

# Deletion of Angiotensin-Converting Enzyme 2 Accelerates Pressure Overload–Induced Cardiac Dysfunction by Increasing Local Angiotensin II

Koichi Yamamoto, Mitsuru Ohishi, Tomohiro Katsuya, Norihisa Ito, Masashi Ikushima, Masaharu Kaibe, Yuji Tatara, Atsushi Shiota, Sumio Sugano, Satoshi Takeda, Hiromi Rakugi, Toshio Ogihara

**Abstract**—Angiotensin-converting enzyme 2 (ACE2) is a carboxypeptidase that cleaves angiotensin II to angiotensin 1-7. Recently, it was reported that mice lacking ACE2 ( $ACE2^{-/-}$  mice) exhibited reduced cardiac contractility. Because mechanical pressure overload activates the cardiac renin–angiotensin system, we used  $ACE2^{-/-}$  mice to analyze the role of ACE2 in the response to pressure overload. Twelve-week-old  $ACE2^{-/-}$  mice and wild-type (WT) mice received transverse aortic constriction (TAC) or sham operation. Sham-operated  $ACE2^{-/-}$  mice exhibited normal cardiac function and had morphologically normal hearts. In response to TAC,  $ACE2^{-/-}$  mice developed cardiac hypertrophy and dilatation. Furthermore, their hearts displayed decreased cardiac contractility and increased fetal cardiac gene induction, compared with WT mice. In response to chronic pressure overload,  $ACE2^{-/-}$  mice developed pulmonary congestion and increased incidence of cardiac death compared with WT mice. On a biochemical level, cardiac angiotensin II concentration and activity of mitogen-activated protein (MAP) kinases were markedly increased in  $ACE2^{-/-}$  mice in response to TAC. Administration of candesartan, an AT<sub>1</sub> subtype angiotensin receptor blocker, attenuated the hypertrophic response and suppressed the activation of MAP kinases in  $ACE2^{-/-}$  mice. Activation of MAP kinases in response to angiotensin II was greater in cardiomyocytes isolated from  $ACE2^{-/-}$  mice than in those isolated from WT mice. ACE2 plays an important role in dampening the hypertrophic response to pressure overload mediated by angiotensin II. Disruption of this regulatory function may accelerate cardiac hypertrophy and shorten the transition period from compensated hypertrophy to cardiac failure. (*Hypertension*. 2006;47:718-726.)

**Key Words:** hypertrophy ■ remodeling ■ angiotensin antagonist ■ receptors, angiotensin

Angiotensin converting enzyme 2 (ACE2) is the first known homologue of ACE.<sup>1,2</sup> Although ACE2 cleaves a single residue from angiotensin I, generating angiotensin 1-9, and a single residue from angiotensin II (Ang II) to generate angiotensin 1-7,<sup>1,2</sup> the catalysis efficiency of Ang II to angiotensin 1-7 by ACE2 is much higher than that of angiotensin I to angiotensin 1-9.<sup>3</sup> Crackower et al<sup>4</sup> reported that 3-month-old mice lacking ACE2 ( $ACE2^{-/-}$  mice) had decreased cardiac contractility and exhibited left ventricle (LV) dilatation, in addition to elevated plasma and cardiac concentrations of Ang II. Another group reported that ACE2 increased angiotensin 1-7 concentrations in failing human heart ventricles.<sup>5</sup> These studies suggest that ACE2 might protect against heart failure by decreasing Ang II concentration. However, the relationship between increased Ang II concentration and impaired heart function in  $ACE2^{-/-}$  mice is still unknown, because no structural or biochemical changes have been observed in the hearts of  $ACE2^{-/-}$  mice.<sup>4</sup>

Pressure overload and humoral factors are the primary signals driving the development of cardiac hypertrophy and dysfunction. Moreover, pressure overload activates a wide variety of humoral factors in cardiac tissue, including Ang II,<sup>6</sup> endothelin-1,<sup>7</sup> and several peptide growth factors.<sup>8</sup> Locally produced Ang II, perhaps more than circulating Ang II, is a potent stimulator of cardiac hypertrophy, and hemodynamic overload induces cardiac hypertrophy by activating the cardiac renin–angiotensin system.<sup>6,9</sup> During cardiac hypertrophy, cardiac synthesis of Ang II increases because of the upregulation of cardiac angiotensinogen and ACE gene transcription.<sup>10,11</sup> ACE inhibitors and angiotensin receptor blockers (ARB) improve pressure-overload–induced cardiac hypertrophy without decreasing arterial pressure.<sup>11–13</sup> Ang II directly induces hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts without increasing vascular resistance or cardiac afterload.<sup>6,9</sup> In this study, we induced

Received October 7, 2005; first decision November 16, 2005; revision accepted January 18, 2006.

From the Department of Geriatric Medicine (T.K., N.I., M.I., M.K., Y.T., A.S., H.R., T.O.), Osaka University Graduate School of Medicine, Suita; Institute of Medical Science (S.S.), University of Tokyo; and the Otsuka GEN Research Institute (S.T.), Otsuka Pharmaceutical Co. Ltd, Tokushima, Japan.

Correspondence to Hiromi Rakugi, Department of Geriatric Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita 565-0871, Japan. E-mail rakugi@geriat.med.osaka-u.ac.jp

© 2006 American Heart Association, Inc.

*Hypertension* is available at <http://www.hypertensionaha.org>

DOI: 10.1161/01.HYP.0000205833.89478.5b

pressure overload in hearts of *ACE2*<sup>-/-</sup> mice by transverse aortic constriction (TAC) to assess whether the deletion of the ACE2 gene might enhance the influence of Ang II on the development of cardiac hypertrophy and dysfunction.

## Methods

### Targeted Disruption of the ACE2 Gene

An expanded Methods section is available in an online supplement available at <http://www.hypertensionaha.org>.

### Pressure Overload by TAC

Twelve-week-old male *ACE2*<sup>-/-</sup> mice and their wild-type (WT) littermates (*ACE2*<sup>+/+</sup>) were divided into 3 groups: sham-operation, TAC, and TAC during candesartan treatment. We performed TAC as described previously, by using a 27-gauge needle.<sup>14</sup> Treatment with candesartan was administered in drinking water initiated 1 day before operation and continued for 2 weeks (1 mg/kg per day). Fourteen days after the operation, surviving animals underwent transthoracic echocardiography and cardiac catheterization to determine cardiac function and proximal aortic pressure. After these experiments, the heart and lungs were excised, weighed, and subjected to histological analysis. The study protocol was approved by the Osaka University Institutional Animal Care and Use Committee and was conducted in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Cell Culture

Neonatal mouse ventricular myocytes were isolated as described previously.<sup>15</sup> Cardiomyocytes were cultured in M199 (Sigma) supplemented with 10% FBS (Equitech-Bio Inc). After 72 hours, the culture medium was changed to M199 without FBS; 24 hours later, cardiomyocytes were stimulated with 10<sup>-8</sup> M or 10<sup>-7</sup> M Ang II for 30 minutes. Control cardiomyocytes were treated with vehicle. After stimulation, cell lysates were extracted for immunoblotting.

### Transthoracic Echocardiography and Cardiac Catheterization

Transthoracic echocardiography was performed using an echocardiography system equipped with a 15-MHz transducer (Toshiba Electronics). The echocardiographer was unaware of the genotype of the mice. Closed-chest cardiac catheterization was performed as described previously.<sup>16</sup> A micro-tip catheter (Millar Instruments) was placed in the LV via the right carotid artery under constant pressure monitoring. The catheter position was verified by registration of typical pressure waves with a pressure transducer. Continuous LV pressure and heart rate were recorded using Powerlab software (AD Instruments).

### Histological Analysis

LV and lung tissue were fixed in 10% formalin, embedded in paraffin, and sectioned with a microtome. Tissue sections were stained with hematoxylin/eosin or Masson trichrome stain.<sup>16</sup>

### Quantitative Real-Time RT-PCR

LV was isolated from cardiac tissue and was quickly stored in an RNA stabilization reagent (RNAlater, Qiagen Inc); RNA was purified using an RNA isolation protocol (SV Total RNA Isolation System, Promega Inc). A TaqMan Gold RT-PCR kit was used according to the manufacturer's instructions (Applied Biosystems). Quantitative PCR was performed using real-time detection technology and analyzed on a model 7900 Sequence Detector (Applied Biosystems) with specific primers and fluorescent probes for ACE, ACE2, Ang II type 1a (AT<sub>1a</sub>) receptor, brain natriuretic peptide (BNP), and atrial natriuretic peptide (ANP) mRNAs (TaqMan Gene Expression Assays, Applied Biosystems). Levels of mRNA were compared at various time points after normalization to concurrent 18S rRNA amplification.

