Differential Regulation of Vascular Angiotensin I–Converting Enzyme in Hypertension

Maria S. Fernández-Alfonso, Reinhold Kreutz, Karin Zeh, Yixian Liu, Detlev Ganten, Martin Paul

Abstract The angiotensin I-converting enzyme (ACE) gene is found on the locus that has been linked to high blood pressure after sodium loading in rats, so in the present study we investigated the role of vascular ACE for the pathophysiology of hypertension in the corresponding parental strains, Wistar-Kyoto (WKY) rats and stroke-prone spontaneously hypertensive rats (SHRSP), in basal conditions at different ages and after sodium loading. Blood pressure was already significantly enhanced in SHRSP from 4 weeks of age, and sodium loading induced an additional increase only in the hypertensive strain. In the aorta, basal ACE gene expression, analyzed by quantitative polymerase chain reaction, and ACE activity were similar in both strains, whereas mRNA levels were elevated in SHRSP after salt compared with WKY rats and correlated with an increase in enzymatic activity. In mesenteric arteries, ACE mRNA levels were significantly enhanced in SHRSP at all ages, although ACE activity was not different between the strains. These results were not modified after sodium loading. These data demonstrate that the level of ACE activity in plasma and vascular tissue can be controlled in a different manner within a rat strain and that in contrast to the soluble form, the membrane-bound ACE may be the one responsible for determining the vasoactive effects of angiotensin II. In addition, ACE undergoes a different regulation in vascular tissues of SHRSP compared with WKY rats, which might be involved in the regulation of blood pressure in these animals. (Hypertension. 1994;24:280-286.)

Key Words • angiotensin I–converting enzyme • blood vessels • hypertension, genetic • rats, inbred SHR, stroke-prone • polymerase chain reaction

Angiotensin I-converting enzyme (ACE) is a zinc metalloproteinase that is expressed throughout the body as a membrane-bound enzyme in particular high concentrations in vascular endothelial cells and intestinal and renal brush borders. In addition, a soluble form of ACE, most likely released from the endothelium through the action of a processing enzyme,1,2 is found in plasma.3 ACE catalyzes the conversion of the inactive decapeptide angiotensin I (Ang I) to the active octapeptide Ang II, which plays a major role in regulating blood pressure as well as in fluid and electrolyte homeostasis. At the vascular level, Ang II induces potent vasoconstriction, either through a direct action on the Ang II receptors of the smooth muscle4 or indirectly by prejunctional5 modulation of norepinephrine release. Moreover, Ang II has also been suggested to induce vascular hypertrophy.6 In addition to its role in Ang II formation, the vasodilator peptide bradykinin is inactivated by ACE through a sequential cleavage of two carboxy-terminal dipeptides.7 Therefore, the functional role of ACE is the release of a potent vasopressor and trophic factor as well as the degradation of a vasodilator.

An increase in the activity of the renin-angiotensin system has been implicated in the pathogenesis of arterial hypertension for a long time, although the causes of this activation remain unclear. Recently, genetic linkage studies have identified a locus associated with high blood pressure after sodium loading in a group of F2 hybrid animals derived from intercrossing stroke-prone spontaneously hypertensive rats (SHRSP) and normotensive Wistar-Kyoto (WKY) rats.8,9 Since the identified region on rat chromosome 10 also contains the gene encoding for ACE, it was proposed as a candidate gene for genetic hypertension in SHRSP. In view of these results, the aim of our study was to analyze first the ontogenic development of vascular ACE and its possible implication for the pathophysiology of hypertension in SHRSP, as suggested from the cosegregation studies, and second the influence of short-term sodium loading on it. For this purpose, we quantified ACE gene expression by polymerase chain reaction (PCR) in aorta and mesenteric arteries of SHRSP compared with WKY rats at different ages and correlated it with plasma and tissue enzyme activity levels in basal conditions and after sodium loading.

