidium (DHE) fluorescence (B), resulting in normalized brain natriuretic peptide (BNP) expression (C) and recovery of systolic dysfunction (D and E). Ang II type 1 receptor (AT1R) blockade and Ang 1-7 supplementation inhibit NADPH oxidase activity (F), reactive oxygen species production (G), mRNA expression of p47phox (H) and NOX2 (gp91phox; I), serine phosphorylation of p47phox determined by immunoprecipitation (J), and expression of gp91phox protein (K) in ACE2KO hearts subjected to pressure-overload. In J, the 2 lanes on the right include a lane without antibody, followed by the last lane with the isotypic control antibody where we detected the IgG heavy chain but not p47phox, n=4 from each group. FS indicates fractional shortening; LVEDD, left ventricular end diastolic dimension; A.U., arbitrary units; R.E., relative expression; gp91 tat, gp91phox ds tat peptide; scr tat, scrambled gp91phox ds tat peptide. #P<0.05 vs all other groups.
effects of Ang II on cardiomyocytes and cardiofibroblasts that can be effectively blocked by either AT1R antagonism or treatment with Ang 1-7.

**Discussion**

Genetic and functional loss of ACE2 is associated with an age-dependent cardiomyopathy, adverse myocardial remodeling in response to myocardial infarction, and pressure-overload and worsens Ang II–induced cardiac dysfunction. The enhanced susceptibility to heart disease in relation to a loss of ACE2 correlates with elevated Ang II and lowered Ang 1-7 levels in the heart. Recombinant human ACE2 attenuates Ang II–induced diastolic dysfunction by lowering the Ang II:Ang 1-7 ratio. Collectively, these results shown that ACE2 plays a key role in metabolizing Ang II into Ang 1-7 in vivo. The Ang II/AT1R axis is a well-known trigger of heart disease, whereas Ang 1-7/Mas is cardioprotective and antagonizes Ang II effects. Thus, it is conceivable that blocking the action of Ang II at the AT1R or enhancing the endogenous levels of Ang 1-7 would result in a reduction of cardiac hypertrophy and improve heart function. In this study, we showed that blocking Ang II/AT1R or enhancing Ang 1-7/Mas action resulted in a marked reduction in the pathological effects in the ACE2KO hearts, illustrating a marked degree of redundancy between these counterregulatory pathways. Treatment with AT1R blocker and Ang 1-7 supplementation increased plasma Ang 1-7 levels, which is consistent with previous studies showing that a systemic increase in Ang 1-7 levels and/or increased Mas receptor expression are cardioprotective. We also showed these effects in isolated cardiomyocytes and cardiofibroblasts, showing a clear cellular basis for the phenotypic rescue in ACE2-deficient hearts independent of blood pressure.

Treatment with irbesartan and Ang 1-7 blocked NADPH oxidase activation, thereby attenuating superoxide formation. This was associated with reduced expression of p47phox and gp91phox and inhibition of p47phox phosphorylation, resulting in reduced NADPH oxidase activation and superoxide formation. Apocynin, an antioxidant and NADPH oxidase inhibitor, ameliorates the pressure-overload–induced dys-

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Figure 3. Suppression of pathological signaling in pressure-overloaded angiotensin (Ang)-converting enzyme 2 knockout (ACE2KO) hearts by irbesartan and Ang 1-7. Western blot analysis showing increased phosphorylation of ERK 1/2 (A), STAT3 (B), Akt (C), glycogen synthase kinase (GSK-3β; D), and increased expression of PKC-α (E) in ACE2KO AB vs sham controls and attenuation of activation of these signaling pathways by Ang II type 1 receptor (AT1R) blockade and Ang 1-7 supplementation. Distinct lines refer to the sham-operated, placebo, irbesartan (IRB), and Ang 1-7 groups, respectively. n=4 from each group; R.E. indicates relative expression; ERK 1/2, extracellular signal-regulated kinase 1/2; STAT3, signal transducer and activator of transcription 3; GSK-3β, glycogen synthase kinase 3β; PKC-α, protein kinase C-α. #P<0.05 vs all other groups.
function in ACE2-null mice, confirming a key role of the NADPH oxidase pathway. Functional interactions exist between Mas and AT1R in the heart and vasculature, wherein the complex formation between Mas and AT1R is inhibitory to AT1R function.16,17,35 Pressure-overload in ACE2KO hearts activated PKC/Akt and ERK1/2 pathways. Interestingly, inhibition of PKC activation observed with AT1R blockade and Ang 1-7 supplementation paralleled changes in NADPH oxidase activity and blunted the hypertrophic response with attenuated activation of both ERK1/2 and Akt. The cardioprotection of AT1R blocker and Ang 1-7 is mediated by interruption of PKC-α–dependent NADPH oxidase activation, resulting in lowered activation of ERK1/2, Akt, glycogen synthase kinase 3β, and attenuation of cardiac hypertrophy. Biomechanical stress and Ang-II–induced generation of ROS are known to activate MMP2 in a p47phox–dependent manner.29,36 Reductions in pro- and active-MMP2 and -MMP9 levels by AT1R blockade and Ang 1-7 represent important mediators of the protective effects of these therapies on heart function.

