Hypertension

Effect of Angiotensin-Converting Enzyme Inhibition and Angiotensin II Receptor Blockers on Cardiac Angiotensin-Converting Enzyme 2

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Background—Angiotensin-converting enzyme 2 (ACE2) has emerged as a novel regulator of cardiac function and arterial pressure by converting angiotensin II (Ang II) into the vasodilator and antitrophic heptapeptide, angiotensin-(1–7) [Ang-(1–7)]. As the only known human homolog of ACE, the demonstration that ACE2 is insensitive to blockade by ACE inhibitors prompted us to define the effect of ACE inhibition on the ACE2 gene.

Methods and Results—Blood pressure, cardiac rate, and plasma and cardiac tissue levels of Ang II and Ang-(1–7), together with cardiac ACE2, neprilysin, Ang II type 1 receptor (AT_1), and *mas* receptor mRNAs, were measured in Lewis rats 12 days after continuous administration of vehicle, lisinopril, losartan, or both drugs combined in their drinking water. Equivalent decreases in blood pressure were obtained in rats given lisinopril or losartan alone or in combination. ACE inhibitor therapy caused a 1.8-fold increase in plasma Ang-(1–7), decreased plasma Ang II, and increased cardiac ACE2 mRNA but not cardiac ACE2 activity. Losartan increased plasma levels of both Ang II and Ang-(1–7), as well as cardiac ACE2 mRNA and cardiac ACE2 activity. Combination therapy duplicated the effects found in rats medicated with lisinopril, except that cardiac ACE2 mRNA fell to values found in vehicle-treated rats. Losartan treatment but not lisinopril increased cardiac tissue levels of Ang II and Ang-(1–7), whereas none of the treatments had an effect on cardiac neprilysin mRNA.

Conclusions—Selective blockade of either Ang II synthesis or activity induced increases in cardiac ACE2 gene expression and cardiac ACE2 activity, whereas the combination of losartan and lisinopril was associated with elevated cardiac ACE2 activity but not cardiac ACE2 mRNA. Although the predominant effect of ACE inhibition may result from the combined effect of reduced Ang II formation and Ang-(1–7) metabolism, the antihypertensive action of AT_1 antagonists may in part be due to increased Ang II metabolism by ACE2. (***Circulation***. 2005;111:2605-2610.)

Key Words: angiotensin ■ receptors ■ inhibitors ■ genetics ■ myocardium

Angiotensin-converting enzyme 2 (ACE2) is a carboxy-monopeptidase with a preference for hydrolysis between proline and carboxy-terminal hydrophobic residues[1,2] that is found both as a membrane-associated and as a secreted enzyme in cardiovascular, neuronal, and reproductive organs.[1,3,4] Although ACE2 shares 42% sequence identity and 61% sequence similarity with ACE,[5–9] ACE2 activity is not prevented by ACE inhibition because the active site of the enzyme in which the S2′ pocket resides is smaller than the corresponding pocket in ACE.[10] The early suggestion that ACE2 participated in the cleavage of angiotensin I (Ang I) into the nonapeptide Ang-(1–9)[1] was not confirmed by more rigorous studies of the kinetics of angiotensin peptide metabolism. Vickers et al[9] and Rice et al[10] showed that Ang I is hydrolyzed very slowly by ACE2, whereas Ang II was hydrolyzed into Ang-(1–7), with the highest catalytic efficiency seen for any of the angiotensin peptides. Genetic

studies and ablation of the ACE2 gene in mice suggest that ACE2 may play a critical role in the regulation of cardiac function, because ACE2-null mice exhibited a phenotype of cardiomyopathy associated with left ventricular dysfunction.[11] Moreover, renal ACE2 gene expression was significantly reduced in salt-sensitive Sabra hypertensive rats and both spontaneously hypertensive and stroke-prone, spontaneously hypertensive rats.[11]

