The American Journal of Pathology, Vol. 172, No. 5, May 2008

DOI: 10.2353/ajpath.2008.070762

Cardiovascular, Pulmonary and Renal Pathology

Angiotensin II Up-Regulates Angiotensin I-Converting Enzyme (ACE), but Down-Regulates ACE2 via the AT1-ERK/p38 MAP Kinase Pathway

Vijay Koka,* Xiao Ru Huang,*† Arthur C.K. Chung,† Wansheng Wang,* Luan D. Truong,‡ and Hui Yao Lan*†

From the Department of Medicine-Nephrology, Baylor College of Medicine, Houston, Texas; the Department of Medicine,† The University of Hong Kong Li Ka Shing Faculty of Medicine, Hong Kong; and the Department of Pathology,‡ Methodist Hospital, Houston, Texas*

The recent discovery of the angiotensin II (Ang II)-breakdown enzyme, angiotensin I converting enzyme (ACE) 2, suggests the importance of Ang II degradation in hypertension. The present study explored the signaling mechanism by which ACE2 is regulated under hypertensive conditions. Real-time PCR and immunohistochemistry showed that ACE2 mRNA and protein expression levels were high, whereas ACE expression levels were moderate in both normal kidney and heart. In contrast, patients with hypertension showed marked ACE up-regulation and ACE2 down-regulation in both hypertensive cardiopathy and, particularly, hypertensive nephropathy. The inhibition of ACE2 expression was shown to be associated with ACE up-regulation and activation of extracellular regulated (ERK)1/2 and p38 mitogen-activated protein (MAP) kinases. *In vitro*, Ang II was able to up-regulate ACE and down-regulate ACE2 in human kidney tubular cells, which were blocked by an angiotensin II (AT)1 receptor antagonist (losartan), but not by an AT2 receptor blocker (PD123319). Furthermore, blockade of ERK1/2 or p38 MAP kinases by either specific inhibitors or a dominant-negative adenovirus was able to abolish Ang II-induced ACE2 down-regulation in human kidney tubular cells. In conclusion, Ang II is able to up-regulate ACE and down-regulate ACE2 expression levels under hypertensive conditions both *in vivo* and *in vitro*. The AT1 receptor-mediated ERK/p38 MAP kinase signaling pathway may be a key mechanism by which Ang II down-regulates ACE2 expression, implicating an ACE/ACE2 imbalance in hypertensive cardiovascular and renal damage. ***(Am J Pathol 2008, 172:1174–1183; DOI: 10.2353/ajpath.2008.070762)***

The prevalence of hypertension is approximately 30% based on National Health and Nutrition Examination Survey data,[1] making it one of the most important risk factors for cardiovascular disease, the major cause of mortality in the United States.

The recent discovery of the angiotensin II (Ang II) breakdown enzyme angiotensin I -converting enzyme (ACE)2 and alternative Ang II-generating pathways such as chymase, in addition to ACE, has increased the complexity of our understanding of Ang II generation and degradation in hypertension.[2,3] ACE2 is a breakdown enzyme responsible for the degradation of Ang II to Angiotensin 1–7 peptide. The later has vasodilatory properties and has its own unique receptor, the Mas receptor.[3] Emerging evidence shows that ACE2 plays an important role in negatively regulating hypertension. In rat models of hypertension, renal ACE2 mRNA and protein are decreased, although this could not be confirmed in human hypertensive nephropathy in a prior study.[4] Further, in rats treated with ACE inhibitors and angiotensin receptor blockers, an increase in local renal ACE2 activity is noted.[5,6] We have shown earlier that ACE is up-regulated in human diabetic nephropathy accompanied with hypertension, a condition associated with high Ang II levels.[7] Taken together, these findings suggest that there may be an alteration in the ACE/ACE2 balance in hypertension in a manner that favors increased Ang II generation (ie, up-regulation of ACE) and decreased Ang II degradation (ie, down-regulation of ACE2) during hypertension.

Supported by grants from the Research Grant Council of Hong Kong (RGC ERCG, HKU 759206, 768207), a Research and Conference Grant of The University of Hong Kong (2006–2007), and grants from the NIH (RO1HL076661, RO1DK062828, and T32DK062706).

Accepted for publication February 6, 2008.

Address reprint requests to Hui Y. Lan, M.D., Ph.D., Department of Medicine, The University of Hong Kong, L839, Laboratory Block, Sassoon Road, Pokfulam, Hong Kong. E-mail: hylan@kucc.hku.hk.

The kidney is an important organ in hypertension. Not only is it a target for end organ damage in hypertension, but several lines of evidence suggest that it may play an active role in the pathogenesis of hypertension. For example, in salt-sensitive hypertension, renal blood flow is decreased with high salt diets[8] and altered-pressure natriuresis curves have been described in essential hypertension.[9] Reduced nephron numbers are also associated with essential hypertension and it is proposed that this may be associated with enhanced Ang II generation.[10,11] Transplantation of kidneys from genetically hypertensive to normotensive rats results in hypertension in renal graft recipients.[12] Conversely, kidneys from normotensive donors lower blood pressure in young transplanted spontaneously hypertensive rats.[13] It has long been recognized that the renin angiotensin system is highly activated within the kidney during hypertension. Furthermore, it is also noted that ACE2 is expressed in normal rat kidney and is reduced in the rat kidney with hypertension. However, it is not known what factors down-regulate renal ACE2 during hypertension.

This study explored the signaling mechanism by which ACE2 is regulated under hypertensive conditions *in vivo* and *in vitro*. This was performed in patients with hypertensive cardiopathy and nephropathy and *in vitro* in a human tubular cell line (HK-2) in response to Ang II.

Materials and Methods

Reagents

Fetal bovine serum (FBS), penicillin/streptomycin/amphotericin B, Dulbecco's modified Eagle's medium, Dulbecco's modified Eagle's medium/F-12K medium, and insulin-transferrin-selenium were obtained from Invitrogen (Carlsbad, CA). Ang II, losartan, and PD 123319 (angiotensin II [AT]2 receptor antagonist) were obtained from Sigma (St. Louis, MO). Antibodies to ACE, ACE2, phosphorylated extracellular regulated (ERK)1/2, and phosphorylated p38 mitogen-activated protein (MAP) kinases were obtained from R&D systems (Minneapolis, MN). Antibodies to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Chemicon (Temecula, CA). ERK1/2 kinase inhibitor PD98059 and the p38 MAP kinase inhibitor SB203580 were purchased from Calbiochem (La Jolla, CA).