Treatment with an AT1R blocker after coronary artery ligation demonstrated an increase in cardiac ACE2 mRNA levels and activity, suggesting that AT1R blocker can partially increase the ACE2 mRNA and formation of Ang 1-7.10 In our model, we demonstrated that AT1R blockade can afford cardioprotection independent of ACE2. Upregulation of the Mas receptor may also contribute toward the cardioprotective effects mediated by AT1R blocker, whereas also facilitating the action of Ang 1-7 in a positive feedback manner.16,17 Ang 1-7 attenuates the development of heart failure postmyocardial infarction14,15 and in response to pressure-overload.12,13 Ang 1-7 also antagonizes the Ang II–induced myocardial hypertrophy and fibrosis.18 Enhancing Ang 1-7 action using the Mas agonist AVE0991 reduces postmyocardial infarction remodeling and cardiac dysfunction.37 The beneficial effects of Ang 1-7 are not limited to cardiomyocytes and include important effects on cardiofibroblasts, such as antifibrotic and antihypertrophic effects associated with reduced ERK1/2 activity. 38,39 These data further suggest that hypertrophy may result from increased action of Ang II and/or loss of Ang 1-7 effects, and supplementation of Ang 1-7 may prove to be effective therapy for pressure-overload–induced heart failure, where the ACE2 system is known to be downregulated.6

Perspectives
The present study demonstrates that AT1R blocker and Ang 1-7 treatment provide comparable cardioprotection in pressure-overload–induced hypertrophy and heart failure in ACE2-null mice. Importantly, the doses of irbesartan and Ang 1-7 tested achieved similar improvement in the structural and functional phenotypes. Our study unveiled a marked
Figure 5. Irbesartan (IRB) and angiotensin (Ang) 1-7 suppresses oxidant stress, signaling, and remodeling induced by Ang II in ACE2-null cardiomyocytes and cardiofibroblasts isolated from pressure-overloaded hearts. Ang II increases oxidant stress, as evident by increased NADPH oxidase activity (A) and dihydroethidium (DHE) fluorescence (B), along with phosphorylation of ERK 1/2 (C) in isolated cardiomyocytes, which were lowered by IRB and Ang 1-7 pretreatment. In cardiofibroblasts, Ang II exposure stimulated mRNA expression of α-SMA (D) with increased phosphorylation of ERK1/2 (E), resulting in increased immunostaining for α-SMA (F), which was prevented by pretreatment with IRB and Ang 1-7 (D through F). n=4 from each group. A.U. indicates arbitrary unit; R.E., relative expression; ERK 1/2, extracellular signal-regulated kinase 1/2; α-SMA, α-smooth muscle actin. #P<0.05 vs all other groups.
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degree of redundancy in the Ang II/AT\textsubscript{R} and Ang 1-7/Mas receptor counterregulatory pathways in the absence of ACE2. Irbesartan is known to upregulate ACE2 mRNA and Mas receptor expression, which may contribute to the cardioprotection in several experimental models of cardiovascular disease. The Ang 1-7/Mas receptor axis plays a key role in cardioprotection against pressure-overload–induced hypertrophy and heart failure, where the ACE2 system is known to be downregulated. This suggests a possible role for Ang 1-7 in human heart failure, and Ang 1-7 can prove to be a potential therapeutic agent. However, redundancy of AT\textsubscript{R} blocker and Ang 1-7 treatment in a wild-type background is unknown and is an important consideration for the translational aspect of Ang 1-7 treatment.

