Effects of enalapril on the expression of cardiac angiotensin-converting enzyme and angiotensin-converting enzyme 2 in spontaneously hypertensive rats

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Received 24 July 2012; received in revised form 22 December 2012; accepted 10 January 2013
Available online 10 April 2013

Summary

Background. — The discovery of angiotensin-converting enzyme 2 (ACE2) has greatly modified understanding of the renin-angiotensin system (RAS).
Aims. — To investigate the cardiac expression of ACE2 and ACE in spontaneously hypertensive rats (SHRs) and the effects of enalapril on them.
Methods. — Fifteen SHRs were randomly assigned to two groups: an SHR control group (n = 7), treated with vehicle; and an enalapril group (n = 8), treated with enalapril (15 mg/kg/day). After 4 weeks of treatment, the rats were killed and the left ventricular tissue was dissected. Reverse transcription-polymerase chain reaction and Western blot protein staining were performed to detect expression of ACE2 and ACE messenger ribonucleic acid (mRNA) and protein. Ten Wistar Kyoto rats (WKYs) served as the normotensive control group, which were treated with vehicle.

Abbreviations: ACE, angiotensin-converting enzyme; RAS, renin-angiotensin system; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; EF, elongation factor; mRNA, messenger ribonucleic acid; PBS, phosphate-buffered solution; SHR, spontaneously hypertensive rat; RT-PCR, reverse transcription-polymerase chain reaction; WKY, Wistar Kyoto.

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http://dx.doi.org/10.1016/j.acvd.2013.01.004
Results. — Compared with in normotensive WKYs, cardiac expression of ACE mRNA and protein in SHRs was increased (1.68 ± 0.34 vs. 0.33 ± 0.12, P < 0.05 and 1.21 ± 0.14 vs. 0.71 ± 0.11, P < 0.05, respectively), whereas cardiac expression of ACE2 mRNA and protein was decreased (0.50 ± 0.15 vs. 1.16 ± 0.24, P < 0.05 and 0.71 ± 0.24 vs. 1.22 ± 0.14, P < 0.05, respectively). After treatment with enalapril, the levels of ACE mRNA and protein were decreased (0.44 ± 0.19 vs. 1.68 ± 0.34, P < 0.01 and 0.87 ± 0.13 vs. 1.21 ± 0.14, P < 0.05, respectively), the level of ACE2 mRNA was increased (1.77 ± 0.49 vs. 0.50 ± 0.15, P < 0.05) but the level of ACE2 protein remained unchanged.

Conclusions. — In SHRs, the expression of cardiac ACE was remarkably increased, whereas ACE2 was notably decreased. Reduction of ACE and elevation of ACE2 might be one of the mechanisms underlying the antihypertensive function of enalapril.

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Background

The discovery of angiotensin-converting enzyme 2 (ACE2) [1,2] has changed our understanding of the renin-angiotensin system (RAS). During the past decade, ACE2 has attracted more and more attention and is gradually becoming a new focus of research. ACE2 is one of the key enzymes in RAS that play important roles in the regulation of blood pressure and maintenance of cardiac function; it has gradually come to be considered as one of the new targets for the treatment of cardiovascular disease [3—6].

There is increasing evidence that ACE2 is closely related to hypertension. Blocking agents of RAS, such as ACE inhibitors (ACEIs) and angiotensin receptor blockers (ARBs), can elevate the level of ACE2 [7].

It will be more informative and persuasive if ACE2 can be studied, not only horizontally together with ACE but also vertically, at the levels of transcription and translation. Moreover, different ACEIs might, to a certain extent, have various pharmacological effects and thereby different effects on ACE2. So it is still necessary to study the different effects of different ACEIs on ACE2/ACE, which may enrich our understanding of the pharmacological effects of ACEIs.

In this study, we used spontaneously hypertensive rats (SHRs) as the animal model and Wistar Kyoto rats (WKYs) as the control group. Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot protein staining were employed to detect cardiac ACE and ACE2, facilitating study of the possible role played by ACE2 in hypertension in SHRs. Moreover, to explore in depth the mechanisms of ACEIs,
enalapril was used to treat SHRs and its effects on cardiac ACE and ACE2 were investigated.