Methods

All constitutional guidelines for animal research were observed. Male SHRSP and WKY rats were obtained from the colony at the Department of Pharmacology of the University of Heidelberg (FRG). Three rat groups were studied under baseline conditions ingesting a normal diet at 4, 12, and 18 weeks of age (n=12 of each age and strain), respectively. For determination of the effects of sodium loading, a group of 16-week-old animals (SHRSP, n=10; WKY, n=10) were subjected to 1% NaCl during 12 days according to the protocol of the previous study by Lindpaintner et al.10 The animals were killed after sodium loading at 18 weeks of age, and plasma and tissue data were compared with the corresponding age group under a normal NaCl diet. Blood pressure was determined in conscious rats following the protocol of the linkage study.10 In the group of 4-week-old rats a modified technique, previously
described for direct blood pressure measurements in conscious mice, was used because of the small body size of these animals.

Blood for the determination of ACE activity was withdrawn from the retro-orbital plexus with rats under light ether anesthesia. The rats were killed by cervical dislocation, and from the retro-orbital plexus with rats under light ether anesthesia. The thoracic aorta was dissected and cleaned of perivascular fat in a similar procedure. These maneuvers did not affect the intactness of endothelium, as had been previously confirmed by NO staining. In addition, aortas were opened longitudinally, and the intimal layer, adventitia, and media were carefully separated under a microscope. The organs were immediately frozen in liquid nitrogen and stored at −80°C until use.

ACE Activity Determination

For ACE activity determination the organs were homogenized in 0.3% Triton solution, sonicated, and centrifuged at 20,000g for 20 minutes at 4°C. Plasma and tissue ACE activities were determined using a modification of the fluorometric method according to Depierre and Roth with carbobenzoxyphénylalanylhistidyl-leucine (Z-Phe-His-Leu) as substrate as previously described. Protein content of the tissue homogenates was analyzed according to Lowry et al. Data are expressed as nanomoles of histidyl-leucine (His-Leu) per minute (plasma) and nanomoles of His-Leu per milligram of protein (tissue homogenates).

ACE mRNA Measurements

Total RNA was isolated with lithium chloride and urea by a modification of the method of Auffray and Rougeon. The precipitated RNA was dissolved in water, and the amount was measured twice by absorbance at 260 nm. The intactness of RNA was checked on an ethidium bromide-stained agarose minigel.

ACE expression was assessed by PCR after reverse transcription (RT-PCR), as previously described. Briefly, 25 PCR amplification cycles were run in the following order: 30 seconds at 94°C for denaturation, 1 minute at 55°C for annealing, and 1 minute at 72°C for primer extension, with an additional 7 minutes at 72°C at the end for final extension. The optimal cycle number for amplification was determined by extracting 5-μL aliquots each 2 cycles, ranging from 19 to 35 cycles, which were then blotted and quantified. The resulting amplification curve showed a linear amplification phase followed by a plateau starting at cycle 31, so that 25 amplification cycles were still on the linear amplification phase. Southern blot analysis was performed for measurement of ACE mRNA expression, and DNA was subsequently cross-linked by UV light. After prehybridization, the blots were hybridized overnight using a 32P-labeled specific primer (GCCTC-CCCAACAAGACTGCGA), washed, and exposed for 2 to 4 hours to an imaging plate (Fuji Photo Film Co). Autoradiograms were scanned twice with a computer-based direct-imaging system (Fujix, Fuji Photo Film Co).

Quantification of ACE mRNA was performed in the presence of a defined concentration of ACE cDNA mutant as an internal standard constructed following the method of Paul et al using two internal primers (antisense primer position 649 to 666; sense primer position 728 to 748 of human ACE cDNA). It contained a deletion of 150 bp between the primer binding sites of the oligonucleotides used for amplification of the endogenous ACE gene product (sense primer position 492 to 502; antisense primer position 860 to 880 of human ACE cDNA) so that primer binding was not affected. The amplification product was 250 bp long. The deletion mutant was added to the PCR mixture after reverse transcription to compete with the endogenous ACE cDNA. One microgram of reverse transcribed RNA was mixed with the appropriate amount of mutant ACE cDNA, ranging from 20 to 1 pg, where neither the endogenous nor the mutant ACE would completely suppress its counterpart. For creation of a standard curve for the estimation of endogenous ACE, this mixture was then serially diluted 1:2 five times. The standard curve was used for quantification and the individual PCR samples, whereby sample-to-sample variations were minimized. The expression was quantified as previously described. To be sure of avoiding unspécific DNA amplification, we performed the PCR in a number of samples without reverse transcription. Additionally, in all the experiments several water samples were run in parallel as negative controls of DNA contamination.