The discovery of ACE2's preferential catalytic activity for Ang II conversion to Ang-(1–7) brought to the forefront the complexity of the biochemical mechanisms that regulate the kinetics of angiotensin peptide formation and metabolism. In addition, identification of ACE2 provided a more solid foundation for a physiological role for Ang-(1–7) in counteracting the vasopressor and growth-promoting cellular actions of Ang II.[12] In this emerging field of ACE2 research, limited information currently exists about the mechanisms for the

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regulation of ACE2 in both physiology and pathology. Evidence of a role for ACE2 in the human heart is provided by (1) the study of Crackower et al[11] in ACE2-null mice, (2) the demonstration that Ang-(1–7) was increased in the hearts of patients receiving ACE inhibitors,[13] and (3) the finding of ACE2 upregulation in ventricular membrane preparations from failing human heart ventricles.[14] On the other hand, a report by Campbell and associates[15] implied that there was a minor role for ACE2 in Ang-(1–7) formation in subjects with heart failure. Technical limitations, the presence of confounding treatments among subjects included in the studies, and the pooling of data from subjects medicated with various ACE inhibitors significantly limit interpretation of these data.

A potential role for ACE2 in contributing to the cardioprotective effect of Ang-(1–7) was demonstrated by Loot et al,[16] who found that long-term infusions of Ang-(1–7) reversed cardiac dysfunction and restored vascular endothelial responses in animals after myocardial infarction. The findings that Ang-(1–7) has antiarrhythmic actions[17] and that ischemia caused significant increases in Ang-(1–7) in myocytes within the noninfarcted regions of the cardiac ventricle[18] indicated a need to investigate a potential role for ACE2 in regulating the formation of Ang-(1–7) from Ang II.

The present investigation is based on our recent finding that administration of either losartan or olmesartan for 28 days after coronary artery ligation was accompanied by upregulation of cardiac ACE2 mRNA associated with increases in plasma Ang-(1–7) levels.[19] The present study was designed to answer 2 questions: (1) Is the effect of long-term Ang II type 1 (AT_1) receptor blockade influenced by the cardiac remodeling induced by myocardial ischemia? (2) Would blockade of Ang II synthesis cause a similar effect? To answer these questions, we examined the independent effects of 12-day administration of the ACE inhibitor lisinopril and the AT_1 receptor blocker losartan on blood pressure and on plasma and cardiac tissue concentrations of Ang II and Ang-(1–7), as well as the expression of ACE, ACE2, neprilysin, and the AT_1 and *mas* receptor mRNAs in the ventricles of Lewis normotensive rats. In addition, similar measures were determined in animals with combined exposure to both lisinopril and losartan. The rigorous examination of these questions included direct assessment of ACE2 activity in cardiac membranes obtained from the same animals given the various medications.

Methods

Animals
Male Lewis rats (270 to 280 g; Charles River Laboratory, Wilmington, Mass) were housed in individual cages (12-hour light/dark cycle) with ad libitum access to rat chow and tap water. Procedures complied with the policies implemented by our institutional Animal Care and Use Committee.

Experimental Protocol
Thirty-six normotensive rats (age range, 8 to 10 weeks) were randomly assigned to drink either tap water (vehicle, n=12) or tap water to which lisinopril (10 mg · kg^{-1} · d^{-1}, n=8), losartan (10 mg · kg^{-1} · d^{-1}, n=8), or both drugs at the same doses (n=8) were added to their drinking water for 12 consecutive days.

During the treatment period, tail-cuff systolic blood pressure was determined for 3 days before and at regular intervals after com-

mencement of the medications. At the end of the treatment period, rats were anesthetized with halothane (1.5%, Halocarbon Laboratories) for direct measurement of arterial pressure and heart rate with a catheter-tip pressure transducer inserted into a carotid artery (model SPR-671, Millar Instrument Co). A 5-mL sample of arterial blood was obtained for laboratory measurements, and after this procedure, deeply anesthetized rats were humanely killed by cardiopulmonary excision. The heart was rinsed in saline, and the cardiac ventricles were separated from the atria, weighed, and cut transversely from apex to base. A segment of the ventricles was quick-frozen in LN_2 and stored at $-80°C$, whereas the remaining tissue was frozen in dry ice for later analysis of angiotensin peptide content.