Heart and Kidney Tissue Collection and Immunohistochemistry

Specimens of normal human and hypertensive kidneys and hearts were obtained from the Department of Pathology, Methodist Hospital, following the approved protocol by Institutional Review Board of Baylor College of Medicine. Among them, 12 patients had been diagnosed with hypertensive nephropathy and 8 with hypertensive cardiomyopathy. All hypertensive patients (seven males and five females; 37 to 80 years of age) with unequivocal hypertension (systolic 141 ± 3.8 mm Hg) were treated with either angiotensin-converting enzyme inhibitor or AT1 receptor blockers. All patients had hypertensive history for up to 15 years. Both kidney and heart tissues were obtained at autopsy. In addition, 12 histologically normal kidneys were obtained from paratumor nephrectomy tissues and 8 normal cardiac tissues from among autopsy samples without cardiovascular diseases. Four-micron sections of the formalin-fixed, paraffin-embedded, human kidney and heart tissues were stained with antibodies to human ACE, ACE2, phosphorylated ERK1/2, and phosphorylated P38 MAP kinases in serial sections using the microwave-based antigen retrieval technique and a modified peroxidase anti-peroxidase method as described previously.[7] Quantitative analyses of ACE and ACE2 expression within the kidney were performed using a quantitative image analysis system (Metamorph, Sunnyvale, CA). Briefly, up to five random areas (×10 power) were chosen from each tissue section and examined. The examined area was outlined, the positive staining patterns were identified, and the percent positive area in the examined area was then measured. Since ACE and ACE2 are expressed by all cardiac cells in normal and hypertensive heart tissues, the intensity score under low power-fields (×10) was used: (0.5) very weak expression with trace positive staining in most cardiac cells; (1) weak expression as determined by a clear but weakly positive immunostaining; (2) moderate expression as identified by positive signals between week and strong scores; and (3) strong expression as demonstrated by a marked immunostaining in most cardiac cells. For quantitative analysis of phospho-ERK1/2 and phospho-38, nuclear positive cells for pERK1/2 and pP38 were identified and percent positive nuclei were counted as previously described.[14] Data were expressed as the percentage of mean ± SEM All examinations were performed blindly on coded slides.

Cell Culture

A human kidney tubular epithelial cell line (HK-2) was obtained from ATCC (Manassas, VA) and maintained in DMEM/F-12 containing 10% FBS. For all experiments, the cells were grown to confluence on 6- or 12-well plates (Falcon, Franklin Lakes, NJ) and made quiescent by incubation in serum-free DMEM for 24 hours before stimulation with Ang II. All reagents used were certified to be endotoxin free. Cells were stimulated with Ang II at 1 μmol/L for 0, 6, 12, 24, and 48 hours, and at doses of 0, 0.1, 0.25, 0.5, 1, 2, and 4 μmol/L for 24 hours, to examine the time and dose response of ACE and ACE2 expression. All cell cultures were performed in the presence of 0.5 mmol/L EDTA to prevent degradation of Ang II in the cell culture medium.

To inhibit binding of Ang II to its type I and type II receptor, losartan (1 μmol/L) and PD 123319 (1 μmol/L) were used. To inhibit Ang II induced ERK1/2 MAP kinase or p38 MAP kinase activities, inhibitors to ERK1/2 (PD98059, 20 μmol/L) or p38 (SB203580, 10 μmol/L) MAP kinases, and dominant negative (DN) Adv-DN-ERK or Adv-DN-p38 adenovirus were used, respectively. Recombinant adenovirus construct containing bacterial β-galactosidase gene (Adv-β-gal) was used as negative

control. The characterization and transfection of these dominant negative vectors, as well as a negative control, has been well described elsewhere.[14,15] Briefly, HK-2 cells were incubated with the adenovirus at multiplicity of infection of 30 in DMEM for 1 hour, and then made quiescent for 24 hours before stimulation with Ang II. Each experiment was repeated at least thrice throughout the study.

Reverse Transcription and Real-Time PCR

Total RNA from cell culture was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA) and total RNA from paraffin-sections of human kidney and heart samples was extracted by High Pure RNA Paraffin Kit (Roche Applied Science, Mannheim, Germany). Template cDNA was prepared using reverse transcriptase. Real-time PCR was performed with Sybgreen (Bio-Rad, Hercules, CA) and the Opticon real-time PCR machine (MJ Research Inc., Waltham, MA). The specificity of real-time PCR was confirmed via routine agarose gel electrophoresis and Melting-curve analysis. Housekeeping gene GAPDH was used as an internal standard. The primers used in this study are as follows: ACE forward 5′-GCAAGGAGGCAGGCTATGAG-3′ and reverse 5′-CGGGTAAAACTGGAGGATGG-3′[16]; ACE2 forward 5′-CATTGGAGCAAGTGTTGGATCTT-3′ and reverse 5′-GAGCTAATGCATGCCATTCTCA-3′; GAPDH forward 5′-CAATGACCCCTTCATTGACC-3′ and reverse 5′-GTTCACACCCATGACGAACATG.

Western Blot Analysis

Cultured HK-2 cells were lysed and protein was extracted, denatured at 99°C for 5 minutes, and then transferred to a nitrocellulose membrane. Nonspecific binding to the membrane was blocked for 1 hour at room temperature with 5% BSA in Tris-buffered saline buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.1% Tween 20). The membranes were then incubated overnight at 4°C with primary antibodies against ACE, ACE2, phosphorylated ERK1/2, phosphorylated p38 MAP kinases, and GAPDH. After being washed extensively, the membranes then were incubated with horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature in 1% BSA/TBST. The signals were visualized by an enhanced chemiluminescence system (Amersham, Piscataway, NJ).

Statistical Analyses

All data are expressed as the mean ± SEM. Statistical significance was determined with one-way analysis of variance. *t*-tests were used for multiple comparisons. Differences were considered statistically significant at value of $P < 0.05$. Statistical analysis was conducted using STATA V6 (College Station, TX) and Microsoft Excel.

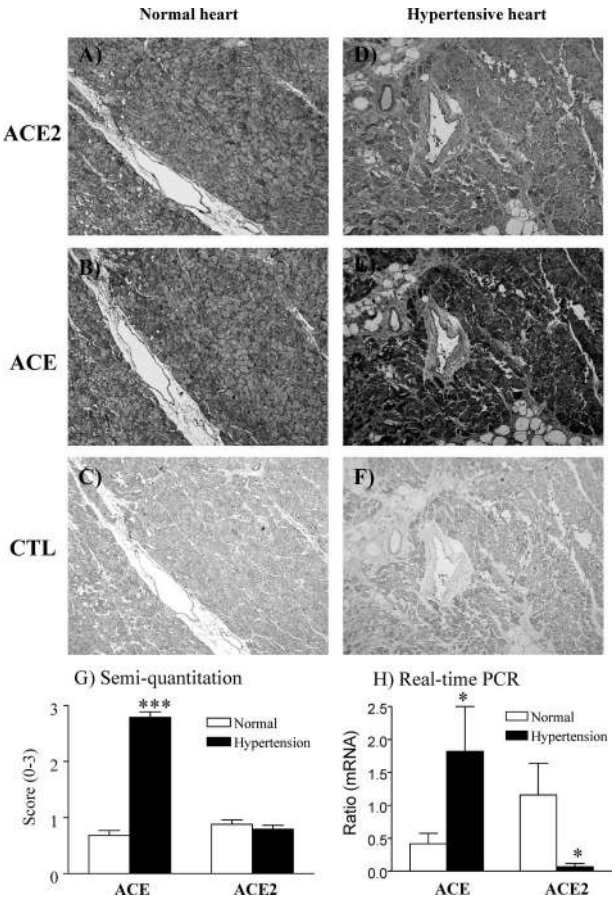

Figure 1. ACE and ACE2 expression in normal and hypertensive heart tissues. Serial sections of normal and hypertensive human hearts are stained with antibodies to ACE and ACE2. **A–C:** Normal human heart tissue stained with antibodies to ACE2 (**A**), ACE (**B**), or a non-immune isotype antibody as a negative control (**C**). **D–F:** A hypertensive human heart tissue stained with antibodies to ACE2 (**D**), ACE (**E**), or a non-immune isotype antibody as a negative control (**F**). **G:** Semiquantitative analysis. Results represent for the mean ± SEM for a group of 8 normal tissues or 12 hypertensive heart tissues. **H:** Results of real-time PCR analysis. Each bar represents the mean ± SEM for a group of six tissues. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to normal. Original magnification ×200.