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Disclosures

None.

References


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Cardioprotective effects mediated by AT1 receptor blockade and enhancing Ang 1-7 in experimental heart failure in ACE2-null mice

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Short Title: AT1R blockade and Ang 1-7 Action in Heart Failure

EXPANDED ONLINE METHODS

Experimental Animals and Protocols. Angiotensin converting enzyme 2 (ACE2) knockout mice (Ace2−/−, ACE2KO) were backcrossed into the C57BL/6 background for at least 8 generations as previously described.1-3 Mice were housed in pathogen-free conditions and had access to sterilized food and water ad libitum. All experiments were performed in accordance with 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), Institutional Guidelines and the Canadian Council on Animal Care. The placebo group was implanted with mini-osmotic pumps filled with saline and was comparable to mice given water ad libitum. All therapies were started at 3 days prior to pressure-overload and over the 2-week post-banding period. Systolic blood pressure was monitored in conscious mice using the tail-cuff methodology.1 The trans-stenotic pressure gradient was determined by echocardiography at 3 days following aortic banding and was found to be equivalent in all groups of mice (n=8): placebo (63.6±6 mmHg), irbesartan (59.3±8 mmHg) and angiotensin 1-7 (62.4±5 mmHg).

Echocardiography. Transthoracic echocardiography was performed and analyzed in a blinded manner as described previously using a Vevo 770 high-resolution imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, Canada).
Tail-Cuff Systolic Blood Pressure. For the measurement of tail-cuff systolic blood pressure (SBP), conscious mice were placed in the restrainers and their body temperature was maintained at ~ 34 °C by the warming chamber. The IITC tail cuff sensor containing both the inflation cuff and the photoelectric sensor was placed on the tail and attached to the restrainer. The cuff was inflated to a pressure of 200 mmHg and then deflated slowly. Upon reappearance of pulse signals, SBP data from the IITC amplifier was recorded, analyzed and reported by the IITC software (IITC Life Science Blood Pressure System, Woodland Hills, CA). The mice were trained on three occasions before actual recordings were made and the corresponding SBPs were averaged from three readings and used for the averaged comparisons.

Isolation and Culture of Adult Cardiomyocytes and Cardiofibroblasts. Adult murine left ventricular cardiomyocytes and cardiofibroblasts were isolated and cultured as previously described. Briefly, ACE2KO-2 Weeks TAC mice were injected with 0.05 ml of 1000USP/ml heparin for 15 min and then anesthetised using 2% isoflurane (1 L/min oxygen flow rate) provided through a nose cone. After opening the chest cavity, the heart was quickly excised and perfused using a Langendorff system within 45 s. Following 3-min perfusion, the heart was then digested with 2.4 mg/mL collagenase type 2 (Worthington) for 7-8 min. After sufficient digestion, the ventricles were removed, dissociated using forceps and transfer pipettes, and resuspended in stopping buffer (10% FBS perfusion buffer). The isolated cardiomyocytes were then exposed to increasing calcium concentrations (100 μM, 400 μM, and 900 μM) for 15 min each before being plated onto laminin coated culture dishes in plating buffer (Eagle’s MEM with 10% FBS, Sigma) and placed at 37 °C in a sterile 2% CO₂ incubator. The discarded stopping buffer was set aside for cardiofibroblasts collection. One hour after plating, the plating buffer was gently aspirated and replaced with culture buffer (serum free Eagle’s MEM with 0.1% BSA) and then placed into the incubator for 18 h before treatment. The discarded stopping buffer is centrifuged at 20 g for 3 min and the resulting supernatant was collected in a 15 mL conical tube. This was then centrifuged at 1500 rpm for 5 min and the pellet was collected and washed in 10% FBS DMEM (GIBCO). The solution is once again centrifuged at 1500 rpm for 5 min and the pellet was washed in 10% FBS DMEM. The cardiofibroblasts were then passaged 2 times and put into 24 h serum free DMEM prior to treatment. IRB (1 µM) and ANG 1-7 (Bachem, Germany; 100 nM) were added to the cardiomyocytes and cardiofibroblasts for 20 min prior to exposure of Ang II (Sigma; 100 nM); Ang II exposure duration was 30 min except for mRNA expression study and immunofluorescence in cardiofibroblasts, where it was 24 hours. Treated cardiomyocytes were used for dihydroethidium (DHE) staining or collected for western blotting analysis or lucigenin-enhanced chemiluminescence assay. Cardiofibroblasts exposed to Ang II (alone or along with IRB or ANG 1-7) for 30 minutes were used for western blotting, whereas cardiofibroblasts exposed for 24 hours were used for mRNA expression analysis and alpha-smooth muscle actin, vimentin double immunofluorescence staining.