Biochemistry
Blood was collected into chilled blood-collection tubes containing a mixture of peptidase inhibitors: 25 mmol/L EDTA, 0.44 mmol/L 1,20-orthophenanthrolene monohydrate (Sigma), 1 mmol/L sodium parachloromercuribenzoate, and 3 μmol/L WFML (rat renin inhibitor: acetyl-His-Pro-Phe-Val-Statine-Leu-Phe). After 30 minutes on ice, blood cells were isolated by centrifugation at 3000g for 20 minutes, and aliquots of plasma were stored at $-80°C$ until use. Frozen tissues were rapidly weighed and homogenized as described elsewhere.[19] Plasma and cardiac tissue homogenates were extracted on Sep-Pack columns. The eluate was divided for the assay of Ang II and Ang-(1–7) as described.[19] Recovery of radiolabeled peptide averaged >65%, and results were corrected for recovery. Ang II was measured with the Alpoc Diagnostics kit. Ang-(1–7) was measured with an antibody produced by our laboratory.[19] The minimum detectable levels of the assays were 0.8 pg/mL for Ang II and 2.5 pg/mL for Ang-(1–7). The intra-assay and interassay coefficients of variation for Ang II were 12% and 22%, and for Ang-(1–7) were 8% and 20%, respectively. ACE activity was determined in plasma samples by a fluorometric assay, as reported elsewhere.[20]

A new method is described here for the measurement of ACE2 activity, based on determining the rate of exogenous ^{125}I-Ang II conversion to ^{125}I-Ang-(1–7) in cardiac membranes isolated from contiguous regions of the left ventricle. Cardiac tissue was weighed and homogenized in 10 mmol/L HEPES (pH 7.4), 120 mmol/L NaCl, and 10 μmol/L $ZnCl_2$ and centrifuged at 30 000g for 20 minutes. The supernatant was removed, and the membranes were resuspended in the aforementioned buffer containing 0.5% Triton X-100 and incubated overnight on ice at 4°C. After centrifugation, the soluble portion was incubated with ^{125}I-Ang II (2×10^6 counts per minute, 2200 Curies/mmol) in the HEPES buffer containing 10 μmol/L amastatin, bestatin, SCH39370 (neprilysin inhibitor), chymostatin, lisinopril, benzyl succinate, and Z-prolyl prolinal at 37°C from 10 to 120 minutes, and the reaction was terminated by addition of 1% phosphoric acid. The samples were centrifuged and the supernatants were filtered through a 0.22-μm membrane before high-performance liquid chromatography (HPLC) analysis. Ang-(1–7) was resolved from Ang II on a reverse-phase HPLC apparatus (Shimadzu) equipped with a Waters NovaPak C18 column (2.1$\times$150 mm). We used a linear gradient from 10% to 25% mobile phase B for 20 minutes and an isocratic phase at 25% for 15 minutes at a flow rate of 0.35 mL/min at ambient temperature. The solvent system consisted of 0.1% phosphoric acid (mobile phase A) and 80% acetonitrile/0.1% phosphoric acid (mobile phase B).[21] The eluted radioactive peaks were identified with an in-line gamma detector (BioScan), and the data were analyzed with Shimadzu (version 7.2.1) acquisition software. ACE2 activity was expressed as the amount of Ang-(1–7) generated per minute per milligram of protein that was inhibited by addition of 10 μmol/L of the ACE2 inhibitor MLN-4760.[22] ^{125}I-Ang II was iodinated by the chloramine T method and purified by HPLC.[21]