### Protein Analysis

Whole cell extracts of LV tissue were separated by SDS-PAGE, and proteins were electrophoretically transferred to nitrocellulose filters. The filters were blocked in Tris-buffered saline solution containing 1% Tween 20 and 5% nonfat dried milk. The filters were washed and incubated with primary antibodies. Primary antibodies were purchased from the indicated sources: rabbit polyclonal anti-ACE2 antibody (Santa Cruz Biotechnology); mouse monoclonal anti- $\alpha$ -actinin antibody (BD Biosciences); rabbit polyclonal anti-phosphorylated extracellular signal-regulated protein kinase (ERK) antibody, rabbit polyclonal anti-phospho ERKs antibody, rabbit polyclonal anti-c-Jun amino-terminal kinase (JNK) antibody, and rabbit polyclonal anti-phospho JNKs antibody (Cell Signaling Technology). Filters were thoroughly washed in Tris-buffered saline solution containing 1% Tween 20 and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham Pharmacia Biotech Inc). Bands were visualized using an enhanced chemiluminescence system (Amersham Pharmacia Biotech Inc).

### Plasma and Heart Ang II Content

Plasma was collected in tubes containing PMSF (final concentration 2.5 mmol/L) and EDTA (final concentration 1 mmol/L). After adequate perfusion with heparinized saline, the isolated LV was dissected and then stored at -80°C until measurement. Each frozen heart was homogenized on ice in 0.9% saline/0.1 mol/L HCl containing 0.1 mol/L aprotinin. Using the Bradford protein assay with bovine serum albumin as a standard (BioRad Protein Assay Reagent, BioRad Laboratories), the total protein content of an aliquot of the homogenate was determined. Peptide extraction was performed as described previously.<sup>17</sup> We measured Ang II concentration by radioimmunoassay using 2 antibodies specific for Ang II (SRL Co). This antibody does not have cross-reactivity to angiotensin 1-7.

### Kaplan-Meier Analysis of Cardiac Death

To determine the effect of TAC on cardiac death, 15 *ACE2*<sup>-/-</sup> mice and 15 WT mice were subjected to TAC. These mice were housed separately for 1 month after TAC. After the mice died, their lungs were weighed to determine the presence or absence of congestive heart failure. We defined lung weight:body weight (LW/BW) higher than mean +4 SD in sham-operated *ACE2*<sup>-/-</sup> and WT mice as the criteria for pulmonary congestion according to previous work.<sup>18</sup> We defined death with pulmonary congestion as cardiac death.

### Statistical Analysis

Data were analyzed with Stat View version 4.51 and are presented as mean  $\pm$  SEM. Statistical analysis was performed by 2-tailed Student *t* test,  $\chi^2$  analysis, and ANOVA where applicable. Multiple group comparison was carried out by ANOVA with Fisher post-hoc comparison. Log-rank test was used to evaluate survival rate. A value of *P* < 0.05 was considered statistically significant.

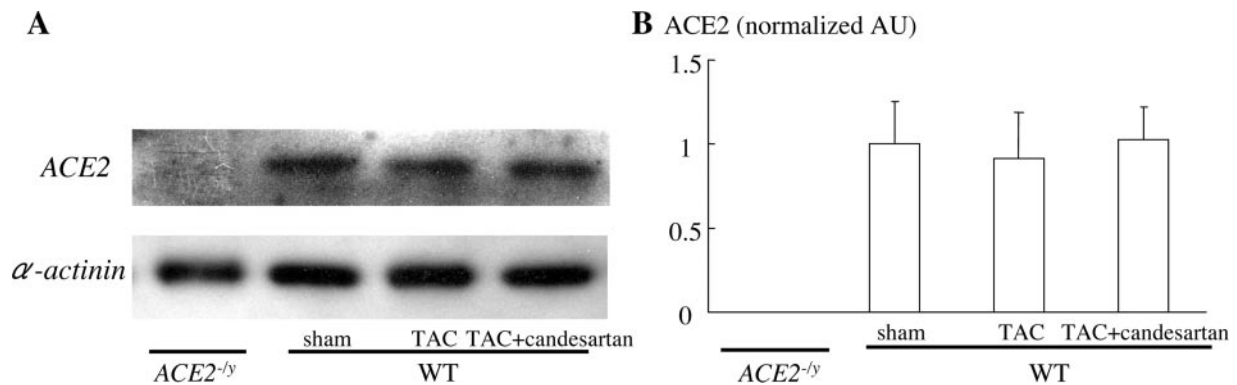
## Results

### Expression of ACE2 Protein and mRNA in *ACE2*<sup>-/-</sup> Mice and WT Mice

ACE2 protein was not detected by immunoblotting protein lysates from *ACE2*<sup>-/-</sup> mice and in WT mice. Expression of ACE2 protein was not altered by TAC with and without candesartan in WT mice (Figure 1A). Expression of ACE2 mRNA in WT mice, as analyzed by real-time PCR, was also not altered by TAC or by TAC with candesartan (Figure 1B).

### Hypertrophic Response of *ACE2*<sup>-/-</sup> Mice to TAC

Fourteen days after TAC, the ratio of heart weight:body weight (HW/BW) in WT mice was increased by 33% from 4.8  $\pm$  0.3 mg/g to 6.4  $\pm$  1.2 mg/g. In *ACE2*<sup>-/-</sup> mice, HW/BW increased by 51% after TAC, from 4.7  $\pm$  0.2 to 7.1  $\pm$  1.4

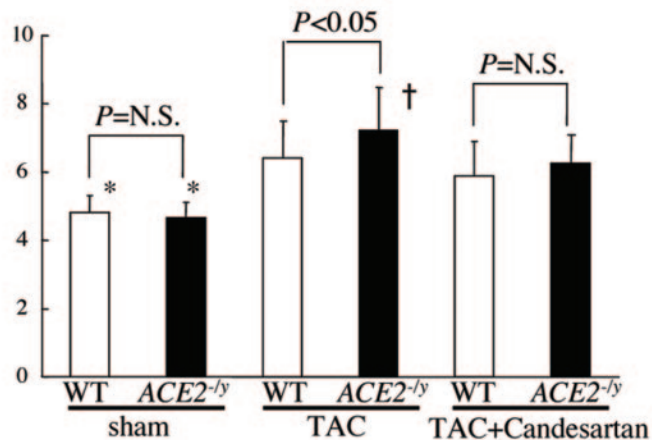


**Figure 1.** Biochemical characterization of ACE2 at 14 days after operation. (A) Protein expression of ACE2 in cardiac tissue. Ventricular lysates were immunoblotted with anti-ACE2 antibody and anti- $\alpha$  actinin antibody for control. ACE2 protein was not observed in ACE2<sup>-/-</sup> mice, and expression of ACE2<sup>-/-</sup> protein was not altered in WT mice. TAC+Candesartan indicates TAC with candesartan administration. (B) mRNA expression of ACE2 in cardiac tissue. Real-time quantitative PCR was performed with specific primers and a fluorescent probe for ACE2. Levels of ACE2 mRNA were corrected using 18s rRNA as an internal control. Expression of ACE2 mRNA was not altered by TAC or TAC with candesartan. Each value represents mean $\pm$ SD (n=10 in each group).