Results

Blood Pressure

Baseline

Blood pressure was measured in all rats at 4, 12, and 18 weeks of age (Fig 1). In the young 4-week-old...
Comparison of ACE mRNA Levels and ACE Activity in 18-Week-Old WKY Rats and SHRSP in Basal Situation and After Sodium Loading

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<th>Aorta</th>
<th>Mesenteric Artery</th>
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<td>WKY</td>
<td>SHRSP</td>
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<td>Basal</td>
<td>Salt</td>
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<td>ACE mRNA, pg/mg total RNA</td>
<td>8±0.8</td>
<td>6.3±0.2</td>
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<td>ACE activity, (nmol/mg)/min</td>
<td>28.6±3</td>
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ACE indicates angiotensin-converting enzyme; WKY, Wistar-Kyoto; and SHRSP, stroke-prone spontaneously hypertensive rats. Values are mean±SEM. *P<.05.

animals, systolic blood pressure values were already significantly enhanced in SHRSP (135±3.2 mm Hg) compared with WKY rats (102.7±2.5 mm Hg; P<.001); this difference increased with age and remained significant in both strains at 12 and 18 weeks of age.

**Sodium Loading**

After 12 days of dietary sodium a significant elevation in blood pressure (Fig 1) was observed only in the SHRSP (systolic, 236±3.4 versus 260±4.9 mm Hg; diastolic, 160.9±2.4 versus 182±3.3 mm Hg; P<.001). No significant changes were observed in the normotensive group.

**ACE mRNA Measurements**

**Basal**

Because of the low amount of RNA that is extracted from blood vessels, ACE mRNA expression was measured by RT-PCR by amplification of 1 g total RNA from aorta and mesenteric arteries. ACE mRNA expression could be localized in all three vascular layers, adventitia, and intima after 25 amplification cycles and in the media after 30 (Fig 2). ACE mRNA in mesenteric arteries showed a significantly higher expression (P<.05) in the hypertensive animals compared with the normotensive at all ages. Aortic ACE mRNA, in contrast, did not show any difference between the strains (Fig 3, Table). In each strain ACE mRNA levels were similar between aorta and mesenteric arteries at all ages, with the exception of the mesenteric bed of the 18-week-old SHRSP, which had twofold higher levels.

**Sodium Loading**

In aorta, ACE mRNA levels were significantly upregulated, whereas in mesenteric arteries the twofold enhancement in basal ACE expression in SHRSP was unmodified (Fig 3, Table). No differences in ACE mRNA could be seen in kidney between the strains either before or after high sodium (Table).

**ACE Activity Determinations**

**Basal**

Measurement of plasmatic ACE activity in all rats at the different ages revealed higher values for WKY rats than for the age-matched SHRSP (Fig 4, Table), showing that there was an inverse relation between blood pressure and plasmatic ACE activity at all ages studied. In contrast, pulmonary ACE activity at 4 and 18 weeks was significantly higher in the hypertensive compared with the normotensive strain (Fig 5).

In blood vessels, ACE activity was measured in aorta and mesenteric arteries at 4 and 18 weeks of age. Comparison between strains showed no apparent difference in ACE activity in either aorta or mesenteric arteries, as shown in Fig 6. However, when the two vascular beds within the same strain were compared, ACE showed an approximately twofold higher activity in the aorta compared with the mesenteric arteries.

**Sodium Loading**

Measurement of ACE activity in plasma (Fig 4) and kidney (Table) did not reveal any difference between either strain compared with basal conditions.
Continued

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<th>Kidney</th>
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In aorta, ACE activity was significantly upregulated in the hypertensive strain, correlating with the increase in expression, whereas in mesenteric arteries no difference in ACE activity could be seen (Figs 3 and 4, Table).

**Discussion**

In recent linkage studies using SHRSP and normotensive WKY rats, the gene encoding for ACE has been proposed as a candidate gene for the pathophysiology of high blood pressure in SHRSP. Based on these results, we carried out the present work to evaluate the relevance of vascular ACE for the development of hypertension in these rats. A recent report shows that the ACE activity differs considerably between various colonies of SHR and WKY rats but not within a strain from a single stock, so the animals used for this study were obtained from the same colony investigated in the previous linkage studies.