RNA Isolation and Reverse Transcriptase/Real-Time Polymerase Chain Reaction
RNA was isolated from heart tissue with Trizol reagent (GIBCO Invitrogen). RNA concentration and integrity were assessed on an
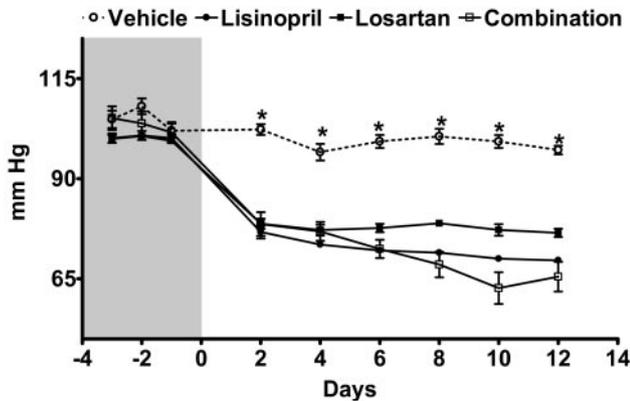
Figure 1. Time course of tail-cuff systolic blood pressure in conscious, normotensive, Lewis rats given vehicle, lisinopril, losartan, or both drugs for 12 days. Values are mean±SEM. *$P<0.05$ compared with all other groups.

Agilent 2100 bioanalyzer with an RNA 6000 Nano LabChip (Agilent Technologies), and real-time polymerase chain reaction was performed. The results were quantified as Ct values, where Ct is defined as the threshold cycle of the polymerase chain reaction at which the amplified product is first detected, and expressed as the ratio of target to control.

Statistical Analysis

All values are expressed as mean±SEM. One-way ANOVA followed by a 2-tailed Student t test was used for comparing the differences at a probability value <0.05.

Results

Comparable decreases in tail-cuff systolic arterial pressure were obtained in Lewis rats medicated with lisinopril, losartan, or both drugs in combination (Figure 1). Treatments had no effect on cardiac rate.

Figure 2 shows the effects of the treatments on plasma concentrations of Ang II and Ang-(1–7). As documented in previous studies,[23] plasma Ang II and Ang-(1–7) levels were comparable in vehicle-treated rats, averaging 54±8 fmol/mL and 31±7 fmol/mL, respectively ($P>0.05$). This was also reflected in the ratio of Ang-(1–7) to Ang II, which in this group averaged 0.83±0.16. Twelve days of ACE inhibitor therapy caused significant decreases in plasma Ang II and a 252% rise in plasma Ang-(1–7) concentration. Losartan-treatment caused a 516% increase in plasma Ang II levels and a 65% rise in plasma Ang-(1–7) when compared with vehicle-treated rats. Combination therapy mimicked the effects obtained in rats medicated with lisinopril alone, because plasma Ang II was again reduced below the values obtained in vehicle-treated rats, whereas plasma Ang-(1–7) levels rose to values not different from those in lisinopril-treated rats. Compared with vehicle-treated rats, the Ang-(1–7):Ang II ratio in lisinopril-treated rats rose to 3.57±0.28 ($P<0.001$), whereas it decreased to 0.16±0.02 in rats medicated with losartan ($P<0.001$). In animals receiving combination therapy, the Ang-(1–7):Ang II ratio averaged 4.87±0.28 ($P<0.05$). Plasma ACE activity in animals given lisinopril or both drugs in combination was reduced by 93% and 92%, respectively, when compared with vehicle-treated animals (57.9±4.8 nmol · min^{-1} · mL^{-1}, $P<0.001$). In rats medicated
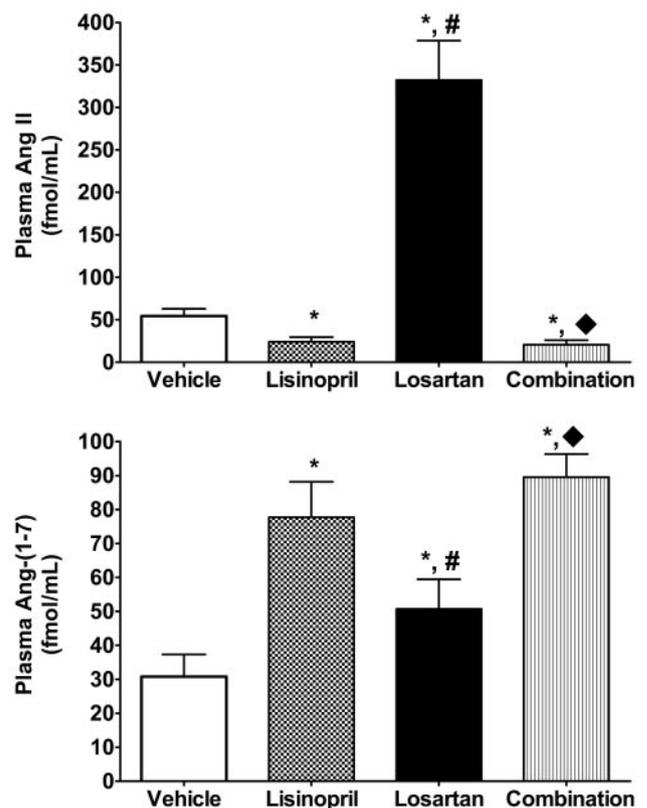