Results

Expression of ACE and ACE2 in Normal and Hypertensive Human Hearts

We first examined the hypothesis whether ACE is up-regulated and ACE2 is down-regulated in human hearts in hypertension. Serial sections of normal and hypertensive human hearts were examined for ACE and ACE2 expression by immunohistochemistry and real-time PCR. As shown in Figure 1, serial sections of normal and hypertensive cardiopathy showed that there was constitutive ACE2 (Figure 1A) and ACE (Figure 1B) expression in the normal human heart. The presence of hypertension did not appear to significantly alter the level of ACE2 expression (Figure 1D), but up-regulated cardiac ACE (Figure 1E). This was further supported by a semiquantitative analysis (Figure 1G). Consistent with the immunohistochemical findings, real-time PCR demonstrated a twofold increase in mRNA expression of ACE in the hy-

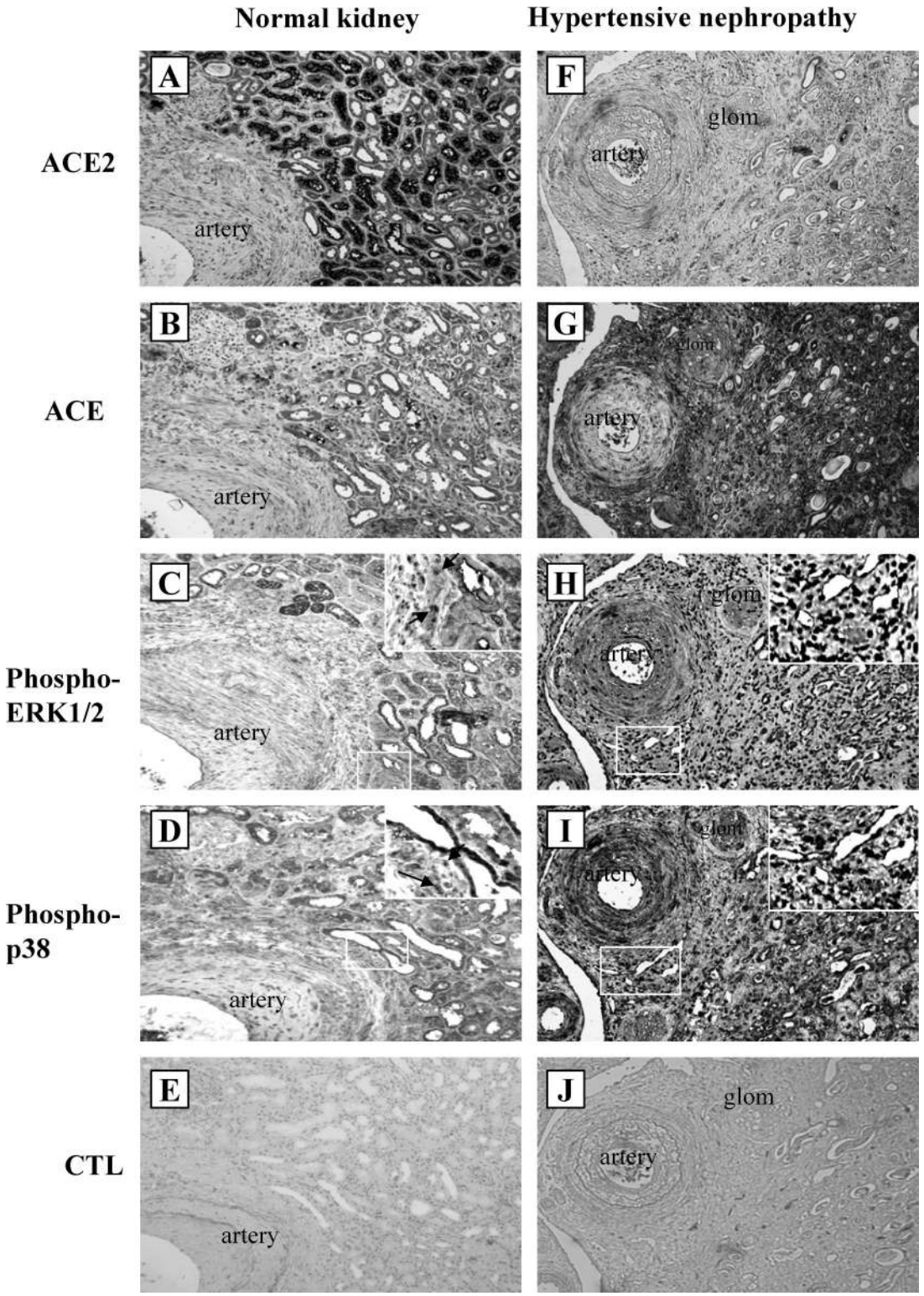

Figure 2. Immunohistochemistry demonstrates that hypertensive nephropathy is associated with an increase in ACE and a decrease in ACE2 expression as well as an increase in ERK1/2 and p38 MAP kinase activation. Serial sections of normal human kidneys and human hypertensive nephropathy are stained with antibodies to ACE, ACE2, phospho-ERK1/2, and phospho-p38 MAP kinases. **A–E:** A normal human kidney tissue stained with antibodies to ACE2 (**A**), ACE (**B**), phospho-ERK1/2 (**C**), phospho-p38 (**D**), or a non-immune isotype antibody as a negative control (**E**). **F–J**: A hypertensive human kidney tissue stained with antibodies to ACE2 (**F**), ACE (**G**), phospho-ERK1/2 (**H**), phospho-p38, (**I**) or a non-immune isotype antibody as a negative control (**J**). It should be noted that a decrease in ACE2 (**F**) and an increase in ACE (**G**) are associated with a marked activation of ERK1/2 and p38 MAP kinases as evidenced by a nuclear staining pattern of phospho-ERK1/2 (**H**) and phospho-p38 (**I**). A clear nuclear staining pattern of both phospho-ERK1/2 and phospho-p38 is further illustrated in the inserted picture (**C, D, H, I**). Original magnification ×200.

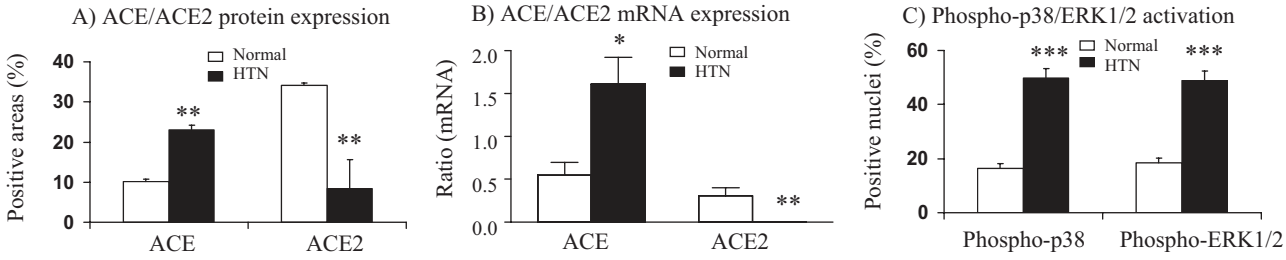

Figure 3. Quantitative analysis of immunohistochemistry and real-time PCR demonstrates that hypertensive nephropathy is associated with an increase in ACE and a decrease in ACE2 expression, as well as an increase in ERK1/2 and p38 MAP kinase activation. **A:** Semiquantitative analysis of ACE and ACE2 immunohistochemical staining. **B:** Detection of renal ACE and ACE2 mRNA expression by real-time PCR. **C:** Semiquantitative analysis of phosphor-p38 and ERK1/2 within the kidney. Each bar represents the mean ± SEM for a group of 8 normal tissues or 12 hypertensive tissues. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to normal.

pertensive heart (Figure 1H). This was associated with a significant decrease in ACE2 mRNA expression (Figure 1H). Antibody controls are shown in Figure 1, C and F.