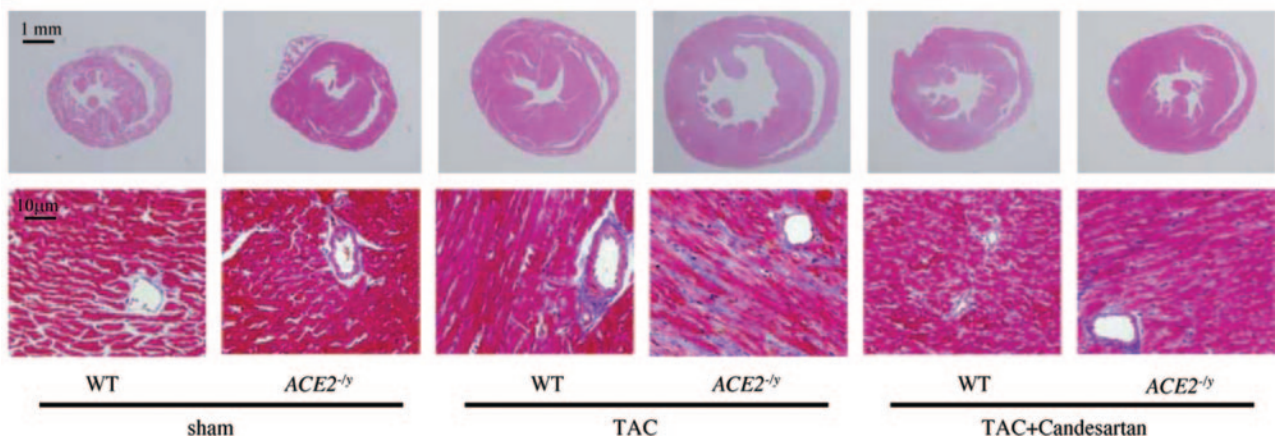
mg/g. HW/BW after TAC in ACE2<sup>-/-</sup> mice was greater than that in WT mice, suggesting that the development of hypertrophy is enhanced by ablation of ACE2 (Figure 2A). Administration of candesartan suppressed the hypertrophic

response in ACE2<sup>-/-</sup> mice. HW/BW after TAC in ACE2<sup>-/-</sup> mice administered candesartan was lower than that in ACE2<sup>-/-</sup> not treated with candesartan. The difference in HW/BW between ACE2<sup>-/-</sup> and WT mice after TAC with

### A. heart weight/body weight (mg/g)

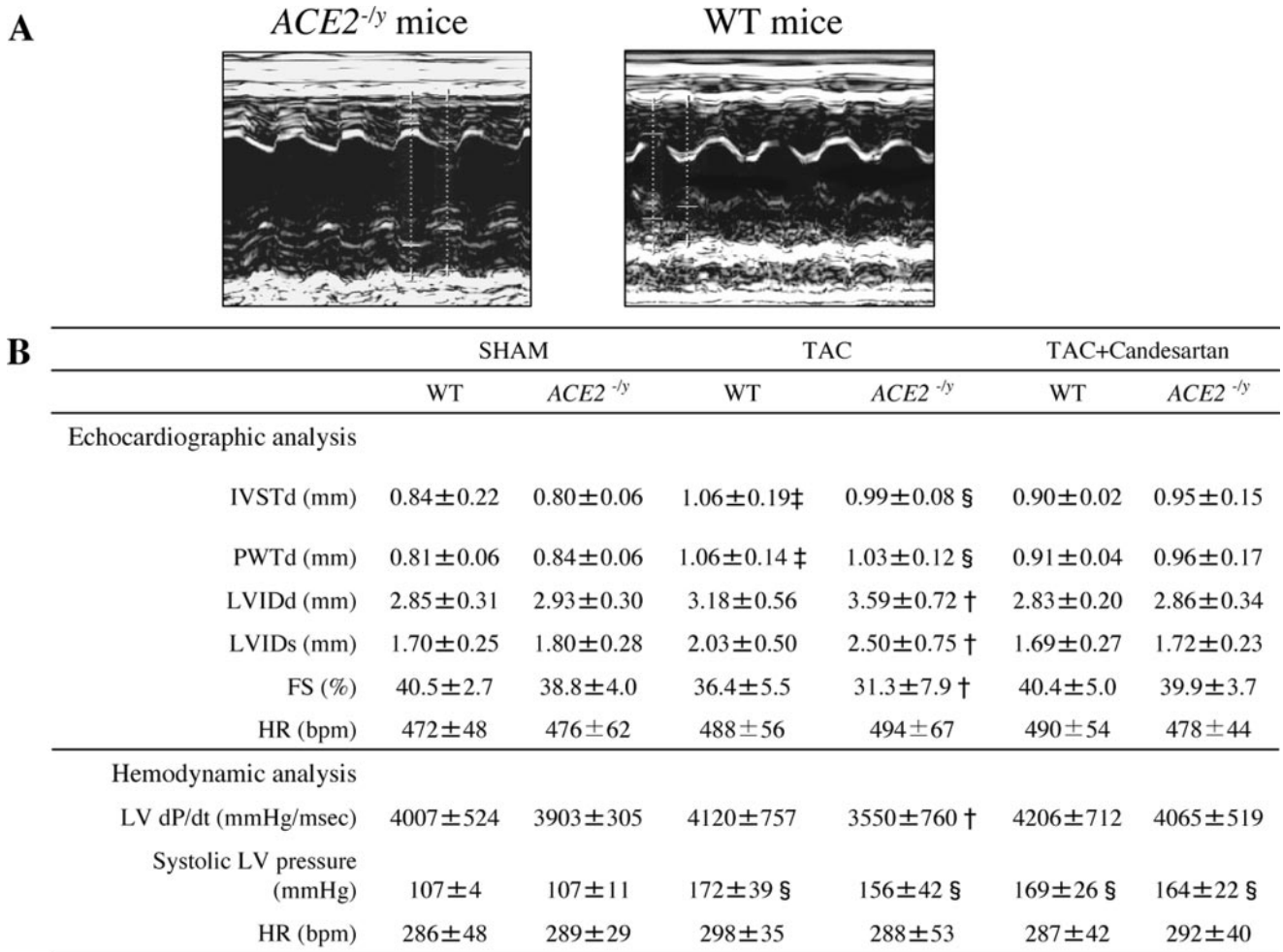


### B



**Figure 2.** Cardiac hypertrophy in ACE2<sup>-/-</sup> mice after TAC. (A) Fourteen days after TAC or sham operation, the cardiac chambers were dissected and weighed to determine HW/BW. TAC+Candesartan indicates TAC with candesartan administration. Each value represents mean $\pm$ SD (n=10 to 15). \* $P<0.01$  vs TAC and TAC+Candesartan.  $\dagger P<0.05$  vs others. (B, top) Ventricular tissue sections stained with hematoxylin/eosin. Marked LV chamber dilatation was observed in ACE2<sup>-/-</sup> mice after TAC. Original magnification was  $\times 2$ . (bottom) Ventricular tissue sections stained with Masson trichrome. ACE2<sup>-/-</sup> mice exhibited decreased myocyte number, myofibrillar disarray, and interstitial and perivascular fibrosis after TAC. Original magnification was  $\times 200$ .





**Figure 3.** Characteristic cardiac findings in *ACE2*<sup>-/-</sup> mice after TAC. Echocardiography and catheterization of *ACE2*<sup>-/-</sup> mice and WT mice were performed 14 days after TAC or sham operation. (A) M-mode echocardiograms after TAC. (B) Echocardiographic results and hemodynamic parameters. PWTd and IVSTd indicate end-diastolic posterior wall and intraventricular septal thickness; LVIDd and LVIDs, end-diastolic and end-systolic LV internal dimensions; FS, fractional shortening; HR, heart rate; TAC+Candesartan, TAC with administration of candesartan. Each value represents mean±SD (n=6 to 8). †*P*<0.05 vs others. ‡*P*<0.05 vs sham-operation and TAC+candesartan. §*P*<0.05 vs sham-operation.

candesartan administration was not statistically significant (Figure 2A).

