Loss of Angiotensin-Converting Enzyme 2 Accelerates Maladaptive Left Ventricular Remodeling in Response to Myocardial Infarction

Zamaneh Kassiri, PhD; Jiuchang Zhong, MD; Danny Guo, BSc; Ratnadeep Basu, MD; Xiuhua Wang, PhD; Peter P. Liu, MD; James W. Scholey, MD; Josef M. Penninger, MD; Gavin Y. Oudit, MD, PhD

Background—Angiotensin-converting enzyme 2 (ACE2) is a monocarboxypeptidase that metabolizes Ang II into Ang 1-7, thereby functioning as a negative regulator of the renin-angiotensin system. We hypothesized that ACE2 deficiency may compromise the cardiac response to myocardial infarction (MI).

Methods and Results—In response to MI (induced by left anterior descending artery ligation), there was a persistent increase in ACE2 protein in the infarct zone in wild-type mice, whereas loss of ACE2 enhanced the susceptibility to MI, with increased mortality, infarct expansion, and adverse ventricular remodeling characterized by ventricular dilation and systolic dysfunction. In ACE2-deficient hearts, elevated myocardial levels of Ang II and decreased levels of Ang 1-7 in the infarct-related zone was associated with increased production of reactive oxygen species. ACE2 deficiency leads to increased matrix metalloproteinase (MMP) 2 and MMP9 levels with MMP2 activation in the infarct and peri-infarct regions, as well as increased gelatinase activity leading to a disrupted extracellular matrix structure after MI. Loss of ACE2 also leads to increased neutrophilic infiltration in the infarct and peri-infarct regions, resulting in upregulation of inflammatory cytokines, interferon-γ, interleukin-6, and the chemokine, monocyte chemotactant protein-1, as well as increased phosphorylation of ERK1/2 and JNK1/2 signaling pathways. Treatment of Ace2−/−MI mice with irbesartan, an AT1 receptor blocker, reduced nicotinamide-adenine dinucleotide phosphate oxidase activity, infarct size, MMP activation, and myocardial inflammation, ultimately resulting in improved post-MI ventricular function.

Conclusions—We conclude that loss of ACE2 facilitates adverse post-MI ventricular remodeling by potentiation of Ang II effects by means of the AT1 receptors, and supplementing ACE2 can be a potential therapy for ischemic heart disease. (Circ Heart Fail. 2009;2:446-455.)

Key Words: myocardial infarction ■ angiotensin ■ heart failure ■ molecular biology ■ angiotensin II

The renin-angiotensin system (RAS) plays a critical role in the pathophysiology of heart failure and many of the key proven pharmacotherapies used in patients with heart failure including angiotensin-converting enzyme (ACE) inhibitors, angiotensin and aldosterone receptor blockers are antagonists of the RAS.1 ACE2 function as a monocarboxypeptidase and is an important negative regulator of the RAS.2–4 Loss of ACE2 enhances lung injury,5 diabetic renal injury,6 and ventricular dilation in response to biomechanical stress7 due in part to increased Ang II-mediated stimulation of AT1 receptors. In the heart, ACE2 is the primary pathway for the metabolism of Ang II and the subsequent formation of Ang 1-7, a peptide known to antagonize the Ang II effects.8–10 Several key pharmacotherapeutic agents with proven efficacy in the treatment of human heart failure including ACE inhibitors, angiotensin receptor and aldosterone receptor blockers, enhance ACE2 activity and/or expression.11,12 Moreover, polymorphisms in the ACE2 gene are linked to the development of pathological myocardial hypertrophy and heart disease in humans.13,14

Clinical Perspective on p 455

To clarify the potential role of ACE2 in cardiac remodeling in response to myocardial infarction (MI), we randomized ACE2 knockout (Ace2−/−) and wild-type littermates (Ace2+/+) mice to either left anterior descending coronary artery ligation or sham surgery. The results demonstrate that loss of ACE2 is associated with increased mortality, infarct expan-
version with adverse ventricular remodeling in a left anterior descending artery (LAD)-ligation model of MI. These changes are associated with greater oxidative stress, matrix metalloproteinase (MMP) activation and inflammation ultimately resulting in adverse early and impaired ventricular remodeling after MI, which are critically dependent on Ang II-mediated stimulation of the AT1 receptors.