ACE2 is Down-Regulated and ACE is Up-Regulated in Hypertensive Nephropathy

Immunohistochemically, serial sections of normal and hypertensive human kidneys revealed that a high expression of ACE2 was noted in the normal human kidney (Figure 2A). This was particularly strong in proximal tubular epithelial cells, glomerular epithelial cells, to a lesser extent in distal tubular epithelial cells, and vascular smooth muscle cells (Figure 2A). However, expression of ACE within the normal kidney was moderate (Figure 2B). In hypertensive nephropathy, ACE2 was markedly decreased, particularly in the tubules with severe tubulointerstitial damage (Figure 2F). Importantly, serial sections of immunohistochemistry demonstrated that a marked decrease in ACE2 in the hypertensive nephropathy was tightly associated with a strong up-regulation of ACE (Figure 2G), particularly in the damaged tubulointerstitium (Figure 2F vs 2G). A semiquantitative analysis of ACE and ACE2 expression in both normal and hypertensive kidney were shown in Figure 3A. Indeed, a significant alteration of the balance of ACE:ACE2 from the normal level (ratio 1:3) to hypertensive nephropathy (ratio 3:1) was evident (Figure 3A).

We also examined the expression of ACE mRNA and ACE2 mRNA in both normal and hypertensive kidney disease by real-time PCR. Consistent with the immunohistochemical findings, compared with the normal kidney, real-time PCR demonstrated a more than twofold increase in mRNA expression of ACE in the hypertensive kidney (Figure 3B). This was associated with a dramatic reduction of ACE2 mRNA, virtually undetectable by real-time PCR (ACE2mRNA/GAPDH mRNA Ratio: 0.3073 ± 0.093 in the normal kidney versus 0.000002 ± 0.000001 in hypertensive nephropathy, Figure 3B).

Ang II Is Able to Up-Regulate ACE and Down-Regulate ACE2 Expression in Vitro

Since up-regulation of ACE and down-regulation of ACE2 were apparent in hypertensive nephropathy, a normal human kidney tubular epithelial cell line (HK-2) was used for studying mechanisms of ACE and ACE2 expression in response to Ang II. Quiescent HK-2 cells were stimulated with Ang II for varying time periods and at varying doses and the mRNA and protein were analyzed for ACE and ACE2 expression. As shown in Figure 4, Ang II (1 μmol/L) induced a significant up-regulation of ACE mRNA (Figure 4A) and protein (Figure 4, C and D) in a time-dependent manner, peaking at 24 hours. This was associated with a significant suppression of ACE2 mRNA (Figure 4B) and protein expression (Figure 4, C and E). Similarly, as shown in Figure 5, both real-time PCR (Figure 5, A and B) and Western blot

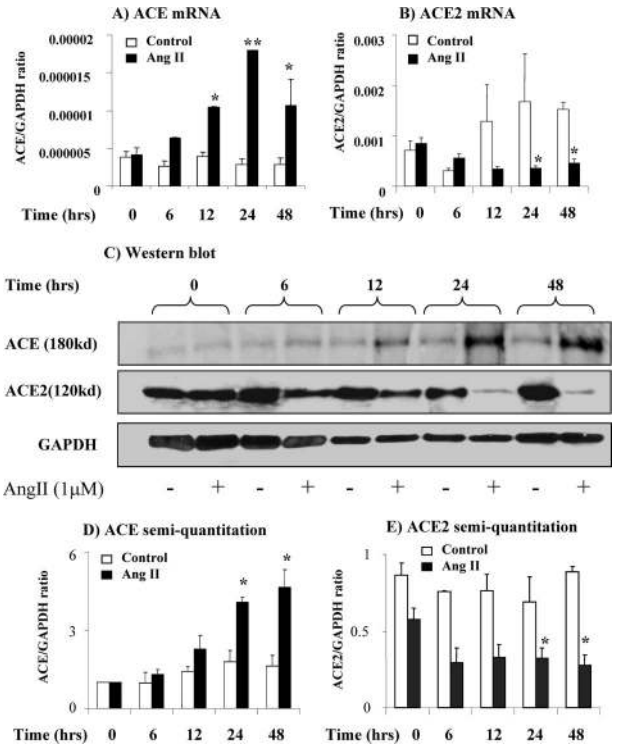

Figure 4. Ang II induces ACE expression and down-regulates ACE2 expression by HK-2 cells in a time-dependent manner. Real-time PCR (**A, B**) and Western blot analyses (**C**; quantification **D, E**) demonstrate that Ang II (1 μmol/L) is able to up-regulate ACE, but down-regulate ACE2 mRNA and protein expression in a time-dependent manner. Data shown represent the mean ± SEM for three independent experiments. Note that in the absence of Ang II stimulation (control) there is no significant change in ACE or ACE2 expression in the entire time course. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the time 0, respectively.

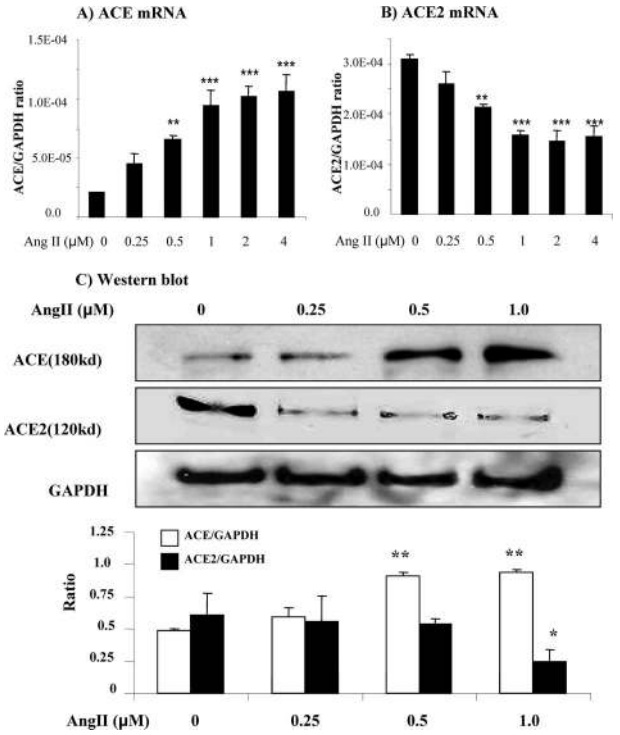

Figure 5. Ang II induces ACE expression and down-regulates ACE2 expression by HK-2 cells in a dose-dependent manner. Real-time PCR (**A, B**) and Western blot analyses (**C**) demonstrate that addition of Ang II is able to up-regulate ACE, but down-regulate ACE2 mRNA (at 24 hours) and protein (at 48 hours) expression in a dose-dependent manner. Note that no further change in ACE and ACE2 mRNA expression is observed beyond 1 μmol/L of Ang II (**A, B**). Data shown represent the mean ± SEM for five independent experiments for mRNA expression and three independent experiments for protein expression. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with time 0, respectively.