**Methods**

**Animals**

Fifteen male SHRs (Shanghai laboratory animal centre), aged 16 weeks and with an average body weight of 39 ± 21 g, were randomized into two groups: an SHR control group \(n = 7\), treated with vehicle; and an SHR enalapril group \(n = 8\), treated with enalapril (15 mg/kg/day). Treatment lasted for 4 weeks. During the treatment period, body weight and tail blood pressure were measured once per week and the enalapril dose was adjusted based upon body weight. Ten WKYS served as the normotensive control group and were also treated with vehicle. At the end of treatment, the rats were killed and the left ventricles were dissected carefully and frozen and stored in liquid nitrogen for RT-PCR and Western blot protein staining.

The study was approved by the Animal Care and Use Committee of Suzhou University and our study followed the principles in the Declaration of Helsinki.

**Isolation of total ribonucleic acid and reverse transcription-polymerase chain reaction**

Total RNA was isolated according to the Trizol reagent manual and reverse transcription was performed using Moloney murine leukaemia virus reverse transcriptase in a mixture containing deoxyribonucleotides, random hexamers and ribonuclease inhibitor in reverse transcriptase buffer. Elongation factor 1α (EF1α) was used as the internal standard. The PCR assay was performed using the primers for ACE, ACE2 and EF1α described by Sakima et al. [8] (Table 1). The PCR amplification conditions (30 cycles) were as follows: denaturing, 94 °C for 90 seconds; annealing, 56 °C for 45 seconds; elongation, 72 °C for 45 seconds. Primers for EF1α were added to all reactions at the cycle number indicated in Table 1. Finally, an elongation was performed at 72 °C for 10 minutes. Amplification products were separated on a 1% agarose gel dyed by Loading Dye I. The bands were visualized and the optical density was quantified by the Bio-Rad computerized densitometry system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The target messenger ribonucleic acid (mRNA) concentration was expressed as the ratio of target gene to the internal standard EF1α gene.

**Western blot**

The membrane protein was isolated using cell lysis buffer containing a proteinase inhibitor mixture. Protein concentration was determined using the Bradford method. Sample proteins (10 µg/lane) and a prestained protein weight marker were loaded and size fractionated by sodium dodecyl sulphate polyacrylamide gels. The proteins were then transferred from the gel onto nitrocellulose membranes and blocked with 1% non-fat skimmed milk in phosphate-buffered solution (PBS) for 45 minutes at room temperature under gentle agitation on a shaker. The membranes were then incubated overnight at 4 °C with the primary antibody (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in PBS containing 0.5% non-fat skimmed milk buffer. On the following day, the membranes were thoroughly washed three times (10 minutes each) using PBS containing 0.1% Tween. Positive bands were developed using the Western Blotting Analysis System (Tiangz, Beijing, China) in which horseradish peroxidase-conjugated secondary antibody was diluted at 1/10,000 and incubated for 1 hour at room temperature. The membranes were then visualized by chemiluminescence and photographed using an x-ray. The protein bands on the x-ray films were quantified by densitometry scanning and corrected for β-actin. The target protein was expressed as the ratio of target protein to β-actin.

**Statistical analysis**

Data were analysed using SPSS 11.0 software. Each value was expressed as mean ± standard error of the mean. Significant differences were obtained when \(P < 0.05\). Differences between two groups were analysed using the \(t\) test. One-way analysis of variance was used to analyse the differences between multiple groups.

| Table 1 | Primer pairs for ACE, ACE2 and EF1α messenger ribonucleic acid. |
|---|---|---|---|
| Gene | Primer sequence | Size (bp) | Total circles\(^a\) |
| ACE | Forward 5′-TTGACGTGAGCACTTCCAG-3′ | 421 | 30 (13) |
| | Reverse 5′-GGCTGACGCTCCTGGTAGT-3′ | | |
| ACE2 | Forward 5′-GTGCAACAGGTGCAATAGG-3′ | 410 | 30 (14) |
| | Reverse 5′-TGTTTCATCAAGGGCAGAGG-3′ | | |
| EF1α | Forward 5′-GGAATGGTGACAAATGCTG-3′ | 347 | | |
| | Reverse 5′-CGTTGAAAGCTACATTGTC-3′ | | |

\(^a\) The number of amplification cycles performed for the specific target gene. The primer pairs for the EF1α control were added after the number of cycles indicated in parentheses.

ACE: angiotensin-converting enzyme; bp: base pairs; EF: elongation factor.
**Results**

**Effects of enalapril on blood pressure in spontaneously hypertensive rats**

Blood pressure in SHRs was markedly higher than that in WKYs \((P<0.05)\). Enalapril significantly reduced blood pressure in SHRs \((P<0.05)\) (Table 2).