**Methods**

**Experimental Animals and Protocols**

Ace2−/− mice have been described previously15,16 and have been backcrossed into a pure C57BL/6 background for 10 generations.16 Only male ACE2-deficient (Ace2−/−) and littermate wild-type (Ace2+/+) mice were used for all experiments. Mice were treated with irbesartan in their drinking water (50 mg/kg/d) for 3 days before MI and during the course of the study. Irbesartan was kindly provided by Bristol-Myers Squibb (Princeton, NJ). All experiments confirmed with Institutional Guidelines and the Canadian Council on Animal Care.

**Myocardial Infarction**

Ten-week-old male mice of either genotype were subjected to MI by ligation of the proximal LAD. Anesthetized and intubated mice underwent left thoracotomy in the fifth intercostal space. The pericardium was opened to expose the left ventricle (LV) and the LAD was encircled and ligated under the tip of the left atrial appendage. Once the LAD ligation was complete, the muscle and skin was closed in layers with use of a 6-0 silk suture. Sham-operated mice were used as baseline controls. Animals were inspected at least 3 times daily for the first 7 postsurgical days. Post-MI mice were anesthetized, hearts were quickly excised, rinsed with ice-cold phosphate-buffered saline, and dissected into infarct, peri-infarct, and noninfarct zones, then flash-frozen separately in liquid nitrogen.

**Autopsy and Infarct Size Measurement**

Autopsy was performed on each mouse found dead throughout the course of the study. Cardiac rupture was confirmed by the presence of a large amount of clotted blood in the chest cavity or in the pericardium. Infarct size was measured at 1 day and 1 week post-MI by perfusing the excised hearts at a constant pressure (60 mm Hg) first with PBS to clear out the blood, and then stained with 1% triphenyl tetrazolium chloride (Sigma, Canada). Hearts were then sectioned in 1-mm slices from apex to the base, and the infarct area was measured using Image Proplus software, and reported as a percentage of the total LV size.

**Echocardiographic Measurements**

For the echocardiographic measurements, mice were lightly anesthetized with isoflurane (1%)/oxygen, and in vivo cardiac function was measured by echocardiography, as described previously.15–17

**TaqMan Real-Time Polymerase Chain Reaction, Western Blot Analysis, Gelatin Zymography, and Gelatinase Activity**

RNA expression levels of various genes were determined by TaqMan real-time polymerase chain reaction as described previously using 18S rRNA as the internal standard.16,17 The primers and probes for Ace2 mRNA expression analysis were based on previously published reports.6,18 Western blot analysis was used to detect phosphorylated and total mitogen-activated protein kinase (MAPK) proteins, ERK1/2, JNK1/2, and p38 using specific antibodies (Cell Signaling Inc., Danvers, Mass).16,19 Pro and cleaved forms of MMP2 and MMP9 were detected by gelatin zymography, and total gelatinase activity was measured using Image Proplus software, and reported as a percentage of the total LV size.
nase activity was measured in myocardial homogenates using a fluorescence-based EnzChek assay kit as before.17

Peptide Measurement
Ang II and Ang 1-7 levels were measured by radioimmunoassay in the Hypertension and Vascular Disease Centre Core Laboratory at Wake Forest University School of Medicine, Winston-Salem, NC, as described previously.15 The infarct and noninfarct regions from 3 hearts were pooled to generate adequate myocardial tissue for a single measurement of Ang II and Ang 1-7; 10 pooled samples were each assayed from the Ace2+/y and Ace2−/y groups.

Measurement of NADPH Oxidase Activity and Superoxide Production
The lucigenin chemiluminescence assay was used to measure reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity using a single-tube luminometer (Berthold FB12, Berthold Technologies, Germany) at 37°C, as described previously.16,20,21 Lucigenin (5 μmol/L) and NADPH (100 μmol/L) were added to the samples and light emission was recorded every 2 minutes during an 8-minute period. The superoxide scavenger, polyethylene glycol-superoxide dismutase (650 U/mL) was used to confirm superoxide generation. To evaluate the production of tissue reactive oxygen species (ROS), freshly frozen left ventricular myocardium was used within 2 to 3 hours and 10-μm slices was incubated for 1 hour at 37°C with dihydroethidium (2 mol/L; Molecular Probes, ●●●) and 5-(6)-chloromethyl-2′,7′-dichlorodihydro-drofluorescein diacetate (4 μmol/L; Molecular Probes), as described previously.22 Negative control sections were preincubated with the superoxide scavenger, titron (10 mmol/L).

Histology and Immunohistochemistry
Hearts were harvested in diastole with 1 mol/L KCl, perfuse fixed with buffered 10% formalin for 20 minutes at 60 mm Hg, followed by incubation in 10% formalin for 48 hours, and then stored in 80% ethanol. Picro-Sirius Red (PSR) and neutrophil staining were carried out, as described previously.16,22 Positive neutrophil pixel counts per frame were determined from 5 frames in 3 separate sections from each heart.