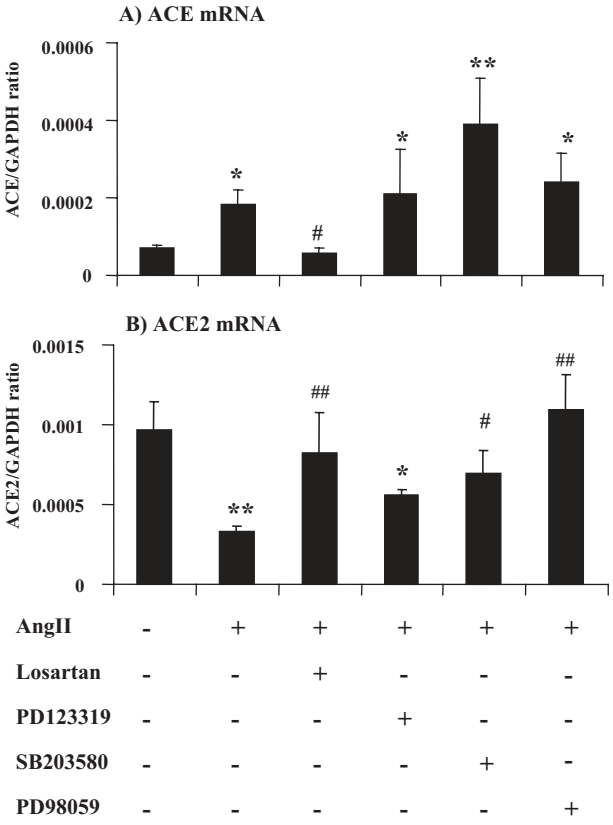

Figure 6. Signaling mechanisms of Ang II-induced up-regulation of ACE and down-regulation of ACE2 at the mRNA levels. Real-time PCR demonstrates that addition of Ang II (1 μmol/L) for 24 hours is able to up-regulate ACE (**A**) and down-regulate ACE2 (**B**) at the mRNA levels, which is blocked by the AT1 receptor antagonist losartan (1 μmol/L), but not by the AT2 receptor antagonist PD 123319 (1 μmol/L). Interestingly, blockade of p38 MAP kinase and ERK1/2 MAP kinase with SB203580 (10 μmol/L) and PD98059 (20 μmol/L) has no effect on Ang II induced up-regulation of ACE mRNA, but abolishes Ang II-induced down-regulation of ACE2 mRNA. Results are expressed as the mean ± SEM for three independent experiments.$^{*}P < 0.05$, $^{**}P < 0.01$ compared to non-Ang II control (the first bar); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to Ang II stimulation alone (the second bar).

analyses (Figure 5C) showed that Ang II significantly up-regulated ACE and down-regulated ACE2 in both mRNA and protein levels in a dose-dependent manner, with the maximal effect at a dose of 1 μmol/L.

Ang II Up-Regulates ACE and Down-Regulates ACE2 Expression via the AT1 Receptor-Mediated, ERK1/2 and p38 MAP Kinases-Dependent Mechanism

Compared to normal kidney (Figure 2A–E), immunohistochemical staining in serial sections showed that up-regulation of ACE and down-regulation of ACE2 in hypertensive nephropathy (Figures 2, F and G, and 3A) was associated with an increase in phosphorylated ERK1/2 (phospho-ERK1/2; Figures 2H and 3C) and phosphorylated p38 (phospho-p38; Figures 2I and 3C; Figure 2J is a negative control) MAP kinases as demonstrated by their nucleated positive pattern. We further tested the hypothesis whether activation of ERK1/2 and p38 Map kinases is responsible for dysregulation of ACE and ACE2 expression in response to Ang II. HK-2 cells were stimulated with Ang II under the presence or absence of losartan (1 μmol/L, a selective AT1 receptor antagonist), PD123319 (1 μmol/L, a selective AT2 receptor antagonist), and inhibitors to ERK1/2 (PD98059, 20 μmol/L) or p38 (SB203580, 10 μmol/L) MAP kinases. As shown in Figures 6 and 7, addition of losartan, but not PD123319, completely blocked Ang II-induced up-regulation of ACE and prevented the reduction of ACE2 in response to Ang II in both mRNA (Figure 6) and protein (Figure 7) levels. Interestingly, blockade of ERK1/2 or p38 MAP kinases with PD98059 or SB203580 prevented Ang II-induced down-regulation of ACE2 in both mRNA and protein levels, but not the effects on Ang II-induced up-regulation of ACE. These observations were further confirmed by inclusion of dominant negative ERK1/2 or p38 adenovirus. As shown in Figures 8 and 9, blockade of ERK1/2 or p38 MAP kinases with Adv-DN-ERK and Adv-DN-P38 was able to abolish Ang II-induced down-regulation of ACE2, but not Ang II-induced up-regulation of ACE.

Discussion

With the discovery of alternative Ang II generating pathways such as chymase and the ACE homologue ACE2 that can degrade Ang I to Ang 1–9 and Ang II to Ang

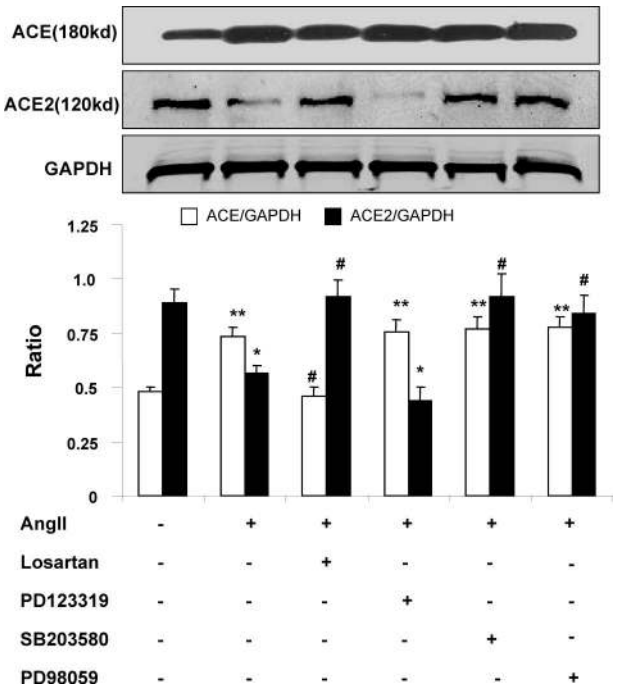

Figure 7. Signaling mechanisms of Ang II-induced up-regulation of ACE and down-regulation of ACE2 at the protein levels. Western blot analysis demonstrates that addition of Ang II (1 μmol/L) for 48 hours is able to up-regulate ACE and down-regulate ACE2 at the protein levels, which is blocked by the AT1 receptor antagonist losartan (1 μmol/L), but not by the AT2 receptor antagonist PD 123319 (1 μmol/L). Blockade of p38 MAP kinase and ERK1/2 MAP kinase with SB203580 (10 μmol/L) and PD98059 (20 μmol/L) has no effect on Ang II-induced up-regulation of ACE, but abolishes Ang II-induced down-regulation of ACE2. Results are expressed as the mean ± SEM for three independent experiments.*$P < 0.05$, **$P < 0.01$ compared to non-Ang II control (the first bar, respectively); #$P < 0.05$ compared to Ang II stimulation alone (the second bar, respectively).