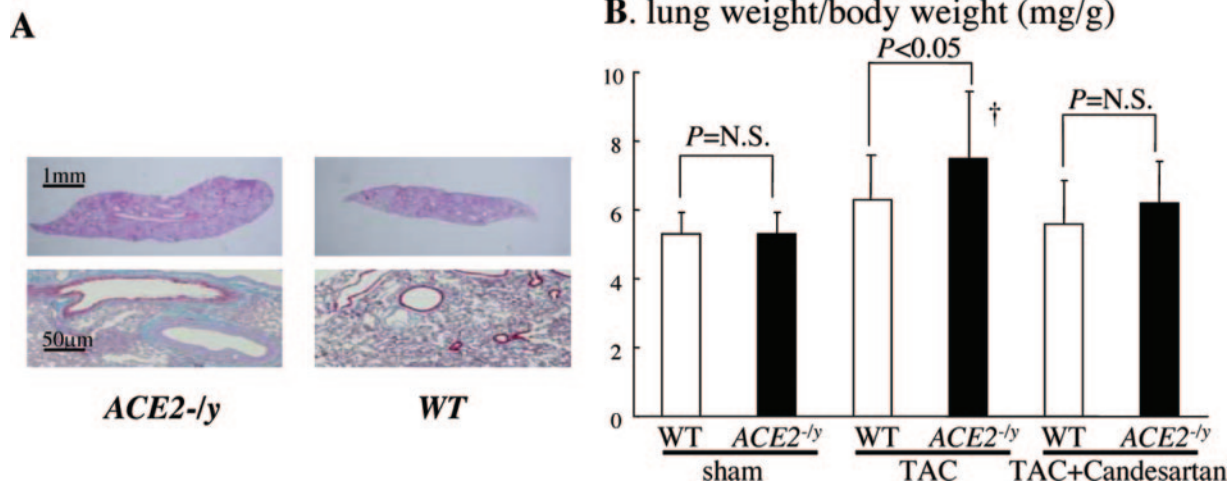
Histological analysis of LV tissue of *ACE2*<sup>-/-</sup> and WT mice was performed 14 days after TAC (Figure 2B). Compared with WT mice, *ACE2*<sup>-/-</sup> mice exhibited obvious LV dilatation, a myofibrillar disarray, and interstitial and perivascular fibrosis after TAC. Administration of candesartan attenuated the fibrosis and relatively preserved the myofibrillar architecture in *ACE2*<sup>-/-</sup> mice after TAC. Figure 3A shows typical echocardiographic findings in *ACE2*<sup>-/-</sup> mice and WT mice. Whereas LV wall thickness after TAC was comparable in *ACE2*<sup>-/-</sup> mice and WT mice, *ACE2*<sup>-/-</sup> mice after TAC exhibited larger LV diastolic dimension and LV systolic dimension than did WT mice after TAC (Figure 3B). In addition, ventricular contractility was decreased in *ACE2*<sup>-/-</sup> mice after TAC, with a fractional shortening of 31%, compared with 36% in WT mice. Administration of candesartan decreased LV chamber dilatation and contractile dysfunction in *ACE2*<sup>-/-</sup> mice after TAC. Hemodynamic analysis revealed a decline in LV dP/dt, an indicator of LV contractility, in *ACE2*<sup>-/-</sup> mice after TAC (Figure 3B). Candesartan did not

lower systolic LV pressure in either *ACE2*<sup>-/-</sup> mice or WT mice that underwent TAC. There were no differences in HW/BW, echocardiographic data, hemodynamic status, and cardiac morphology between sham-operated *ACE2*<sup>-/-</sup> and WT mice.

### Heart Failure in *ACE2*<sup>-/-</sup> Mice After TAC

Pressure overload induced congestive heart failure, as manifested by an increase in lung weight (Figure 4A). LW/BW (mg/g) 14 days after TAC was higher in *ACE2*<sup>-/-</sup> mice than in WT mice (Figure 4B). Administration of candesartan decreased LW/BW in *ACE2*<sup>-/-</sup> mice, and no significant difference in LW/BW was found between candesartan-treated TAC mice and sham-operated mice (Figure 2B). After TAC, the incidence of pulmonary congestion was 46% (7 of 15) in *ACE2*<sup>-/-</sup> mice, which was higher than the 13% (2 of 15) in WT mice (*P*<0.05).

In survival analysis, 5 of 15 WT mice and 10 of 15 *ACE2*<sup>-/-</sup> mice died within 30 days of TAC. Three mice in each group died within 48 hours of TAC without pulmonary congestion. We diagnosed cardiac death with pulmonary congestion in



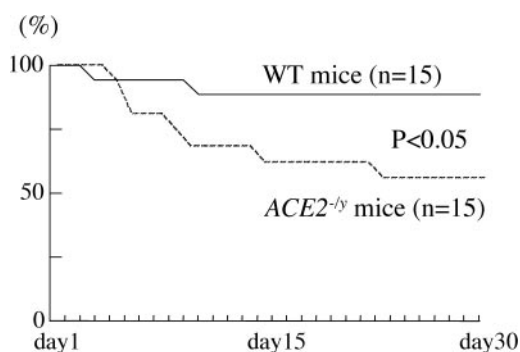
**Figure 4.** Congestive heart failure in  $ACE2^{-/-}$  mice following TAC. (A) Masson trichrome staining of lung tissue with  $ACE2^{-/-}$  (left) and WT (right) mouse excised 14 days after TAC. Original magnification was  $\times 2$  in top and  $\times 40$  in bottom. (B) 14 days after TAC or sham operation, the lungs were dissected and weighed to determine LW/BW. TAC+Candesartan indicates TAC with candesartan administration. Each value represents mean  $\pm$  SD ( $n=10$  to  $15$ ).  $\dagger P < 0.05$  vs others.

the remaining 2 WT and 7  $ACE2^{-/-}$  mice. The Kaplan–Meyer analysis showed that the incidence of cardiac death was significantly high in  $ACE2^{-/-}$  mice compared with WT mice (Figure 5).

#### Gene Expression in $ACE2^{-/-}$ Mice After TAC

Pressure overload leads to a variety of alterations in cardiac gene expression. We used real-time, quantitative RT-PCR to assess the expression of hypertrophic marker genes in mice after TAC. In both WT and  $ACE2^{-/-}$  mice, BNP and ANP expression was increased 14 days after TAC (Figure 6A and 6B). BNP, which is highly sensitive for detecting overt heart failure with decompensated left ventricular function,<sup>19</sup> was more distinctly increased in  $ACE2^{-/-}$  mice than in WT mice after TAC. Administration of candesartan attenuated ANP and BNP expression after TAC in  $ACE2^{-/-}$  mice (Figure 6A and 6B).

We also measured cardiac expression of ACE and  $ATB_{1B}$  receptor. ACE gene expression was upregulated by TAC and was attenuated by administration of candesartan in WT mice, as reported previously (Figure 6C).<sup>10,20</sup> A similar alteration of the ACE gene by TAC or administration of candesartan was



**Figure 5.** Kaplan–Meyer analysis of cardiac death. TAC was performed in 15  $ACE2^{-/-}$  mice and 15 WT mice. These mice were housed separately until 1 month after TAC, and the presence of congestive heart failure was confirmed after death.

observed in  $ACE2^{-/-}$  mice.  $ATB_{1B}$  receptor gene expression was comparatively high in sham-operated  $ACE2^{-/-}$  mice and was downregulated by TAC (Figure 6D). Administration of candesartan did not affect  $AT1$  receptor expression.