**Effects of enalapril on cardiac angiotensin-converting enzyme and angiotensin-converting enzyme2 messenger ribonucleic acid in spontaneously hypertensive rats**

The cardiac expression of ACE mRNA was markedly higher and of ACE2 mRNA was significantly lower in SHRs than in WKYs \((1.68±0.34 \text{ vs. } 0.33±0.12, P<0.05 \text{ and } 0.50±0.15 \text{ vs. } 1.16±0.24, P<0.05, \text{ respectively})\). Enalapril markedly reduced the expression of ACE mRNA \((0.44±0.19 \text{ vs. } 1.68±0.34, P<0.05)\) and significantly increased the expression of ACE2 mRNA \((1.77±0.49 \text{ vs. } 0.50±0.15, P<0.05)\) (Fig. 1).

**Effects of enalapril on cardiac angiotensin-converting enzyme and angiotensin-converting enzyme2 proteins in spontaneously hypertensive rats**

The cardiac expression of ACE protein was markedly higher and of cardiac ACE2 protein was significantly lower in SHRs than in WKYs \((1.21±0.14 \text{ vs. } 0.71±0.11, P<0.05 \text{ and } 0.71±0.24 \text{ vs. } 1.22±0.14, P<0.05, \text{ respectively})\). Enalapril markedly reduced the expression of ACE protein \((0.87±0.13 \text{ vs. } 1.21±0.14, P<0.05)\) but had no statistically significant impact on the expression of ACE2 protein \((0.42±0.22 \text{ vs. } 0.71±0.24, P>0.05)\) (Fig. 2).

**Discussion**

In this study, we investigated the cardiac expression of ACE and ACE2 mRNA and protein in SHRs; the results showed that cardiac expression of ACE was significantly higher and of ACE2 was markedly lower in SHRs than in WKYs. These data suggest that the imbalance between ACE and ACE2 might play a critical role in the pathophysiology of hypertension in SHR.

### Table 2  Blood pressure before and after treatment in different groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Blood pressure (mmHg)</th>
<th>Base value</th>
<th>1st week</th>
<th>2nd week</th>
<th>3rd week</th>
<th>4th week</th>
<th>Pa</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY group</td>
<td>10</td>
<td>122 ± 14</td>
<td>124 ± 10</td>
<td>118 ± 13</td>
<td>0.664</td>
<td>120 ± 15</td>
<td>0.774</td>
<td>116 ± 4</td>
</tr>
<tr>
<td>SHR control group</td>
<td>7</td>
<td>188 ± 24</td>
<td>187 ± 17</td>
<td>189 ± 18</td>
<td>0.421</td>
<td>185 ± 15</td>
<td>0.401</td>
<td>187 ± 11</td>
</tr>
<tr>
<td>SHR enalapril group</td>
<td>8</td>
<td>195 ± 16</td>
<td>150 ± 11</td>
<td>140 ± 9</td>
<td>&lt; 0.001</td>
<td>140 ± 12</td>
<td>&lt; 0.001</td>
<td>138 ± 11</td>
</tr>
</tbody>
</table>

Blood pressure data are mean ± standard error of the mean. SHR: spontaneously hypertensive rat; WKY: Wistar Kyoto rat.

*a* Compared with blood pressure before treatment within the same group. Data were analyzed with pairwise comparisons.

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**Figure 1.** Cardiac angiotensin-converting enzyme (ACE) 2 and ACE messenger ribonucleic acid (mRNA) in the Wistar Kyoto rat group (W), the spontaneously hypertensive rat (SHR) control group (C) and the SHR enalapril group (E). ACE mRNA elevated and ACE2 mRNA decreased in SHRs \((P<0.05)\). Enalapril significantly reduced the expression of ACE mRNA and elevated the level of ACE2 mRNA \((P<0.05)\). Data were analyzed with analysis of variance and pairwise comparisons. \* \(P<0.05\) vs. C. \(\triangle P<0.05\) vs. W.
In 2000, ACE2—a human homologue of ACE—was discovered and reported by two groups, independently of each other, using differing genomic-based strategies [1,2]. ACE2 is a zinc metalloprotease and its gene maps to the X chromosome. Like ACE [9], ACE2 is a strong candidate for a hypertensive quantitative trait locus. In early research, ACE2 was found to be expressed in heart and kidney [1]. Late studies indicated that it was also expressed in the testes [10], gastrointestinal tract [11] and lungs [12].