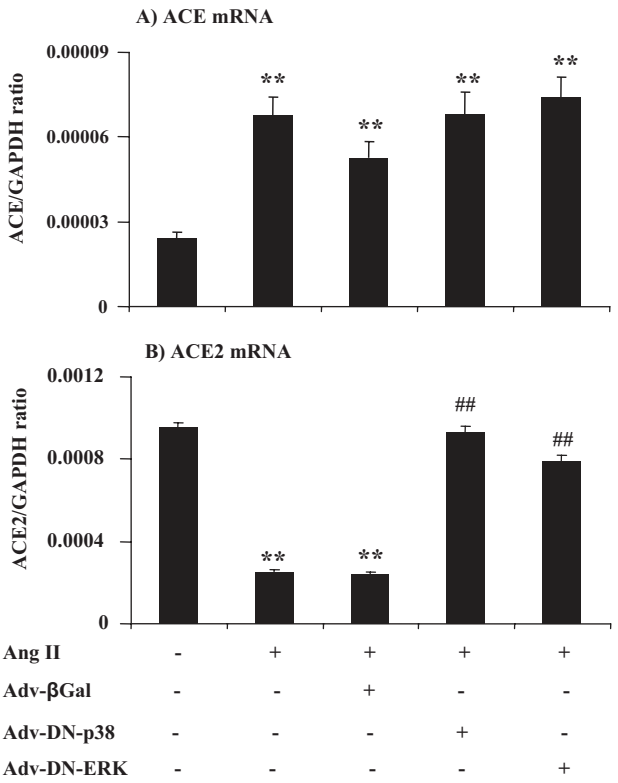

Figure 8. Blockade of ERK1/2 MAP kinase and p38-MAP kinase by dominant negative adenovirus abolishes Ang II-induced down-regulation of ACE2 mRNA. Real-time PCR demonstrates that blockade of ERK1/2 and P38 MAP kinases by Adv-DN-ERK and Adv-DN-P38 (MOI of 30) abolishes Ang II (1 μmol/L)-induced down-regulation of ACE2 (**B**), but has no effect on Ang II-induced up-regulation of ACE mRNA (**A**) at 24 hours. Results are expressed as mean ± SEM for three independent experiments. **$P < 0.01$, ***$P < 0.001$ compared to non-Ang II control (the first bar, respectively); ##$P < 0.01$ compared to Ang II stimulation alone (the second bar, respectively).

1–7,[2,3,17] it is clear that the rennin-angiotensin system is much more complex than once thought. The interaction between ACE and ACE2 is demonstrated by the observation that ACE inhibition results in elevation of Ang 1–7 *in vivo* and blockade of Ang 1–7 with a specific antagonist reverses the antihypertensive effects of lisinopril.[4,6,18] Furthermore, a recent study demonstrated that lentiviral delivery of ACE2 can reverse cardiac hypertrophy in rats, suggesting not only that ACE2 is beneficial, but that manipulation of ACE2 may have therapeutic potential.[19] Direct evidence for ACE2 in the development of hypertensive cardiopathy and kidney disease comes from the ACE2 gene knockout mice. Mice lacking ACE2 exhibit an increase in blood pressure with the development of cardiopathy and glomerulosclerosis in the age-dependent manner.[20–22] These are associated with an increase in local Ang II generation and Ang II-mediated hypertension.[22,23] However, mechanisms of regulating ACE2 under hypertensive conditions remain largely unclear. We sought to address this unknown question in kidney tubular epithelial cells because we noted that normal kidney proximal tubular epithelial cells constitute a rich source of both ACE and ACE2 and, particularly, the kidney is a vital organ in the genesis of hypertension and more so in the context of human disease. The heart is also thought to be an important source of ACE and ACE2. Indeed, we found that up-regulation of ACE, but down-regulation of ACE2 mRNA was noted in hypertensive heart by real-time PCR. However, we could not find a significant reduction in ACE2 expression in the hypertensive heart, immunohistochemically. The discrepancy between mRNA and protein expression of ACE2 may be associated with detecting methods used since real-time PCR is much more sensitive and quantitative technique than the immunohistochemical analysis. In addition, the time length before the tissues were collected and fixed with fixatives and the length of fixation time may also cause this discrepancy between the ACE2 mRNA and protein in the heart.

There were several novel observations in this study. Firstly, the down-regulation of ACE2 expression and up-regulation of ACE in hypertensive nephropathy observed by both real-time PCR and immunohistochemistry suggested that the balance between these two enzymes was altered in hypertension. In the normal kidney, immunohistochemistry demonstrated that there was a threefold increase in ACE2 compared to ACE. However, there was a threefold decrease in ACE2, which was associated with a twofold increase in ACE in the hypertensive nephropathy. Consistent with the immunohistochemical finding, real-time PCR also showed a loss of ACE2 was associated with a marked up-regulation of renal ACE in hypertensive nephropathy. These observations imply that the balance between ACE and ACE2 is critical in the pathogenesis of

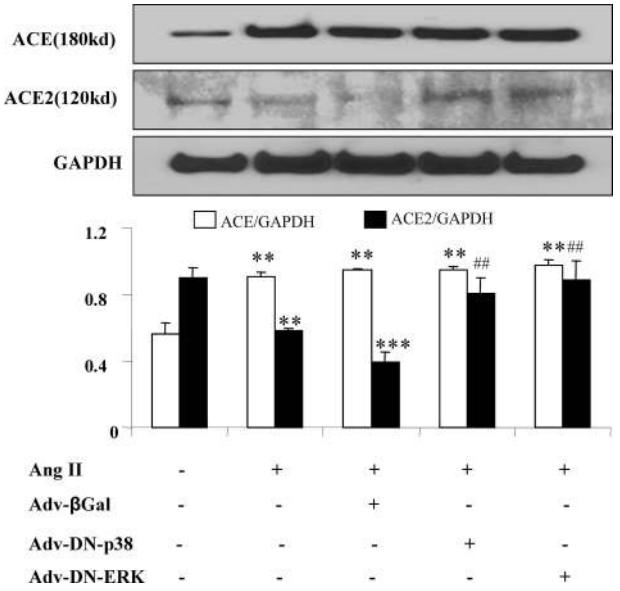

Figure 9. Blockade of ERK1/2 MAP kinase and p38-MAP kinase by dominant negative adenovirus abolishes Ang II-induced down-regulation of ACE2 protein. Western blot analysis demonstrates that blockade of ERK1/2 and P38 MAP kinases by Adv-DN-ERK and Adv-DN-P38 (MOI of 30) abolishes addition of Ang II (1 μmol/L)-induced down-regulation of ACE2 protein, but has no effect on Ang II-induced up-regulation of ACE at 24 hours. Results are expressed as mean ± SEM for three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to non-Ang II control (the first bar, respectively); $^{\#}P < 0.05$ compared to Ang II stimulation alone (the second bar, respectively).