#### Ang II Concentration in $ACE2^{-/-}$ Mice

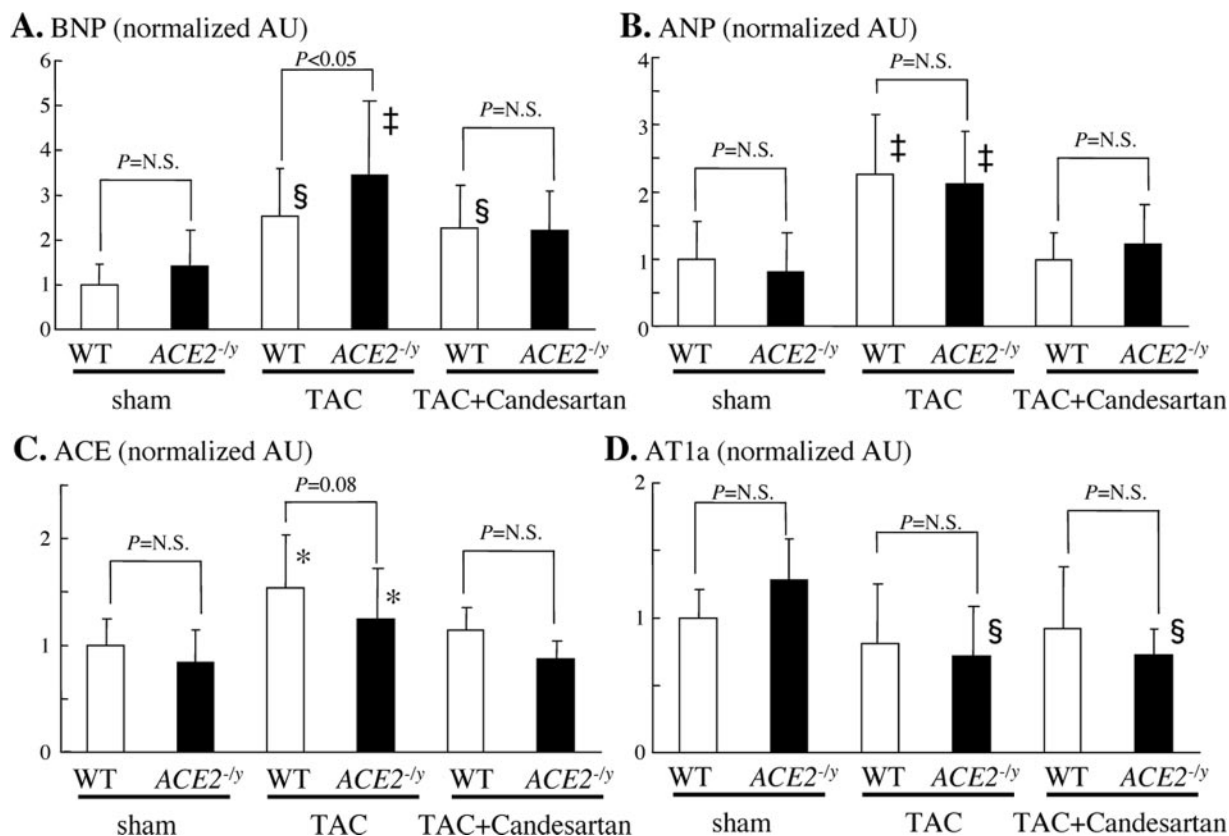
In our study, there were no differences in plasma and LV Ang II concentration between sham-operated  $ACE2^{-/-}$  and WT mice (Figure 7A). TAC increased the Ang II concentration in both  $ACE2^{-/-}$  and WT mice. In particular, a marked elevation of Ang II concentration was observed in  $ACE2^{-/-}$  mice LV tissue. In WT mice, TAC increased LV Ang II concentration  $\approx 5$ -fold from  $0.35 \pm 0.09$  pg/mg protein to  $1.73 \pm 0.43$  pg/mg protein; however, in  $ACE2^{-/-}$  mice, TAC increased Ang II concentration in LV tissue 7.5-fold from  $0.40 \pm 0.19$  pg/mg protein to  $3.03 \pm 1.19$  pg/mg protein. LV Ang II concentration was higher in  $ACE2^{-/-}$  mice than in WT mice 2 weeks after TAC. The difference in plasma Ang II concentration was statistically not significant between  $ACE2^{-/-}$  mice and WT mice after TAC.

#### Analysis of Mitogen-Activated Protein Kinase Activation in $ACE2^{-/-}$ Mice After TAC

We analyzed activation of mitogen-activated protein (MAP) kinases in LV tissue in response to pressure overload. ERKs and JNKs increased in LV tissue from both WT and  $ACE2^{-/-}$  mice 2 weeks after TAC (Figure 7B). The activation of ERKs and JNKs was higher in  $ACE2^{-/-}$  mice than in WT mice after TAC. In both  $ACE2^{-/-}$  and WT mice, the activation of ERKs and JNKs was reduced by administration of candesartan, suggesting that activation of these MAP kinase cascades is regulated by  $AT_1$  receptor-mediated signal transduction.

#### Analysis of Ang II-Induced MAP Kinase Activation in Cardiomyocytes From $ACE2^{-/-}$ Mice

To determine the role of Ang II-mediated signal transduction in  $ACE2^{-/-}$  mice, we stimulated cardiomyocytes with Ang II for 30 minutes and analyzed the activation of ERKs and JNKs by



**Figure 6.** Distinct gene expression in *ACE2*<sup>-/-</sup> mice after TAC. Ventricular tissue was obtained 14 days after TAC or sham operation from *ACE2*<sup>-/-</sup> and WT mice, and RNA was purified from the tissue samples. Gene expression was analyzed by use of quantitative real-time RT-PCR with specific primers and probes. 18s rRNA was used as an internal control in all cases. (A) BNP gene expression in *ACE2*<sup>-/-</sup> and WT ventricular tissue. (B) ANP gene expression in *ACE2*<sup>-/-</sup> and WT ventricular tissue. (C) ACE gene expression in *ACE2*<sup>-/-</sup> and WT ventricular tissue. (D) AT<sub>1</sub>a receptor gene expression in *ACE2*<sup>-/-</sup> and WT ventricular tissue. TAC+Candesartan indicates TAC with candesartan administration; AU, arbitrary unit. Each value represents mean±SD (n=10 in each group). ‡*P*<0.05 vs sham operation and TAC+Candesartan. §*P*<0.05 vs sham operation.

assessment of the phosphorylation of these kinases (Figure 8). Treatment with Ang II increased ERK and JNK phosphorylation in the cardiomyocytes of both WT and *ACE2*<sup>-/-</sup> mice in a dose-dependent manner. Although the phosphorylation of ERKs and JNKs was similar in the cardiomyocytes of WT and *ACE2*<sup>-/-</sup> mice treated with vehicle, the increase in ERK and JNK phosphorylation of the cardiomyocytes after treatment with Ang II was greater in *ACE2*<sup>-/-</sup> mice than in WT mice.

### Discussion

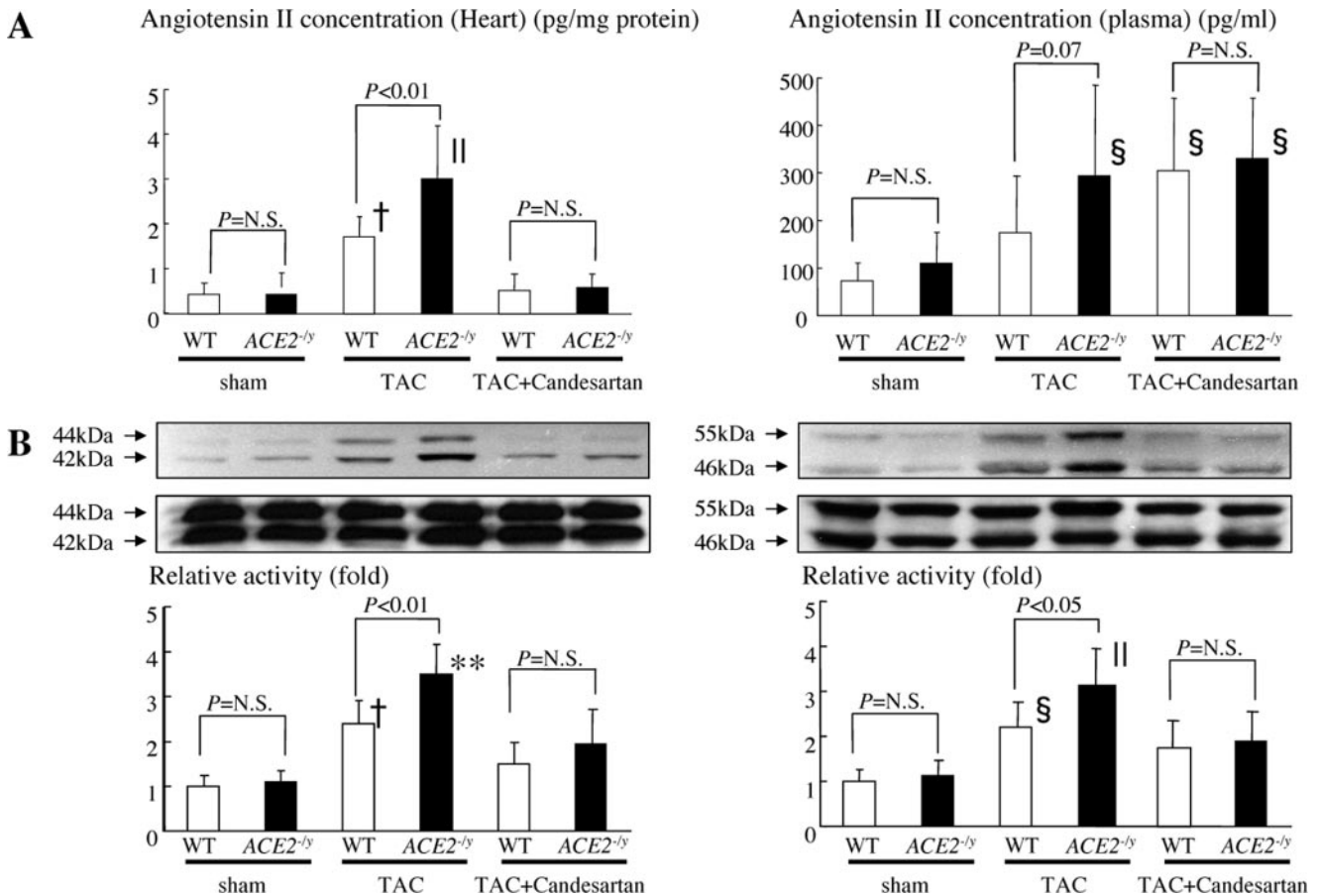
In the present study, we demonstrated that *ACE2*<sup>-/-</sup> mice develop severe cardiac hypertrophy with a reduction in cardiac contractility and dilatation of the left ventricular cavity in response to pressure overload. Morphologically, their hearts exhibited marked perivascular and interstitial fibrosis and disarray. These results suggested that disruption of ACE2 function might accelerate cardiac hypertrophy and shorten the transition period to heart failure in response to pressure overload. Indeed, 2 weeks after TAC, *ACE2*<sup>-/-</sup> mice began to exhibit heart failure that led to death within 1 month.