Blood pressure was already significantly enhanced in SHRSP from the age of 4 weeks. At this young age the hypertensive animals showed a difference in systolic blood pressure of approximately 30 mm Hg, which is considerable if we take into account the small weight (50 g) of these rats. After sodium loading, blood pressure was significantly enhanced only in the hypertensive strain, as has been previously shown by other authors.

Thus suggesting that the effects observed in F1 rats in the linkage studies might be specific for SHRSP.

It has been demonstrated that high levels of plasmatic ACE, such as found in human pathologies such as sarcoidosis, Gaucher's disease, leprosy, silicosis, and berylliosis, are not associated with high blood pressure. This is further supported by our study, showing a striking difference in plasmatic ACE activity between SHRSP and WKY rats, namely, an inverse relation with high blood pressure at all ages investigated. In addition, despite the enhancement in blood pressure in SHRSP after sodium loading, plasmatic ACE activity remained unchanged, once more suggesting that plasmatic ACE levels are not indicative of and probably not relevant for blood pressure regulation. As has been shown from the good correlation between the ACE activity measured in SHR and the quantity of [3H]ramiprilat bound to the enzyme, this lower ACE activity observed in the SHR strain corresponds to a lower enzyme concentration in plasma rather than to any change in the enzyme activity or enzyme characteristics in the hypertensive animals. On the other hand, although it has been suggested that plasmatic ACE is most likely released by the pulmonary endothelial cells, in our rats we did not find any correlation between pulmonary and plasmatic ACE, because SHRSP with low plasmatic ACE levels have higher ACE activity and expression in lung than WKY rats with high plasmatic ACE. For this reason, we have to exclude the lung as the principal source for plasmatic ACE in these rats. One possibility is that lower plasmatic ACE levels in hypertensive animals are due to an enhanced clearance of the enzyme. Second, it is conceivable that plasmatic ACE may represent a form of the enzyme, which is not a processing product of the membrane form but is secreted via a different constitutive pathway. Nevertheless, independently of the origin of plasmatic ACE, we could recently demonstrate that the difference in plasmatic ACE levels in these rats appears to be due to a primary genetic abnormality affecting the regulation of the ACE gene rather than to a regulatory modulation of the enzyme.

Many studies have analyzed the role of ACE in cardiovascular tissues through either ACE activity determination or, indirectly, measurement of the conversion of Ang I to Ang II in reactivity experiments. Few studies, however, have correlated gene expression and enzyme activity levels of ACE in the vasculature. This is in part due to the low yield in RNA that can be extracted from blood vessels, which makes the determination and even more the quantification of mRNA very difficult with conventional techniques. Therefore, in the present study ACE mRNA levels were assessed with the PCR assay.

Using this method, we found ACE gene expression in adventitia and intima of aorta from both SHRSP and WKY rats, as suggested previously by several authors using in vitro autoradiography and immunohistochemistry. However, none of these authors have described the presence of ACE in the medial layer of the arteries studied. In the present study we assessed ACE gene expression in the media after surgical removal of all adventitia under a microscope. Although this procedure does not assure complete removal of adventitia, and enough residual adventitia could remain to give a PCR signal, there are apparent quantitation differences. Ex-
traendothelial conversion of Ang I to Ang II, which is blocked by ACE inhibitors, has been reported by measurement of the isometric contractions in endothelium-denuded rings after exogenous administration of Ang I. Other authors have measured ACE activity in intact and denuded perfused rat mesenteric arterial beds, finding that although the ACE activity was significantly reduced, approximately 80% of the ACE activity remained after endothelium removal. Together with these results, the present finding that the ACE gene is expressed in the medial layer of the vascular wall increases the functional importance of this enzyme, even when the endothelium is damaged.