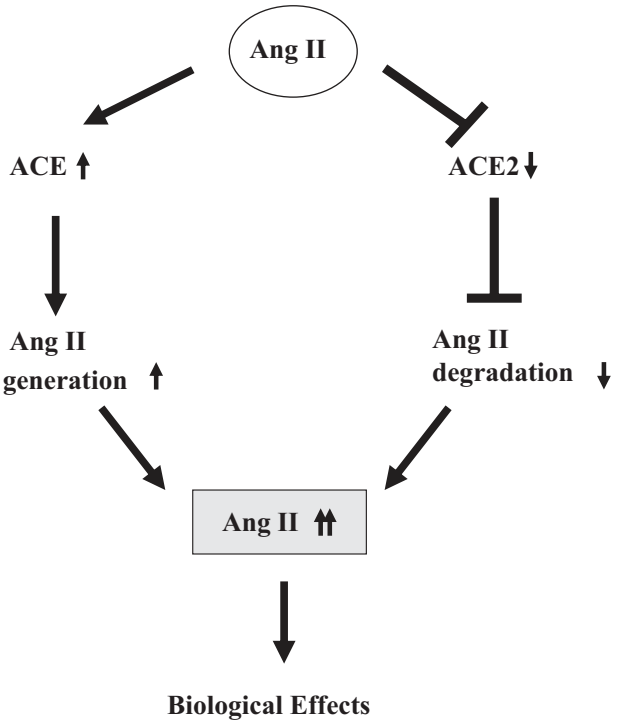

Figure 10. Scheme for Ang II autoregulatory feedback loop leading to its biological effects. Ang II signals through the AT1 receptor to up-regulate the ACE-dependent Ang II generating pathway and down-regulate the ACE2-mediated Ang II degradation pathway, ultimately leading to the elevation of Ang II levels and hypertension.

hypertension in terms of Ang II generation versus degradation within the hypertensive kidney, although other Ang II-generating pathways may be also involved. In the normal kidney, constitutive high levels of ACE2 with higher ratio of ACE2/ACE (3:1) may be associated with an increase in the Ang II breakdown system compared to Ang II generation, which may be important in maintaining the normal physiological and biological effects of Ang II. In contrast, increased ACE and decreased ACE2 with higher ratio of ACE/ACE2 (3:1) in the hypertensive kidney may favor Ang II generation, leading to hypertensive cardiovascular and renal damage. This is consistent with our previous observation that ACE is up-regulated in diabetic nephropathy, particularly in those with hypertension.[7] Indeed blockade of Ang II by either ACE inhibitors or angiotensin receptor blockers does improve progression of hypertensive cardiovascular and kidney diseases.[24–26] Moreover, an association of increased Ang II degradation products with inhibition of ACE activities also suggests the importance of the balance of ACE/ACE2 in hypertension. The present study provides direct evidence that up-regulation of ACE is associated with the down-regulation of ACE2 in human hypertensive kidney disease. This implies that the alteration of ACE/ACE2 ratio may promote the disease progression in hypertension. However, it should be pointed out that there is limitation regarding the findings from human autopsy tissues. This is because the enzyme degradation occurs after death and it is unknown the time lag between the death and tissue collection in these patients. In addition, since all of the patients were treated with either ACE inhibitors or AT1 receptor blockers, it is difficult to exclude the potential effects of anti-hypertensive treatment on expression of ACE and ACE2 within the diseased kidney and heart. These may account for the discrepancy between mRNA and protein expression of ACE2 in the hypertensive heart, although this discrepancy may also be caused by the difference between the detecting methodologies used as discussed above. Nevertheless, the alteration of ACE/ACE2 ratio could be a valuable index for progression of hypertensive disease.

More importantly, we showed that Ang II was able to up-regulate ACE and down-regulate ACE2 in renal tubular epithelial cells and this occurred via the AT1 receptor, because blockade of AT1 receptor, but not AT2 receptor, abolished Ang II-induced up-regulation of ACE and down-regulation of ACE2. This novel finding suggests that there is an Ang II autoregulatory loop within the kidney, which is illustrated in Figure 10. Indeed, the current view is that ACE and ACE2 act to counter-regulate one another.[27,28] Down-regulation of ACE2 by ANG II represents a novel positive feed-forward system as noted in the brain.[29] Once released, Ang II may signal through the AT1 receptor to up-regulate the ACE-dependent Ang II generating pathway and down-regulate the ACE2-mediated Ang II degradation pathway, ultimately leading to the elevation of Ang II levels and hypertension. Thus, Ang II could potentially upset the normal balance between the two ACEs, leading to conditions favoring excess Ang II generation and reduced Ang II breakdown. This would likely lead to more deleterious effects on progression of renal and cardiovascular disease, which commonly coexist in the setting of advanced hypertension and ne-

phropathy. The magnitude order of dose-dependent effect of Ang II on up-regulation of ACE but down-regulation of ACE2 further supports this notion and implicates the interplay between two ACEs in local Ang II generation in response to Ang II. This is consistent with recent findings that loss of ACE2 or inhibition of ACE2 promotes glomerular injury in association with up-regulation of ACE expression in type-1 diabetic mice.[30,31] Nevertheless, breakdown of this autoregulating pathway may be one mechanism of beneficial effects on hypertensive complications with ACE inhibitors and AT1 receptor antagonists.

It should be pointed out that although the present study has identified Ang II as a critical mediator in differentially regulating ACE and ACE2 expression *in vitro*, with significant effect at a dose of 0.5 μmol/L. This does not imply directly to the patients with hypertension because plasma levels of Ang II is normally much lower (10 pmol/L) and may not be elevated during hypertension.[32] However, the doses of Ang II used *in vitro* may relate more to the local effect of Ang II in the kidney, because studies from human and experimental models of hypertensive kidney disease have clearly demonstrated that a marked increase in intrarenal Ang II levels that far exceed plasma Ang II levels occurs.[32] In animal models of hypertension, for example, addition of Ang II infusion results in a severe renal injury with a marked increase in intrarenal Ang II (5740 $\pm$ 260 pmol/L) when compared to plasma Ang II levels (181 $\pm$ 30 pmol/L) after Ang II infusion.[33] This suggests that local production of renal Ang II is much more important in the development of hypertensive nephropathy than the plasma Ang II levels. Indeed, inhibition of Ang II-induced renal injury by AT1 receptor blockade that is associated with a decrease in intrarenal kidney Ang II contents, while enhancing plasma Ang II concentrations, further supports a critical role of local Ang II in hypertensive renal injury.[33,34]

Furthermore, the present study also found that although both induction of ACE and suppression of ACE2 expression in response to Ang II occur through the AT1 receptor, disparate intracellular signaling pathways may operate. Ang II-induced down-regulation of ACE2 was mediated via the ERK1/2 and p38 MAP kinase pathways. This was supported by the observation that reduced ACE2 expression in response to Ang II was abolished by blocking ERK1/2 and p38 MAP kinases with both specific pharmacological inhibitors and Adv-DN-ERK and Adv-DN-P38. In contrast, blockade of ERK/p38 MAP kinases by either specific inhibitors or Adv-DN-ERK/p38 produced no effect on ACE expression in response to Ang II, indicating that an alternative Ang II signaling pathway may be responsible for ACE expression. Although the signaling pathways of ACE are well established,[35] signaling mechanisms of Ang II –induced ACE remain largely unclear and require further investigation.

In summary, we have demonstrated that Ang II, once released, can act to up-regulate ACE but down-regulate ACE2 via the AT1 receptor-mediated mechanism. Activation of the ERK1/2 and p38 MAP kinase pathway may represent a key mechanism by which Ang II down-regulates ACE2.