What is the main mechanism accelerating pressure-overload-induced cardiac remodeling and dysfunction in *ACE2*<sup>-/-</sup> mice? Because administration of an ARB attenuated the onset of cardiac failure in *ACE2*<sup>-/-</sup> mice that underwent TAC, ACE2 may function as a cardioprotective enzyme against

pressure overload by decreasing AT<sub>1</sub> receptor-mediated signal transduction. Pressure overload activates the cardiac renin-angiotensin system and increases local Ang II concentration.<sup>10,11</sup> Our experiments also demonstrated that both serum and cardiac concentrations of Ang II were increased by TAC, and the rise in intracardiac Ang II was higher in *ACE2*<sup>-/-</sup> than in WT mice. Because it has been demonstrated that the catalysis efficiency of Ang II to angiotensin 1-7 by ACE2 is much higher than that of angiotensin I to angiotensin 1-9,<sup>3</sup> the large rise in Ang II concentration in *ACE2*<sup>-/-</sup> mice after TAC may be mainly because of the block in conversion of Ang II to angiotensin 1-7.

Although stretching of neonatal cardiomyocytes results in upregulation of the AT<sub>1</sub> receptor, AT<sub>1</sub> expression is reduced in the chronic phase of pressure overload after TAC; this reduction results from the negative regulation of AT<sub>1</sub> expression by Ang II.<sup>21</sup> Similar mechanisms might be present in our study: increases in Ang II induced by pressure overload might downregulate AT<sub>1</sub> receptor expression in *ACE2*<sup>-/-</sup> mice that underwent TAC. Consistent with a previous report suggesting that cardiac expression of ACE is increased in response to pressure overload, ACE expression in response to TAC showed an increase in both WT and *ACE2*<sup>-/-</sup> mice.<sup>20</sup>

There is abundant evidence that ERKs and JNKs, the family of MAP kinases, are involved in Ang II-mediated activation of



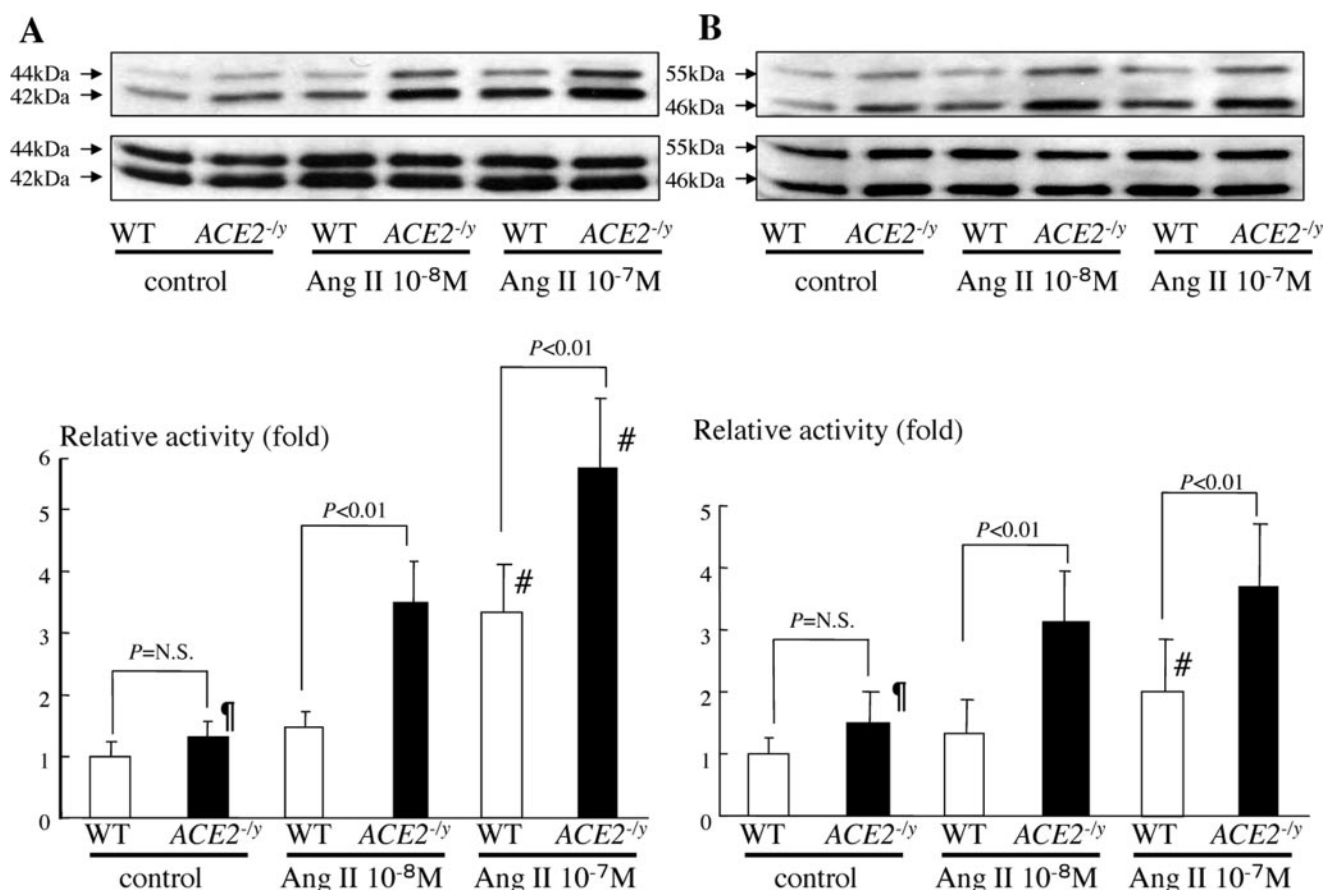
**Figure 7.** Altered angiotensin II concentration and MAP kinase activation in *ACE2*<sup>-/-</sup> mice after TAC. (A) Ventricular (left) and plasma (right) angiotensin II concentration. Ventricular tissue and plasma were obtained from *ACE2*<sup>-/-</sup> and WT mice 14 days after TAC or sham operation. Angiotensin II concentration was measured by radioimmunoassay using 2 antibodies specific for angiotensin II (n=5 in each group). (B) Activation of MAP kinases. Ventricular tissue was obtained from *ACE2*<sup>-/-</sup> and WT mice 14 days after TAC or sham operation, and ventricular lysates were separated by SDS-PAGE and examined by immunoblotting. (left) Analysis of protein levels of ERKs, consisting of p42 ERK and p44 ERK, in cardiac tissue from *ACE2*<sup>-/-</sup> and WT mice. (top) Anti-phospho ERKs immunoblot. (middle) Anti-ERKs immunoblot of control for protein content. (bottom) Relative activity of ERKs in ventricular lysates by densitometric analysis of immunoreactive bands. (n=4 in each group). (right) Analysis of protein levels of JNKs, consisting of p46 JNK and p55 JNK, in cardiac tissue from *ACE2*<sup>-/-</sup> and WT mice. (top) Anti-phospho JNKs immunoblot. (middle) Anti-JNKs immunoblot of control for protein content. (bottom) Relative activity of JNKs in ventricular lysates by densitometric analysis of immunoreactive bands (n=4 in each group). TAC+Candesartan indicates TAC with candesartan administration. Each value represents mean±SD. †P<0.05 vs others. §P<0.05 vs sham operation. ||P<0.01 vs others.