Figure 2. Plasma concentrations of Ang II and Ang-(1–7) in normotensive Lewis rats given either vehicle or medication(s) for 12 days. Values are mean±SEM. *$P<0.05$ vs vehicle; #$P<0.05$ vs lisinopril; ◆$P<0.05$ vs losartan.

with losartan, plasma ACE activity (49.8±4.1 nmol · min^{-1} · mL^{-1}) was not different from that in vehicle-treated controls ($P>0.05$).

Figure 3 shows that in vehicle-treated rats, cardiac levels of both Ang II and Ang-(1–7) were present at concentrations approximately 10-fold lower than those in plasma. In vehicle-treated rats, cardiac Ang II and Ang-(1–7) were present at comparable concentrations, as reflected by a 1.07±0.13 Ang-(1–7):Ang II ratio. ACE inhibition had no effect on the concentrations of Ang II and Ang-(1–7) in the left ventricle, although the Ang-(1–7):Ang II ratio increased to 1.57±0.37 ($P>0.05$). Blockade of AT_1 receptors with losartan was associated with significant increases in left ventricular levels of both Ang II and Ang-(1–7) and, indicative of their parallel increase, the Ang-(1–7):Ang II ratio rose to 1.21±0.17. Combination therapy reduced the cardiac concentration of Ang II to values comparable to those found in vehicle- and lisinopril-treated rats, whereas cardiac Ang-(1–7) levels remained significantly elevated compared with those in vehicle-treated animals. As a result of the inverse changes in Ang II and Ang-(1–7), the Ang-(1–7):Ang II ratio in the left ventricles of rats medicated with both lisinopril and losartan rose to 2.30±0.92.

Figure 4 documents that the various medications had no significant effect on the expression of ACE mRNA in the left ventricles of Lewis rats, whereas cardiac ACE2 mRNA increased by 4.7-fold or 2.8-fold in rats medicated with either
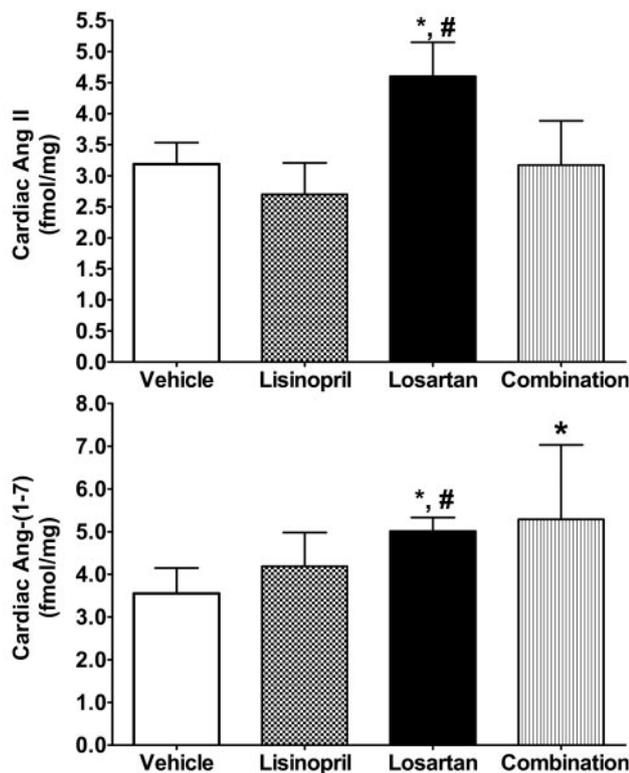
Figure 3. Cardiac tissue concentrations of Ang II and Ang-(1–7) in normotensive Lewis rats given either vehicle or medication(s) for 12 days. Values are mean±SEM. *$P<0.05$ vs vehicle; #$P<0.05$ vs lisinopril.