Statistical Analysis
All data are shown as mean±SEM. All statistical analyses were performed using SPSS software (version 10.1, SPSS Inc, Chicago, Ill). Survival data were analyzed using the Kaplan-Meier method, and the log rank test was used to test for statistical significance in the survival distribution. The effects of genotype and irbesartan were evaluated using ANOVA followed by the Student Neuman-Keuls test for multiple comparison testing and comparison between 2 groups.

Results
Spatial and Temporal Changes in Ace2 mRNA Expression and ACE2 Protein Levels
In wild-type mice, Ace2 mRNA levels are significantly reduced at 3 days, 1 week, and 4 weeks after MI in the infarct, peri-, and noninfarct zones compared with sham-operated mice (Figure 1A). In contrast, Western blot analysis revealed a marked increase in ACE2 protein levels in the infarct-related zone at 3 days (Figure 1B), and after a transient decrease at 1 week was significantly increased at 4 weeks post-MI (Figure 1C and 1D). These results indicate that myocardial ACE2 protein increases in a region and time-dependent manner after MI by means of a posttranscriptional mechanism.

Loss of ACE2 Enhances Susceptibility to Myocardial Infarction
To examine the role of ACE2 in MI, we subjected Ace2−/y mice to MI. The mortality rate in Ace2−/y mice was significantly greater post-MI compared with wild-type mice (Figure 2A; n=180) versus 36.3±2.4% (Ace2−/y) versus 38.5±3.6% (Ace2−/y); n=8; P=0.311), whereas at 1 week post-MI, there was clear evidence for greater infarct expansion in infarcted Ace2−/y hearts (Figure 2C). To examine whether ACE2 deficiency also affects cardiac function
post-MI, we performed transthoracic echocardiography. Although sham mice from either genotypes had comparable heart function, Ace2−/−-MI hearts exhibited significantly greater reductions in fractional shortening and more severe LV dilation compared with Ace2+/− MI (Figure 2D, Table). In Ace2-deficient hearts, the early adverse remodeling persisted at 8 weeks after MI with increased LV end-diastolic dimension and worsening systolic performance (Table). These data show clearly that loss of ACE2 leads to increased mortality, infarct expansion, and greater adverse post-MI ventricular remodeling.

Loss of ACE2 Increases Ang II Levels and Superoxide Production

In vitro experiments have shown that ACE2 can metabolize Ang II into Ang 1-7.8,23 Hence, we measured angiotensin peptide levels in the infarct and noninfarct regions to assess the impact of ACE2 deficiency on peptide levels. We found that Ang II levels are significantly elevated, whereas Ang 1-7 levels are markedly reduced in the Ace2−/− myocardium (Figure 3A). In contrast, these peptide levels did not change appreciably in the noninfarct regions of either genotype. Ang II is a key activator of ROS (Figure 3B). Polyethylene glycol-superoxide dismutase normalized superoxide production in ACE2-deficient hearts is predominantly because of Ang II-mediated activation of the NADPH oxidase system.

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HR indicates heart rate; LVEDD, LV end diastolic dimension; LVESD, LV end systolic dimension; FS, fractional shortening; VCFc, velocity for circumferential shortening corrected for HR.

*P<0.01 compared with corresponding sham-operated group.
†P<0.05 compared with Ace2−/−+ MI group.

Increased MMP Activation, Inflammation, and Activation of MAPK in ACE2-Deficient Hearts

Ang II-induced ROS can activate a number of MMP, which can lead to adverse ventricular remodeling.25,26 Gelatin zymography shows markedly increased MMP2 and MMP9 levels at 3 days and 1 week in the infarct and peri-infarct regions in ACE2-deficient hearts (Figure 4). Increased total MMP activity was confirmed independently by a fluorescent-based gelatinase activity assay, which showed substantially greater increases in the Ace2−/− infarct-related region (Figure 4B). Although we have not provided direct evidence for collagen degradation, this increase in MMP activities provides strong evidence for increased degradation of the extracellular matrix in the Ace2−/−-MI heart as assessed by picrosirius red staining and confocal microscopy (Figure 4C).