early growth response genes and cell proliferation in cardiomyocytes.<sup>22,23</sup> Mechanical stretch also activates the ERK and JNK signaling pathways through activation of AT<sub>1</sub> receptor.<sup>24</sup> Our studies in primary cell culture revealed that MAP kinase activation in response to Ang II was greater in cardiomyocytes of *ACE2*<sup>-/-</sup> mice than in those of WT mice. These data suggest that ACE2 degrades Ang II, reversing the activation of MAP kinases. However, as mechanical stretch activates various kinds of humoral factors in addition to Ang II, regulation of the activation of intracellular signal transduction in mechanical stretch is more complicated than stimulation with Ang II alone. Recent experiments using mice lacking AT<sub>1</sub> receptors demonstrated that activation of protein kinases was evoked without involvement of the AT<sub>1</sub> receptor in pressure-overload-induced hypertrophy.<sup>25,26</sup> In our study, pressure overload increased the activation of MAP kinases to a greater extent in cardiac tissue from *ACE2*<sup>-/-</sup> mice than from WT mice. Furthermore, administration of candesartan attenuated the activation of these kinases in *ACE2*<sup>-/-</sup> mice, indicating a role for the receptor in their activation.

Zou et al<sup>27</sup> reported that mechanical stress activates the AT<sub>1</sub> receptor independent of Ang II, suggesting that the AT<sub>1</sub> receptor can function as a mechanical sensor and convert mechanical stress into biochemical signals in cells. Candesartan is reported to block this stretch-induced activation of the AT<sub>1</sub> receptor by serving as an inverse agonist.<sup>27</sup> Therefore, it is impossible to distinguish between the effect of candesartan as an inverse agonist on AT<sub>1</sub> receptor and its inhibition of Ang II on pressure overload-induced hypertrophy in *ACE2*<sup>-/-</sup> mice. However, because the degree of aortic constriction was the same in *ACE2*<sup>-/-</sup> and WT mice, mechanical stress on cardiomyocytes that results in ligand-independent activation of AT<sub>1</sub> receptor ought to be the same in both groups after TAC. Thus, our results indicate that excessively increased cardiac Ang II plays a primary role in the activation of signal transduction in pressure overload-induced hypertrophy of *ACE2*<sup>-/-</sup> mice.

In this study, there were no differences in left ventricular function and cardiac morphology between sham-operated





**Figure 8.** Activation of MAP kinases in response to Ang II stimulation. Neonatal cardiomyocytes were treated with 10<sup>-8</sup> or 10<sup>-7</sup> M Ang II for 30 minutes. Control cardiomyocytes were treated with vehicle. Cellular lysates were separated by SDS-PAGE and examined by immunoblotting. (A) Analysis of protein levels of ERK1/2, consisting of p42 ERK and p44 ERK, in cardiomyocytes of ACE2<sup>-/-</sup> and WT mice. (top) Anti-phospho ERK1/2 immunoblot. (middle) Anti-ERK1/2 immunoblot. (bottom) Relative activity of ERKs in cellular lysates by densitometric analysis of immunoreactive bands. (B) Analysis of protein levels of p46 JNK and p55 JNK in cardiomyocytes of ACE2<sup>-/-</sup> and WT mice. (top) Anti-phospho JNKs immunoblot. (middle) Anti-JNKs immunoblot. (bottom) Relative activity of JNKs in cellular lysates by densitometric analysis of immunoreactive bands. TAC+Candesartan indicates TAC with candesartan administration. Each value represents mean±SD (n=3 in each group). ¶P<0.01 vs Ang II 10<sup>-8</sup> M and Ang II 10<sup>-7</sup> M. #P<0.01 vs control and Ang II 10<sup>-8</sup> M.

ACE2<sup>-/-</sup> and WT mice. Moreover, plasma and cardiac concentrations of Ang II were comparable between sham-operated ACE2<sup>-/-</sup> and WT mice. These observations are somewhat different from those of a previous study by Crackower et al,<sup>4</sup> who demonstrated by echocardiography that 3-month-old mice lacking ACE2 had impaired cardiac contractility. They also reported that plasma and cardiac concentrations of Ang II were elevated in mice lacking ACE2. Another group reported that there were no differences in left ventricular function and plasma Ang II concentration between 1-year-old ACE2<sup>-/-</sup> and WT mice.<sup>28</sup> The reasons for these differences are still unclear.

**Study Limitations**

We demonstrated an important role of ACE2 to catalyze activated Ang II in the pressure-overloaded heart. In contrast, we could not detect the role of ACE2 to catalyze Ang II in sham-operated ACE2<sup>-/-</sup> and WT mice, although ACE2 mRNA and protein levels in the heart were similar to those in mice with TAC operation. It may be a possible reason that levels of Ang II, a substrate of ACE2, in mice without TAC were close to the detectable limit by radioimmunoassay. Therefore, difference of cardiac Ang II between ACE2<sup>-/-</sup> and

WT mice without TAC might be masked by considerable variation of the data.

ACE2 causes several biochemical changes in addition to catalyzing Ang II.<sup>2,3</sup> There are several reports suggesting that angiotensin 1-7 acts as a cardioprotective agent.<sup>29,30</sup> However, it is unlikely that angiotensin 1-7 contributes as a major molecule to the differences between WT and ACE2<sup>-/-</sup> mice after TAC, because most of those differences were eliminated by candesartan, an AT1 blocker, and angiotensin 1-7 does not bind to AT1 receptor. However, we cannot deny the role of angiotensin 1-7 in the difference between WT and ACE2<sup>-/-</sup> mice, because we have not performed the experiment using specific antagonist for angiotensin 1-7. Furthermore, the loss of the ability of ACE2 to catalyze several kinds of humoral factors other than Ang II may contribute to the phenotype of ACE2<sup>-/-</sup> mice: ACE2 hydrolyzes apelin-13, dynorphin A 1-13, and des-Arg-bradykinin with high catalytic efficiency in vitro.<sup>3</sup> The functions of their hydrolytic products in cardiac tissue are also undetermined. Additional investigations will be required to determine the role of these factors in pressure overload.

**Perspectives**

The present study demonstrated that ACE2 might suppress the development of cardiac hypertrophy and congestive heart failure



induced by pressure overload. Our results exhibit the definite role of ACE2 that suppresses the activated Ang II in vivo. Recently it was recognized that organ distribution of ACE2 is more ubiquitous than previously expected.<sup>31</sup> The tissue renin-angiotensin system is considered to have an important role in several organs, such as blood vessel, brain, adipose, and kidney, as well as heart. The role of ACE2 in such organs is still unknown. Functional analysis of ACE2 in these organs will, furthermore, clarify the significance of this enzyme in vivo.

### Acknowledgments

We are most grateful to Seiko Kaji, and Kazuko Iwasa at Osaka University; and Dr Naoki Nishino, Sawako Muroi, Takashi Wadatsu, Norihiro Miyazawa, and Takahiro Sato at Otsuka GEN Research Institute for their excellent technical assistance. We thank Dr Paul Kretchmer at San Francisco Edit for his assistance in editing this article.