lisinopril or losartan, respectively. Combination therapy reduced cardiac tissue ACE2 mRNA levels to values not different from those in vehicle-treated rats. The various treatments had no effect on neprilysin mRNA, whereas cardiac *mas* receptor mRNA, implicated as a site for Ang-(1–7) binding,[24] was decreased significantly in rats given losartan (0.58±0.12 versus 1.04±0.13 U in vehicle-treated rats, $P<0.02$). Cardiac AT_2 mRNA was not detected in any of the rats assigned to the various regimens.

Figure 5 contrasts the effects of lisinopril and losartan, given alone or in combination, on cardiac ACE2 activity. Rats medicated with losartan or the combination of losartan and lisinopril showed significant increases in ACE2 activity, whereas ACE2 activity did not change in the lisinopril-treated group.

Discussion

In this study in Lewis rats, we rigorously examined the comparative effect of 12-day treatment with an ACE inhibitor, an AT_1 receptor blocker, or both drugs combined on plasma and cardiac levels of Ang II and Ang-(1–7) and the expression of ACE and ACE2 in their hearts. Although the findings of the present study are consistent with our previous demonstration that blockade of AT_1 receptors is accompanied by a significant increase in cardiac ACE2 mRNA,[19] we now report that inhibition of Ang II synthesis also increases cardiac ACE2 gene transcription. Although ACE2 activity is not inhibited by ACE inhibitors,[10] the new finding suggests
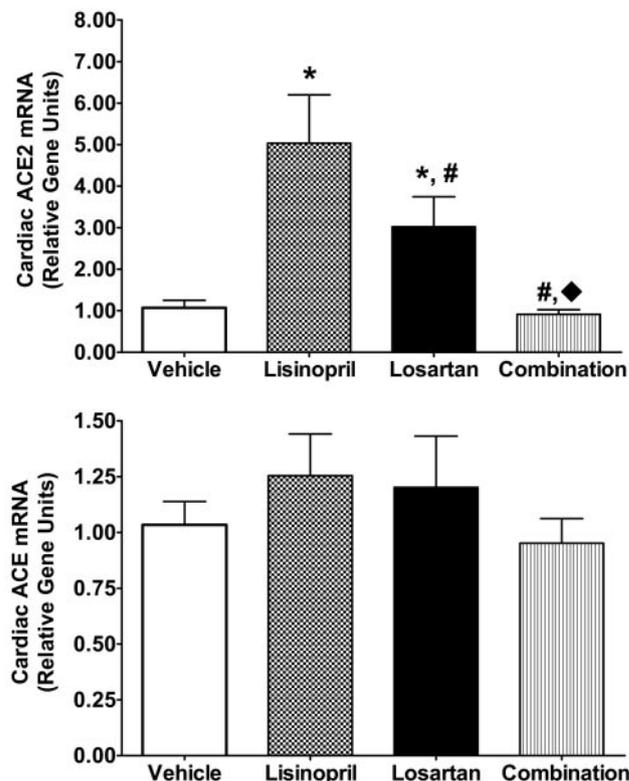

Figure 4. Expression of ACE2 and ACE genes in left ventricle of Lewis rats given either the vehicle or various medication(s) for 12 days. Values are mean±SEM. *$P<0.05$ vs vehicle; #$P<0.05$ vs lisinopril; ◆$P<0.05$ vs losartan.