Histology. For heart morphometry, hearts were arrested with 1M KCl, perfuse-fixed with buffered 10% formalin, and embedded in paraffin. Masson Trichrome stained sections were used to study cardiac morphology and hypertrophy. Picro-sirius red (PSR) staining and visualization were carried out as previously described and myocyte cross sectional area was determined using the Olympus IX81 and MetaMorph Software.
TaQMan Real-time PCR. For various genes, RNA expression levels were determined by TaqMan Real-time PCR as previously described.\textsuperscript{2,4} Total RNA was extracted from flash-frozen kidney tissue using TRIzol reagent, and cDNA was synthesized from 1 µg RNA by using random hexamers. For each gene, a standard curve was generated using known concentrations of cDNA (0.625, 1.25, 2.5, 5, 10 and 20 µg) as a function of cycle threshold (CT). Expression analysis of the reported genes was performed by TaqMan Real-time PCR using ABI 7900 Sequence Detection System. The SDS2.2 software (integral to ABI7900 real-time machine) fits the CT values for the experimental samples and generates values for cDNA levels. All samples were run in triplicates in 384 well plates. 18S rRNA was used as an endogenous control.

Lucigenin-Enhanced Chemiluminescence. The activities of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in heart tissues of mice were quantified by lucigenin-enhanced chemiluminescence as previously described.\textsuperscript{3,4} The heart homogenates (200 µg total proteins) were collected in 100 µl of phosphate buffer solution (PBS) mixture with protease inhibitor (Calbiochem, San Diego, USA) and phosphatase inhibitor cocktails (Sigma-Aldrich, Oakville, Canada) and then centrifuged at 1000 g for 10 min. The supernatants were then collected and added NADPH (1 mM) and lucigenin (50 µM) for NADPH oxidase activities assay with FB-12 luminometer, modified to maintain the temperature at 37 °C. The specific peptide inhibitor of NADPH oxidase, gp91phox ds tat (50 µM), was used to confirm superoxide generation from NADPH oxidase. Data were calculated as the change in the rate of luminescence per minute per milligram of tissue.

Dihydroethidium Fluorescence. We used oxidative fluorescent dye dihydroethidium (DHE) to measure superoxide (O$_2^-$) levels in heart tissues from ACE2KO mice as previously described.\textsuperscript{2,4} For kidney samples, 20 µm fresh frozen tissue sections were washed with hanks balanced salt solution (HBSS) with magnesium and calcium and then incubated at 37 °C for 30 min with DHE (20 µM) in HBSS. For a separated experiment, kidney tissue sections from mice with Ang II pumps were incubated with polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) (500U/mL) at 37 °C for 30 min prior to 30-min exposure of DHE (20 µM). The tissue slides were wrapped with foil to minimize them exposure to light. Fluorescent images were observed with an Olympus Fluoview laser-scanning confocal microscope mounted on an Olympus microscope selected with CY3 (red) channel. One tissue slide was kept without DHE for blank control. Fluorescence was quantified by the ImageJ software (U.S. National Institutes of Health, Bethesda, MD).

Western Blot Analysis. Western blot analysis were carried out as previously described.\textsuperscript{1} Total protein was extracted from frozen heart tissue by homogenization in EDTA-free RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP40, 0.1% SDS including protease inhibitor (Calbiochem, San Diego, USA) and phosphatase inhibitor cocktails (Sigma-Aldrich, Oakville, Canada) and quantified using the BCA Protein Array Kit (Pierce, Rockford, IL, USA). Protein samples were separated by 8%–12% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane (Millipore). The membrane was blocked with 5% milk in Tris-Buffered Saline Tween-20 (TBST) for 2 h and then incubated overnight at 4 °C with primary antibody against PKCα (80 kDa), PKCβ1 (79 kDa), ACE2 (90 kDa), β-actin (45 kDa) and total and phosphorylated ERK1 (44 kDa) and ERK2 (42 kDa) (Santa Cruz and Cell Signaling Inc.) as previously described.\textsuperscript{1} After washing 3 times in TBST buffer, the membrane was then incubated
with an secondary antibody at a 1:5000 dilution in TBST for 2 h at room temperature, then washed 3 times with TBST for 15 min each.