### References

- Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, Turner AJ. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem*. 2000;275:33238–33243.
- Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res*. 2000;87:E1–E9.
- Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, Godbout K, Parsman T, Baronas E, Hsieh F, Acton S, Patane M, Nichols A, Tummino P. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem*. 2002;277:14838–14843.
- Crackower MA, Sarao R, Oudit GY, Yagil C, Koziereadzi I, Scanga SE, Oliveira-dos-Santos AJ, da Costa J, Zhang L, Pei Y, Scholey J, Ferrario CM, Manoukian AS, Chappell MC, Backx PH, Yagil Y, Penninger JM. Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature*. 2002;417:822–828.
- Zisman LS, Keller RS, Weaver B, Lin Q, Speth R, Bristow MR, Canver CC. Increased angiotensin-(1-7)-forming activity in failing human heart ventricles: evidence for upregulation of the angiotensin-converting enzyme Homologue ACE2. *Circulation*. 2003;108:1707–1712.
- Baker KM, Booz GW, Dostal DE. Cardiac actions of angiotensin II: Role of an intracardiac renin-angiotensin system. *Annu Rev Physiol*. 1992;54:227–241.
- Ito H, Hirata Y, Hiroe M, Tsujino M, Adachi S, Takamoto T, Nitta M, Taniguchi K, Marumo F. Endothelin-1 induces hypertrophy with enhanced expression of muscle-specific genes in cultured neonatal rat cardiomyocytes. *Circ Res*. 1991;69:209–215.
- Simpson P. Norepinephrine-stimulated hypertrophy of cultured rat myocardial cells is an  $\alpha$  1 adrenergic response. *J Clin Invest*. 1983;72:732–738.
- Lindpaintner K, Ganten D. The cardiac renin-angiotensin system: a synopsis of current experimental and clinical data. *Acta Cardiol*. 1991;46:385–397.
- Schunkert H, Dzau VJ, Tang SS, Hirsch AT, Apstein CS, Lorell BH. Increased rat cardiac angiotensin converting enzyme activity and mRNA expression in pressure overload left ventricular hypertrophy. Effects on coronary resistance, contractility, and relaxation. *J Clin Invest*. 1990;86:1913–1920.
- Baker KM, Chernin MI, Wixson SK, Aceto JF. Renin-angiotensin system involvement in pressure-overload cardiac hypertrophy in rats. *Am J Physiol*. 1990;259:H324–H332.
- Regan CP, Anderson PG, Bishop SP, Berecek KH. Pressure-independent effects of AT1-receptor antagonism on cardiovascular remodeling in aortic-banded rats. *Am J Physiol*. 1997;272:H2131–H2138.
- Schunkert H, Jackson B, Tang SS, Schoen FJ, Smits JF, Apstein CS, Lorell BH. Distribution and functional significance of cardiac angiotensin converting enzyme in hypertrophied rat hearts. *Circulation*. 1993;87:1328–1339.
- Liao Y, Ishikura F, Beppu S, Asakura M, Takashima S, Asanuma H, Sanada S, Kim J, Ogita H, Kuzuya T, Node K, Kitakaze M, Hori M. Echocardiographic assessment of LV hypertrophy and function in aortic-banded mice: necropsy validation. *Am J Physiol Heart Circ Physiol*. 2002;282:H1703–H1708.
- Narmoneva DA, Vukmirovic R, Davis ME, Kamm RD, Lee RT. Endothelial cells promote cardiac myocyte survival and spatial reorganization: implications for cardiac regeneration. *Circulation*. 2004;110:962–968.
- Rogers JH, Tamirisa P, Kovacs A, Weinheimer C, Courtois M, Blumer KJ, Kelly DP, Muslin AJ. RGS4 causes increased mortality and reduced cardiac hypertrophy in response to pressure overload. *J Clin Invest*. 1999;104:567–576.
- Allred AJ, Chappell MC, Ferrario CM, Diz DI. Differential actions of renal ischemic injury on the intrarenal angiotensin system. *Am J Physiol Renal Physiol*. 2000;279:F636–F645.
- Liao Y, Takashima S, Asano Y, Asakura M, Ogai A, Shintani Y, Minamino T, Asanuma H, Sanada S, Kim J, Ogita H, Tomoike H, Hori M, Kitakaze M. Activation of adenosine A1 receptor attenuates cardiac hypertrophy and prevents heart failure in murine left ventricular pressure-overload model. *Circ Res*. 2003;93:759–766.
- Langenickel T, Pagel I, Hohnel K, Dietz R, Willenbrock R. Differential regulation of cardiac ANP and BNP mRNA in different stages of experimental heart failure. *Am J Physiol Heart Circ Physiol*. 2000;278:H1500–H1506.
- Weinberg EO, Lee MA, Weigner M, Lindpaintner K, Bishop SP, Benedict CR, Ho KK, Douglas PS, Chafizadeh E, Lorell BH. Angiotensin AT1 receptor inhibition. Effects on hypertrophic remodeling and ACE expression in rats with pressure-overload hypertrophy due to ascending aortic stenosis. *Circulation*. 1997;95:1592–1600.
- Schultz D, Su X, Wei CC, Bishop SP, Powell P, Hanks GH, Dillon AR, Rynders P, Spinale FG, Walcott G, Ideker R, Dell'Italia LJ. Downregulation of ANG II receptor is associated with compensated pressure-overload hypertrophy in the young dog. *Am J Physiol Heart Circ Physiol*. 2002;282:H749–H756.
- Zou Y, Komuro I, Yamazaki T, Aikawa R, Kudoh S, Shiojima I, Hiroi Y, Mizuno T, Yazaki Y. Protein kinase C, but not tyrosine kinases or Ras, plays a critical role in angiotensin II-induced activation of Raf-1 kinase and extracellular signal-regulated protein kinases in cardiac myocytes. *J Biol Chem*. 1996;271:33592–33597.
- Murasawa S, Mori Y, Nozawa Y, Gotoh N, Shibuya M, Masaki H, Maruyama K, Tsutsumi Y, Moriguchi Y, Shibazaki Y, Tanaka Y, Iwasaka T, Inada M, Matsubara H. Angiotensin II type I receptor-induced extracellular signal-regulated protein kinase activation is mediated by Ca2+/calmodulin-dependent transactivation of epidermal growth factor receptor. *Circ Res*. 1998;82:1338–1348.
- Yamazaki T, Komuro I, Kudoh S, Zou Y, Shiojima I, Mizuno T, Takano H, Hiroi Y, Ueki K, Tobe K, Kadowaki T, Nagai R, Yazaki Y. Angiotensin II partly mediates mechanical stress-induced cardiac hypertrophy. *Circ Res*. 1995;77:258–265.
- Hamawaki M, Coffman TM, Lashus A, Koide M, Zile MR, Oliverio MI, DeFreyte G, Cooper Gt, Carabello BA. Pressure-overload hypertrophy is unabated in mice devoid of AT1A receptors. *Am J Physiol*. 1998;274:H868–H873.
- Harada K, Komuro I, Shiojima I, Hayashi D, Kudoh S, Mizuno T, Kijima K, Matsubara H, Sugaya T, Murakami K, Yazaki Y. Pressure overload induces cardiac hypertrophy in angiotensin II type 1A receptor knockout mice. *Circulation*. 1998;97:1952–1959.
- Zou Y, Akazawa H, Qin Y, Sano M, Takano H, Minamino T, Makita N, Iwanaga K, Zhu W, Kudoh S, Toko H, Tamura K, Kihara M, Nagai T, Fukamizu A, Umemura S, Iiri T, Fujita T, Komuro I. Mechanical stress activates angiotensin II type 1 receptor without the involvement of angiotensin II. *Nat Cell Biol*. 2004;6:499–506.
- Gurley SB, Allred A, Le TH, Mao L, Donoghue M, Breitbart R, Acton SL, Rockman HA, Coffman TM. Altered blood pressure response and normal cardiac phenotype in ACE2-deficient mice (abstract). *Hypertension*. 2004;44:501.
- Ferreira AJ, Santos RA, Almeida AP. Angiotensin-(1-7): cardioprotective effect in myocardial ischemia/reperfusion. *Hypertension*. 2001;38:665–668.
- Loot AE, Roks AJ, Henning RH, Tio RA, Suurmeijer AJ, Boomsma F, van Gilst WH. Angiotensin-(1-7) attenuates the development of heart failure after myocardial infarction in rats. *Circulation*. 2002;105:1548–1550.
- Gembardt F, Sterner-Kock A, Imboden H, Spaltheholz M, Reibitz F, Schultheiss HP, Siems WE, Walther T. Organ-specific distribution of ACE2 mRNA and correlating peptidase activity in rodents. *Peptides*. 2005;26:1270–1277.