that a product resulting from inhibition of the hydrolytic activity of ACE regulates ACE2 mRNA but not ACE2 activity. Direct assessment of the endogenous metabolism of Ang II to Ang-(1–7), as reflected by the measurement of ACE2 activity in cardiac membranes from the same animals, showed that the increase in cardiac ACE2 mRNA was correlated with increased cardiac ACE2 activity in animals medicated with losartan and in those receiving both medications. The increase in cardiac ACE2 mRNA induced by blockade of Ang II production or activity was not caused by a hemodynamic effect of the drugs, because all treatment
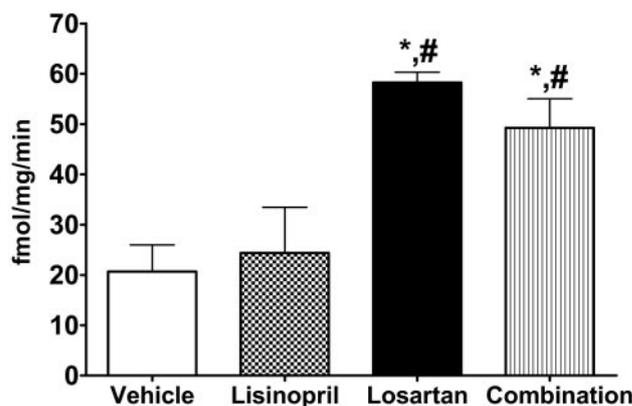

Figure 5. Effects of various treatments on ACE2 activity in Lewis rats. Values are mean±SEM. *$P<0.05$ vs vehicle; #$P<0.05$ versus lisinopril.

combinations produced comparable decreases in blood pressure. Furthermore, neither losartan nor lisinopril induced changes in cardiac neprilysin mRNA (data not shown), a primary pathway for the conversion of circulating Ang I to Ang-(1–7).[25] Although the effect of the treatments on cardiac ACE2 mRNA may be best explained by a negative-feedback signal mediated by AT_1 receptor modulation of the ACE2 gene, our findings do not exclude the possibility of a more complex signaling mechanism involving a negative effect of Ang-(1–7) on AT_1 receptors or alternatively, an action of another unidentified product hydrolyzed by ACE2.

Blockade of AT_1 receptors with either losartan or olmesartan induced significant increases in ACE2 mRNA in the viable myocardium of rats given the drugs for the first 28 days after coronary artery ligation.[19] The present experiments confirmed a direct effect of the AT_1 receptor in the modulation of cardiac ACE2 mRNA while now also documenting a comparable effect of ACE inhibition on cardiac ACE2 mRNA. Correlative determinations of ACE2 activity in cardiac membranes from left ventricles showed increased ACE2 activity in rats medicated with either losartan or the combination of lisinopril and losartan. Because cardiac ACE2 activity was measured as the rate at which Ang II was converted to Ang-(1–7), these findings suggest a key role for ACE2 in the metabolism of cardiac Ang II after blockade of AT_1 receptors. The specificity of the ACE2 assay was further demonstrated by inhibition of Ang II metabolism in the presence of the selective ACE2 inhibitor MLN-4760.[22] Therefore, our findings are concordant with the suggestion that ACE2 is involved in the conversion of Ang II to Ang-(1–7).