**Immunoprecipitation.** Briefly, 200 μg protein was subjected to immunoprecipitation with polyclonal rabbit anti-p47\textsuperscript{phox} and protein A sepharose for 16 h at 4°C. Immunoprecipitates were analyzed by Western blot with anti-phosphoserine antibody as well as p47\textsuperscript{phox} using a second monoclonal anti-p47 antibody (p47\textsuperscript{phox} (A-7):sc-17844). The relative densities of phospho-p47\textsuperscript{phox} to total p47\textsuperscript{phox} were compared.

**Immunofluorescence.** After treatment, the cardiofibroblasts were washed several times with PBS and fixed with 4% formaldehyde for 20 minutes. Cardiofibroblasts were then permeabilized with 0.25% Triton-X100 in PBS for 5 minutes, followed by incubation with 1% bovine serum albumin. They were then incubated with mixture of primary antibodies against alpha-smooth muscle actin (1:100) and vimentin (1:1000) overnight at 4°C (Abcam). Cells were incubated with mixture of different fluorophore conjugated secondary antibodies for 1 hour at 37°C (Invitrogen). The cardiofibroblasts were visualized under fluorescence microscope (IX81, Olympus) after mounting with ProlongGold antifade mounting medium with DAPI (Invitrogen). The cells were washed with PBS, three times for 5 minutes each, in between each steps.

**References**

SUPPLEMENTAL TABLE

Table S1. Echocardiographic and hemodynamic data in ACE2KO mice subjected to pressure overload.

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<thead>
<tr>
<th>Parameter</th>
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<td>N</td>
<td>HR (bpm)</td>
<td>LVEDD (mm)</td>
<td>LVFS (%)</td>
</tr>
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<td>494±12</td>
<td>512±15</td>
<td>504±9</td>
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<tr>
<td></td>
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<td>512±15</td>
<td>4.88±0.19</td>
<td>3.65±0.10</td>
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<td>32.3±1.70</td>
<td>16.3±2.19*</td>
<td>29.8±2.69</td>
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<td>0.60±0.05</td>
<td>0.79±0.08*</td>
<td>0.62±0.0.06</td>
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<td>4.98±2.51</td>
<td>17.89±4.99*</td>
<td>7.50±3.03</td>
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<td>10998±111.3</td>
<td>6002±59.0*</td>
<td>9007±91.7</td>
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<td>10002±98.4</td>
<td>4998±52.4*</td>
<td>8246±85.0</td>
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Sham=sham-operated; AB=aortic banding; IRB=irbesartan, ANG 1-7=Angiotensin 1-7; HR=heart rate; LVEDD=Left Ventricular End Diastolic Diameter; LVFS=LV Fractional Shortening; LVPWT=LV Posterior Wall Thickness; LVEDP=LV End Diastolic Pressure; +dp/dt\text{max}=maximum first derivative of the LV pressure; –dP/dt\text{min}=minimum first derivative of the LV pressure. *p<0.05 compared with all other groups.
Figure S1. Tail cuff systolic blood pressure (TC-SBP) in conscious aortic banded (AB) ACE2 knockout mice at baseline, 1 week and 2 weeks (A) and invasive mean proximal aortic blood pressure at 2 weeks post-AB (B) in response to irbesartan (IRB) and angiotensin 1-7 (ANG 1-7). n=8 per group; *p<0.05 compared with IRB and ANG 1-7; *p<0.05 compared with ACE2KO Sham group.
**Figure S2.** Plasma (A) and myocardial (B) angiotensin 1-7 (ANG 1-7) levels in ACE2 knockout mice in response to pressure-overload and the impact of AT1 receptor blockade using irbesartan (IRB) and ANG 1-7 supplementation. Data are mean ± SEM; n=10 per group. *p<0.05 compared with placebo; #p<0.05 compared with IRB group.