References

1. Cheung BM, Ong KL, Man YB, Lam KS, Lau CP: Prevalence, awareness, treatment, and control of hypertension: United States National Health and Nutrition Examination Survey 2001–2002. J Clin Hypertens 2006, 2:93–98
2. 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
3. Crackower MA, Sarao R, Oudit GY, Yagil C, Kozieradzki I, Scanga SE, Oliveira-dos-Santos AJ, da CJ, 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
4. Lely AT, Hamming I, van Goor H, Navis GJ: Renal ACE2 expression in human kidney disease. J Pathol 2004, 204:587–593
5. Ferrario CM, Jessup J, Chappell MC, Averill DB, Brosnihan KB, Tallant EA, Diz DI, Gallagher PE: Effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin-converting enzyme 2. Circulation 2005, 20:2605–2610
6. Ferrario CM, Jessup J, Gallagher PE, Averill DB, Brosnihan KB, Ann Tallant E, Smith RD, Chappell MC: Effects of renin-angiotensin system blockade on renal angiotensin-(1–7) forming enzymes and receptors. Kidney Int 2005, 68:2189–2196
7. Huang XR, Chen WY, Truong LD, Lan HY: Chymase is up-regulated in diabetic nephropathy: implications for an alternative pathway of angiotensin II-mediated diabetic renal and vascular disease. J Am Soc Nephrol 2003, 14:1738–1747
8. Campese VM: Salt sensitivity in hypertension. Renal and cardiovascular implications. Hypertension 1994, 23:531–550
9. Guyton AC: Kidneys and fluids in pressure regulation. Small volume but large pressure changes. Hypertension 1992, 19:I2–I8
10. Keller G, Zimmer G, Mall G, Ritz E, Amann K: Nephron number in patients with primary hypertension. N Engl J Med 2003, 348:101–108
11. Brenner BM, Chertow GM: Congenital oligonephropathy and the etiology of adult hypertension and progressive renal injury. Am J Kidney Dis 1994, 23:171–175
12. Rettig R, Stauss H, Folberth C, Ganten D, Waldherr B, Unger T: Hypertension transmitted by kidneys from stroke-prone spontaneously hypertensive rats. Am J Physiol 1989, 257:F197–F203
13. Patschan O, Kuttler B, Heemann U, Uber A, Rettig R: Kidneys from normotensive donors lower blood pressure in young transplanted spontaneously hypertensive rats. Am J Physiol 1997, 273:R175–R180
14. Koka V, Wang W, Huang XR, Kim-Mitsuyama S, Truong LD, Lan HY: Advanced glycation end products activate a chymase-dependent angiotensin II-generating pathway in diabetic complications. Circulation 2006, 113:1353–1360
15. Hamaguchi A, Kim S, Yano M, Yamanaka S, Iwao H: Activation of glomerular mitogen-activated protein kinases in angiotensin II-mediated hypertension. J Am Soc Nephrol 1998, 9:372–380
16. Studer R, Reinecke H, Muller B, Holtz J, Just H, Drexler H: Increased angiotensin-I converting enzyme gene expression in the failing human heart. Quantification by competitive RNA polymerase chain reaction. J Clin Invest 1994, 94:301–310
17. Doggrell SA, Wanstall JC: Vascular chymase: pathophysiological role and therapeutic potential of inhibition. Cardiovasc Res 2004, 61:653–662
18. Igase M, Strawn WB, Gallagher PE, Geary RL, Ferrario CM: Angiotensin II AT1 receptors regulate ACE2 and angiotensin-(1-7) expression in the aorta of spontaneously hypertensive rats. Am J Physiol Heart Circ Physiol 2005, 289:H1013–H1019
19. Huentelman MJ, Grobe JL, Vazquez J, Stewart JM, Mecca AP, Katovich MJ, Ferrario CM, Raizada MK: Protection from angiotensin II-induced cardiac hypertrophy and fibrosis by systemic lentiviral delivery of ACE2 in rats. Exp Physiol 2005, 90:783–790
20. Oudit GY, Kassiri Z, Patel MP, Chappell M, Butany J, Backx PH, Tsushima RG, Scholey JW, Khokha R, Penninger JM: Angiotensin II-mediated oxidative stress and inflammation mediate the age-dependent cardiomyopathy in ACE2 null mice. Cardiovasc Res 2007, 75:29–39
21. Oudit GY, Herzenberg AM, Kassiri Z, Wong D, Reich H, Khokha R, Crackower MA, Backx PH, Penninger JM, Scholey JW: Loss of an-

giotensin-converting enzyme-2 leads to the late development of angiotensin II-dependent glomerulosclerosis. Am J Pathol 2006, 168:1808–1820
22. Yamamoto K, Ohishi M, Katsuya T, Ito N, Ikushima M, Kaibe M, Tatara Y, Shiota A, Sugano S, Takeda S, Rakugi H, Ogihara T: Deletion of angiotensin-converting enzyme 2 accelerates pressure overload-induced cardiac dysfunction by increasing local angiotensin II. Hypertension 2006, 47:718–726
23. Gurley SB, Allred A, Le TH, Griffiths R, Mao L, Philip N, Haystead TA, Donoghue M, Breitbart RE, Acton SL, Rockman HA, Coffman TM: Altered blood pressure responses and normal cardiac phenotype in ACE2-null mice. J Clin Invest 2006, 116:2218–2225
24. Weir MR, Dzau VJ: The renin-angiotensin-aldosterone system: a specific target for hypertension management. Am J Hypertens 1999,12: 205S–213S
25. Remuzzi G, Perico N, Macia M, Ruggenenti P: The role of renin-angiotensin-aldosterone system in the progression of chronic kidney disease. Kidney Int 2005, 15(Suppl):S57–S65
26. Burnier M, Zanchi A: Blockade of the renin-angiotensin-aldosterone system: a key therapeutic strategy to reduce renal and cardiovascular events in patients with diabetes. J Hypertens 2006, 24:11–25
27. Brosnihan KB, Neves LA, Chappell MC: Does the angiotensin converting enzyme (ACE)/ACE2 balance contribute to the fate of angiotensin peptides in programmed hypertension. Hypertension 2005, 46:1097–1099
28. Iyer SN, Ferrario CM, Chappell MC: Angiotensin-(1-7) contributes to the antihypertensive effects of blockade of the renin-angiotensin system. Hypertension 1998, 31:356–361
29. Gallagher PE, Chappell MC, Ferrario CM, Tallant EA: Distinct roles for ANG II and ANG-(1-7) in the regulation of angiotensin-converting enzyme 2 in rat astrocytes. Am J Physiol Cell Physiol 2006, 290:C420–C426
30. Soler MJ, Wysocki J, Ye M, Lloveras J, Kanwar Y, Batlle D: ACE2 inhibition worsens glomerular injury in association with increased ACE expression in streptozotocin-induced diabetic mice. Kidney Int 2007, 72:614–623
31. Wong DW, Oudit GY, Reich H, Kassiri Z, Zhou J, Liu QC, Backx PH, Penninger JM, Herzenberg AM, Scholey JW: Loss of angiotensin-converting enzyme-2 (Ace2) accelerates diabetic kidney injury. Am J Pathol 2007, 17:438–451
32. Kobori H, Nangaku M, Navar LG, Nishiyama A: The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. Pharmacol Rev 2007, 59:251–287
33. Nishiyama A, Seth DM, and Navar LG: AT_1 receptor-mediated augmentation of renal interstitial fluid angiotensin II in angiotensin II-induced hypertension. J Hypertens 2003, 21:1897–1903
34. Kobori H, Prieto-Carrasquero MC, Ozawa Y, Navar LG: AT1 receptor mediated augmentation of intrarenal angiotensinogen in angiotensin II-dependent hypertension. Hypertension 2004, 43:1126–1132
35. Fleming I: Signaling by the angiotensin-converting enzyme. Circ Res 2006, 98:887–896