 Activation of the RAS has also been linked to inflammation, another key determinant of adverse post-MI ventricular remodeling.27 Neutrophil-specific staining showed greater neutrophil infiltration in the infarct and peri-infarct regions of the Ace2−/− myocardium (Figure 5A) at 3 days post-MI, which was quantified using the positive pixel count of neutrophil staining (5.03×104±356 [Ace2−/−] versus...
1.54×10^5±395 [Ace2^-y]; n=4; P<0.05). The increased neutrophil infiltration was associated with significantly higher expression of several inflammatory cytokines including interleukin-6, interferon-γ, and monocyte chemoattractant protein-1 (Figure 5B), but not tumor necrosis factor-α (data not shown), in ACE2-deficient hearts. The activation of MAPK signaling pathways mediates maladaptive responses in the heart and is characteristic of adverse ventricular remodeling.27,28 Increased ERK1/2 and JNK1/2 phosphorylation in the infarct and peri-infarct regions is consistent with
increased Ang II levels, oxidative damage, and inflammation in ACE2-deficient hearts (Figure 5C).

**AT1 Receptor Blockade Rescues Ace2<sup>+/y</sup>-MI Hearts**

Ang II is known to mediate its pathological effects by means of the activation of AT1 receptors. To test this hypothesis, we treated Ace2<sup>+/y</sup> mice with the specific AT1 receptor blocker, irbesartan. Blockade of AT1 receptors prevented the increased NADPH oxidase activity and infarct size (Figure 6A) and the excess ventricular dilation and deterioration in systolic function (Figure 6B) in ACE2-deficient hearts at 1 week post-MI. These beneficial effects of irbesartan treatment on the ventricular function of Ace2<sup>+/y-MI</sup> hearts was associated with normalization of the increased MMP levels (Figure 6C) and prevention of the increase in proinflammatory cytokines, interleukin-6, and interferon-γ, without affecting the expression of monocyte chemoattractant protein-1 (Figure 6D). Collectively, our data demonstrate that loss of ACE2 leads to an AT1 receptor-mediated increase in NADPH oxidase activity, MMP activation and increased inflammation ultimately resulting in adverse ventricular remodeling and heart disease.

**Discussion**

The monocarboxypeptidase, ACE2, shuttles angiotensin metabolism away from the formation of Ang II leading to the formation of Ang 1-7 thereby functioning essentially as a negative regulator of the RAS. In human heart failure, there is an upregulation of ACE2<sup>+/y</sup> whereas increased serum ACE2 activity is associated with worsening LV systolic function and worsening symptoms. In this study, we define a unique and important role of ACE2 in the cardiac response to MI. We demonstrate that in the absence of ACE2, MI results in enhanced activation of the RAS resulting in increased mortality driven in part by increased free LV wall...
rupture, and adverse ventricular remodeling characterized by infarct expansion, greater ventricular dilation, and reduced systolic performance. These changes are linked to increased Ang II levels in the setting of reduced Ang 1-7 levels, enhanced activation of NADPH oxidase and increased MMP activities and myocardial inflammation. The ability of Ang II to mediate adverse ventricular remodeling in ACE2-deficient hearts is likely facilitated by reduced Ang 1-7 levels because Ang 1-7 is a physiological antagonist of Ang II signaling and has been shown to mediate important cardioprotective effects. Our study supports an in vivo role of ACE2 in the generation of myocardial Ang 1-7 and validates aggregate biochemical evidence showing that ACE2 cleaves Ang II into Ang 1-7.

Figure 5. Increased inflammation and activation of MAPK signaling in ACE2-deficient hearts at 3 days post-MI. A, Increased neutrophilic infiltration in the infarct and per-infarct regions as shown using rat antineutrophil antibody staining in the Ace2−/− hearts following 1 week of MI. B, Increased expression of proinflammatory cytokines, interleukin-6, interferon-γ, and monocyte chemoattractant protein-1 following MI in ACE2-deficient hearts (n=6 for all groups). *P<0.05 compared with the sham group; #P<0.05 compared with the Ace2+/+ group. C, Western blot analysis of MAPK pathways with quantification shown on the right illustrating increased phosphorylation of ERK1/2 and JNK1/2 predominantly in the infarct regions in Ace2−/− hearts (n=4 for each group). *P<0.05 compared with Ace2+/+ group.