Although ACE and ACE2 are homologues, their functions are notably different. Studies showed that the average blood pressure in mice with the ACE2 gene knocked out was 10 mmHg higher than in controls [13]. Moreover, in SHRs and stroke-prone SHRs, ACE2 expression was lower than that in normotensive WKYs. On the other hand, WKYs had high expression of ACE2 [14]. The decrease in ACE2 can lead to acceleration of the progression of hypertension in Goldblatt hypertensive rats [3]. All of these findings suggest that ACE2 has antihypertensive effects. From our study, we inferred that the reduced ACE2 in SHRs can lead to impaired antihypertensive effects of ACE2, which is conducive to hypertension.

The main function of ACE2 is to produce Ang-1–7, which has peripheral vasodilative and antiproliferative actions [15]. ACE2 cleaves a single amino acid from angiotensin II to generate Ang-1–7 [16]. ACE2 also cleaves an amino acid from the C-terminal of angiotensin I to generate Ang-1–9. Ang-1–9 is afterwards cleaved by ACE or another dipeptidase into Ang-1–7 and other angiotensin peptides. Ang-1–7 reduces artery pressure dose-dependently [17], amplifies the vasodilator actions of bradykinin [18], accelerates the degradation of angiotensin II [5] and attenuates the formation of reactive oxygen species [4]. Through a bradykinin/nitric oxide-mediated mechanism that stimulates cyclic guanosine monophosphate/protein kinase G signalling, Ang-1–7 reduces the release of norepinephrine [15] and increases the release of nitric oxide from platelets [19], thereby dilating the vessels indirectly.

In addition to blood pressure-lowering effects, Ang-1–7 can also prevent angiotensin II-mediated cardiac remodelling [20]. One study showed that subcutaneous administration of the ACE2 inhibitor MLN-4760 (30 mg/kg/day) via mini-osmotic pumps for 28 days increased the level of angiotensin II by 24%, which exacerbated cardiac hypertrophy and fibrosis [21]. ACE2 deficiency due to ACE2 gene knockout exacerbated hypertension, myocardial hypertrophy, fibrosis and diastolic dysfunction, which were induced by angiotensin II infusion. In summary, ACE2 is an important negative regulator of angiotensin II-induced heart disease [22].

Our study also explored the effects of enalapril on cardiac expression of ACE and ACE2 mRNA and protein. The results showed that enalapril reduces the expression of ACE at transcriptional and translational levels, increases ACE2 expression at transcriptional level alone but has no marked effects on ACE2 protein. In other words, upregulation of cardiac ACE2 mRNA did not lead to upregulation of ACE2 protein.

This lack of parallel between mRNA, protein and enzymatic activity has been reported previously. For example, Ferrario et al. [7] explored the effects of lisinopril and losartan, alone and in combination, on cardiac ACE and ACE2 in normotensive Lewis rats. The results showed that losartan significantly increased ACE2 mRNA and activity. Lisinopril also upregulated ACE2 mRNA but had no marked effects on ACE2 activity. When lisinopril and losartan were used in combination, although ACE2 activity increased as in the group in which losartan was used alone, no evident increase in ACE2 mRNA was found. Additional research is needed to further define the mechanisms behind the absence of parallel between ACE2 mRNA and protein.

ACE and ACE2 are two products with opposite functions and together they maintain normal blood pressure. In hypertension, ACE is increased and ACE2 is decreased. It is reasonable for enalapril, an ACEI, to inhibit the activity of ACE. Why did enalapril increase ACE2 mRNA? In our study, after treatment with enalapril, the expression of ACE2 mRNA increased even beyond the values in normotensive WKYs. This implied that the increase in ACE2 was more than a reflection of the down-regulation of ACE. However, the mechanisms by which ACEIs increase ACE2 are still unclear. In terms of pathology, the decrease in ACE and the increase in ACE2 favour the control of blood pressure. This sort of effect that enalapril has on ACE and ACE2.
might play a critical role in the antihypertensive functions of ACEIs.

**Conclusions**

Our study showed that in SHRs, the cardiac expression of ACE mRNA and protein was remarkably increased, whereas that of ACE2 mRNA and protein was notably decreased. Enalapril markedly reduced ACE and significantly elevated ACE2. These findings suggest that the antihypertensive and cardiac protective functions of ACEIs may be due, in part, to elevation of ACE2.

**Disclosure of interest**

The authors declare that they have no conflicts of interest concerning this article.

**Acknowledgments**

This study was carried out, in part, in the Cardiovascular Research Institute, Affiliated First Hospital of Suzhou University, Suzhou. We appreciate the assistance and support from staff members there.

**References**