There was a lack of concordance between increased ACE2 mRNA and ACE2 activity in rats medicated with lisinopril alone or in those receiving combination therapies. In rats medicated with lisinopril, increased cardiac ACE2 gene transcription did not elicit a comparable increase in ACE2 activity. The addition of lisinopril to animals receiving losartan abolished the increase in ACE2 mRNA, although ACE2 activity remained as high as that found in animals given losartan alone. The absence of parallelism between gene expression and protein levels has been documented in many studies; therefore, it is not surprising that we did not find a direct correlation between cardiac ACE2 mRNA and cardiac ACE2 activity. Although our experiments do not shed light on the mechanism(s) that may account for these differences, some insight may be gained by examining the effects of lisinopril on plasma and cardiac tissue levels of Ang II and Ang-(1–7). As reported elsewhere,[26,27] ACE inhibition alone or in combination with losartan[20] increases plasma Ang-(1–7) while abating plasma Ang II. This was reflected by a 358% increase in the Ang-(1–7):Ang II ratio. Because ACE is the primary pathway for the metabolism of Ang-(1–7),[25] blood levels of Ang-(1–7) may predominantly reflect inhibition of Ang-(1–7) metabolism by ACE. Rice et al[10] showed that Ang-(1–7) did not inhibit cleavage of Ang II by ACE2. In agreement with these findings, we showed that Ang II downregulates ACE2 mRNA in astrocytes and neonatal rat myocytes in culture, an action that is blocked by the AT_1 receptor antagonists losartan and valsartan but not the AT_2

receptor antagonist PD123319.[4,28] These findings suggest that reduced plasma Ang II levels during ACE therapy may be a stimulus for increased ACE2 mRNA. Further work will be necessary to elucidate the mechanisms by which lisinopril influences transcriptional control of ACE2 without changes in ACE2 activity. The reduced ACE2 gene expression when lisinopril was added to losartan suggests that ACE inhibitor therapy can override the signal that modulates ACE2 mRNA. It should not be ignored that ACE2 can act on several substrates, although the efficiency of the catalytic conversion for Ang II is among the highest.[9,10] We did not measure plasma or cardiac tissue levels of Ang-(1–9), because recent studies indicate that ACE2 makes little if any contribution to Ang I metabolism.[9,10,15]

It could be argued that if ACE2 plays a critical role in Ang II metabolism, changes in the tissue concentration of the angiotensins would reflect the influence of ACE2 on peptide concentrations within the heart. In human atrial tissue, ACE inhibition did not alter cardiac levels of Ang-(1–7), although it increased plasma Ang-(1–7) levels.[26] In accord with previous findings,[29–32] the contrasting effects of the therapies on plasma and tissue angiotensins suggest that the cardiac and circulating renin-angiotensin systems are independently regulated. Nevertheless, in our experiments, both losartan and the combination treatments produced significant increases in cardiac tissue Ang-(1–7) in parallel with augmented cardiac ACE2 activity. These data suggest that ACE2 was involved in increasing the content of cardiac Ang-(1–7) through increased Ang II metabolism after AT_1 blockade.

The effects of 12-day therapy with lisinopril and losartan on cardiac ACE2 mRNA are rather specific, because cardiac ACE and neprilysin mRNAs were not changed in any of the treatment conditions. Further corroboration of the selective role of the AT_1 receptor in mediating the changes in cardiac ACE2 mRNA was gained by measures of *mas* receptor mRNA, which only changed in rats medicated with losartan. In a previous study, we found that increased expression of ACE2 mRNA in the heart after AT_1 receptor blockade could not be explained by an effect of Ang II on AT_2 receptors because concomitant administration of PD123319 had no effect.[19] Therefore, these data suggest that Ang II modulates ACE2 mRNA by interacting with AT_1 receptors, although this is not the only mechanism that may account for the changes observed in the present experiments.

In summary, 12-day administration of agents that either inhibit the synthesis of circulating Ang II or block the activity of Ang II at the AT_1 receptor induced an increase in cardiac ACE2 mRNA, accompanied by increases in cardiac membrane ACE2 activity in rats medicated with either losartan or both losartan and lisinopril. The data presented in this study suggest that the beneficial effects of ACE inhibitors or AT_1 receptor blockers in terms of tilting the balance between vasopressor and vasodilator mechanisms wherein Ang-(1–7) is implicated may occur through different mechanisms. Although the predominant effect of ACE inhibition may result from the combined effect of reduced Ang II formation and Ang-(1–7) metabolism, the antihypertensive action of AT_1 antagonists may in part be due to increased Ang II metabolism by ACE2.

Acknowledgments

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