Aortic ACE gene expression was not different between the strains, in contrast to the higher activity in aorta of SHR reported by others, and mRNA levels correlated well with the ACE activity measured. However, in mesenteric arteries, although the ACE gene was significantly more highly expressed in SHRSP at all ages studied, there was no apparent difference in ACE activity compared with WKY rats. One possibility, caused by the high plasmatic ACE activity relative to the ACE activity within the blood vessel, is that small amounts of plasma contamination could mask the difference, leading to an overestimated mesenteric ACE activity in WKY rats. On the other hand, according to our result, other investigators have not found a direct correlation between the ACE mRNA levels and enzymatic activity in various tissues studied. This apparent discrepancy between the higher ACE mRNA levels and the lower ACE activity measured might be reflecting an enhanced turnover rate of the membrane-bound enzyme in mesenteric arteries in contrast to the lung or kidney, in which a direct correlation between expression and activity exists, and therefore the vascular ACE activity seems to be equal between the two rat strains, as suggested previously by others. An enhanced turnover is also supported by the fact that although the ACE mRNA levels in mesenteric arteries are similar compared with the aorta, the activity in this vascular bed is much lower. Tissue levels of ACE are a balance between ACE gene expression, ACE synthesis and incorporation into the membrane, and ACE release to the plasma, and this balance seems to be regulated in a different way in resistance blood vessels than in big vessels. It remains to be established, however, if this is reflected in vivo as an enhanced Ang II synthesis and/or bradykinin degradation.

The fact that ACE expression increases significantly in 4-week-old animals when blood pressure is already increased does not allow one to conclude that the changes observed in ACE are an epiphenomenon of rather than the cause for hypertension. However, no increases in ACE could be assessed in other organs, such as kidney, suggesting that it is specific for blood vessels rather than an unspecific pressure-induced phenomenon.

Another interesting finding is the different regional distribution of ACE in these rats. ACE activity is approximately twofold higher in aorta than in mesenteric arteries of either strain, which stresses the importance of ACE in large vessels. This has already been
suggested by others, who report that 20% of total ACE activity is localized in large vessels. Regarding this, the finding that ACE expression and activity are significantly enhanced in aorta of SHRSP after sodium loading is very attractive, because several authors have reported that the therapeutic efficacy of ACE inhibitors seems to be modulated in part by sodium status. It may be suggested that the increase in the aortic ACE activity, which would lead to an enhanced vascular tone of this vessel in the hypertensive strain, might contribute to the increase of blood pressure after sodium loading. Similarly, Shiota and coworkers have shown that ACE expression and activity are increased in aorta of rats with two-kidney, one clip hypertension and that this enhancement is associated with the development of high blood pressure in these rats. On the other hand, the contribution of large vessels to systemic resistance is low, and in the present study no modification of ACE expression or activity could be observed after sodium loading in mesenteric arteries. One explanation is that the level of stimulation of the mesenteric ACE is already maximal in SHRSP in basal status and that no further stimulation of the system can be achieved in these vessels after sodium loading. The mechanism by which ACE might be upregulated after sodium loading still remains unclear. Although it is well known that in vitro ACE activity is upregulated by chloride at supra-physiological concentrations, plasmatic chloride concentrations are not strongly modified in vivo after sodium loading.

In summary, we have demonstrated that plasmatic ACE activity is much lower in SHRSP than in WKY rats, with a negative relation between plasmatic ACE and high blood pressure. In basal conditions, ACE mRNA is already enhanced only in resistance vessels from SHRSP from 4 weeks of age, although ACE activity is similar in blood vessels of both strains. In addition, sodium loading raises blood pressure only in SHRSP, which is paralleled by an increase in ACE expression and activity in aorta but not in mesenteric arteries. Therefore, we suggest that the level of ACE activity in plasma and vascular tissue seems to be controlled in a different manner within each strain and that in contrast to the soluble form, the membrane-bound ACE may be responsible for the vasoactive effects of Ang II. In addition, ACE undergoes a different regulation in vascular tissues of SHRSP compared with WKY rats, which might be implicated in the regulation of blood pressure in these animals.

In conclusion, on the basis of our findings we cannot rule out the pathophysiological relevance of ACE for hypertension in SHRSP. Although genetic studies in human primary hypertension are still inconclusive, the role for ACE as a candidate gene has been recently underlined by a crossbreeding study using a second hypertensive animal model, the Dahl salt-sensitive rat.

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