There is marked discrepancy in the reported changes in Ace2 mRNA levels in post-MI rats with either no change or a marked increase being reported in the noninfarcted myocardium. In control wild type mice, we observed a marked initial decrease in Ace2 mRNA expression in the infarct-related zones at 1 day post-MI, which is consistent with observations in rats. In contrast, we do not observe a subsequent increase in Ace2 mRNA in the infarct zone, which may relate to species-related differences in the transcriptional control of Ace2. Increased ACE2 protein levels in the infarct region occurs in the setting of reduced Ace2 mRNA levels suggesting that myocardial mRNA and protein can be uncoupled possibly through posttranscriptional regulation, such as proteolytic processing by ADAM-17/TACE.
In ACE2-deficient hearts, increased myocardial Ang II levels in the infarct region was clearly associated with increased NADPH oxidase activity and enhanced formation of ROS, which are consistent with findings in the age-dependent cardiomyopathy4,15 and pressure-overload induced heart failure7 in ACE2-deficient mice. Ang II-mediated activation of the NADPH oxidase complex by means of AT1 receptor in cardiomyocytes, vascular, and endothelial cells results in the formation of ROS,20,21,37,38 facilitates the negative inotropic effects of Ang II in heart failure39,40 and plays a key role in the adverse ventricular remodeling after MI.41,42 Consistent with the increased oxidative stress in the myocardium, loss of ACE2 also leads to Ang II-dependent renal oxidative damage and glomerular injury in aged mice.43 ACE2 deficiency leads to increased pro-MMP2 and MMP9 levels and activation of MMP2 in the infarct and peri-infarct regions in response to MI. However, we cannot rule out contribution from other MMPs such as neutrophil-specific MMP8, macrophage-specific MMP12, and MMP14 (MT1-MMP). Ang II-mediated NADPH oxidase activation is also a
major stimulus of the myocardial MMP system because of increased synthesis and/or activation of MMPs leading to degradation of the extracellular matrix. \(^{26,44}\) Indeed, MMP2 is known to contain a redox-sensitive catalytic center, which confers susceptibility to increased ROS formation leading to its activation. \(^{45}\)

We observed a marked increase in Ang II-dependent infiltration of neutrophils into the myocardium in ACE2-deficient hearts, which may perpetuates the free radical-induced myocardial injury \(^{46,47}\) and degradation of the extracellular matrix thereby compromising systolic function. \(^{17}\)

Activation of the MAPK pathway is a characteristic finding in heart disease and plays a key role in progression to heart failure. \(^{28}\) Indeed, increased phosphorylation of ERK1/2 and JNK1/2 pathways in the infarct zone of Ace2 \(^{-/-}\) mice is consistent with increased action of Ang II, oxidative stress, and inflammation. The AT1 receptor blocker, irbesartan, prevented the adverse ventricular remodeling in ACE2-deficient mice, reduced NADPH oxidase activity, MMP activation, and curtailed myocardial inflammation proving that Ang II mediated activation of the AT1 receptor is a key pathophysiological event in the post-MI ACE2-deficient heart.

In summary, this study shows that absence of ACE2 leads to an amplification of Ang II-mediated stimulation of AT1 receptors resulting in increased mortality and infarct size expansion, adverse ventricular remodeling and greater systolic dysfunction after MI. Collectively, these results support the notion that ACE2 is a negative regulator of the RAS. Enhancing ACE2 activity may serve to minimize the adverse post-MI ventricular remodeling as recently shown in rats. \(^{32}\)

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Disclosures

Dr Oudit is a Clinician-Investigator Scholar of the Alberta Heritage Foundation for Medical Research, and Dr Kassiri is a New Investigator of the Heart and Stroke Foundation of Canada.

References


Coronary artery disease is now the most common cause of heart failure in Westernized nations. Angiotensin-converting enzyme 2 (ACE2) functions as a membrane-bound carboxypeptidase and directly metabolizes Ang II to generate Ang 1-7. Ang 1-7 functions essentially as a physiological antagonist of Ang II signaling in the cardiovascular system. Our study shows that mice lacking the ACE2 gene had greater mortality and infarct expansion after left anterior descending artery ligation. These were linked to increase Ang II and lower Ang 1-7 myocardial levels resulting in increased reduced nicotinamide-adenine dinucleotide phosphate oxidase activity, inflammation, activation of the matrix metalloproteinases and resultant degradation of the extracellular matrix. Collectively, these changes in ACE2-deficient mice were associated with greater ventricular dilation and reduction in systolic function after myocardial infarction. Blockade of the AT1 receptors prevented the adverse biochemical and functional changes seen in the ACE2 knockout mice. Our results identify ACE2 as a potential new target in myocardial infarction and suggest that strategies to enhance ACE2 expression/activity can minimize the characteristic adverse ventricular remodeling after myocardial infarction. ACE inhibitors, angiotensin II, and aldosterone receptor blockers have all been shown to increase myocardial ACE2 expression and/or activity, which may partially account for their therapeutic benefits in patients with heart disease.
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