Interaction of mRNAs for Angiotensin II Type 1 and Type 2 Receptors to Vascular Remodeling in Spontaneously Hypertensive Rats

Shoji Otsuka, Masahiro Sugano, Naoki Makino, Shojiro Sawada, Tomoji Hata, Yoshiyuki Niho

Abstract—We administered angiotensin II (Ang II) receptor type 1 (AT1) blockade (losartan, 40 mg · kg⁻¹ · d⁻¹), type II receptor (AT2) blockade (PD123319, 100 mg · kg⁻¹ · d⁻¹), or angiotensin-converting enzyme (ACE) inhibitor (enalapril, 30 mg · kg⁻¹ · d⁻¹) to spontaneously hypertensive rats (SHR) from 10 to 20 weeks of age. Control SHR and Wister-Kyoto rats (WKY) received a placebo for the same period. At the end of treatment, losartan and enalapril were both found to have significantly reduced the arterial systolic blood pressure and the collagen concentration to the level of WKY, whereas PD123319 had no effect. Enalapril and PD123319 significantly reduced the media cross-sectional area of the aorta in comparison to that of untreated SHR, which was still larger than that of the WKY; however, losartan did not change it. Using reverse transcription–polymerase chain reaction, we next examined the mRNA expressions for ACE, AT1 receptor, and AT2 receptor in experimental animals. We observed significantly enhanced mRNA expression for AT1 and AT2 receptors and ACE in untreated SHR compared with WKY. The AT1 mRNA level was also significantly decreased in the SHR treated with either losartan or enalapril, whereas the AT2 mRNA level was significantly decreased in the SHR treated with either PD123319 or enalapril in comparison to untreated SHR. The level of ACE mRNA was significantly decreased only in the SHR treated with enalapril. These results indicate that AT1 receptor, but not AT2 receptor, plays a crucial role in the remodeling of matrix tissue, while AT2 receptor may play a role in the development of hypertrophy of smooth muscle in aorta in SHR, and that the reduction of hypertrophy of smooth muscle does not fully account for the suppression of hypertension. (Hypertension. 1998;32:467-472.)

Key Words: angiotensin II receptors, angiotensin II hypertrophy, vascular collagen rats, inbred SHR

Long-term hypertension is reported to be associated with cardiovascular remodeling, which consists of cardiovascular hypertrophy and an increase in the extracellular matrix (especially collagen).1,2 In spontaneously hypertensive rats (SHR), both left ventricular hypertrophy and the properties of characteristic vascular resistance are already present early in life, based on the results of a comparison with normotensive Wister-Kyoto rats (WKY).3,4 Angiotensin-converting enzyme (ACE) inhibitors can lower the blood pressure in SHR mainly by reducing the production of angiotensin II (Ang II).5 Ang II is considered to act as a growth-promoting factor in aortic smooth muscle cells,6,7 while it also increases collagen synthesis in smooth muscle cells.8 ACE inhibitors suppress such vascular remodeling in experimental models, possibly through blood pressure–independent mechanisms.9,10 Recently, a selective Ang II receptor antagonist has been developed that inhibits the renin-angiotensin system (RAS) more specifically than ACE inhibitors. Two main Ang II receptor subtypes, AT1 and AT2, have been identified, and some other subtypes have also been described.11,12 The antihypertensive action of losartan is based on the blockade of AT1 receptors, which are believed to mediate most of the cardiovascular actions of Ang II.13 AT1 receptor is also involved in maintaining the systemic blood pressure and responsiveness of the cardiovascular system to Ang II.14,15 Two controversial reports have recently been published. Levy et al16 reported that only AT2 blockade, and not AT1 blockade, prevented the increased aortic media thickness and increased extracellular matrix in Ang II–induced hypertensive rats. On the other hand, Benetos et al17 concluded that spironolactone attenuates both the aortic media thickness and the development of collagen through the inhibition of AT1 receptor in SHR. However, there is little information regarding the interaction of mRNAs for AT1 and AT2 receptors and ACE to the extracellular matrix and vascular hypertrophy. In the present study, we therefore examined the effects of the AT1 antagonist losartan, the AT2 antagonist PD123319, or the ACE inhibitor enalapril to selectively block the RAS on vascular remodeling. We also investigated the molecular expression of the aortic mRNA levels for AT1 and AT2 receptors and ACE. These studies provide information on how the RAS affects the extracellular matrix and vascular...
hypertrophy, while also shedding some light on how vascular RAS inhibition may attenuate vascular remodeling.

Methods

Animals and Experimental Protocols
Male SHR at 5 weeks of age (n=32) and age-matched male WKY (n=36) were used for this study. All animals were housed in a room where the temperature, humidity, and light were controlled, and a standard rat diet plus water was provided ad libitum. Systolic blood pressure and heart rate were measured once a week using the tail-cuff method. The ACE inhibitor enalapril, the AT1 receptor antagonist losartan, or the AT2 receptor antagonist PD123319 was then administered daily to SHR from 10 to 20 weeks of age. The dose of losartan, or the AT2 receptor antagonist PD123319 was then administered to SHR from 10 to 20 weeks of age. All drugs were administered daily through a stomach tube to the rats. At the end of the treatment with each drug, body weight and blood pressure were measured. The rats were killed by decapitation, and blood samples were drawn into chilled tubes (4°C) containing 0.1% EDTA to determine the plasma ACE activity. After the immediate removal of the thoracic aorta, the aorta was freed from the adventitia and was washed with ice-cold 10 mmol/L potassium phosphate buffer (pH 8.3) for the determination of the collagen content and RNA isolation. The aortas were then frozen in liquid nitrogen and kept for up to 2 weeks at -80°C until the assays were performed. For the histological examination, the lower part of the descending thoracic aorta was fixed in 10% formaldehyde in saline and embedded in Parafilm. Losartan was a gift of Merck, Sharp & Dohme Research Laboratories (Rahway, NJ). PD123319 was kindly provided by Parke-Davis Pharmaceutical Research Division (Ann Arbor, Mich).

Measurement of Plasma ACE Activity
Plasma ACE activity was measured using the modified method of Hayakari et al. as described previously. The assay for the ACE activity was performed in a 150-μL incubation mixture containing 80 mmol/L potassium phosphate buffer, pH 7.5, 600 mmol/L sodium chloride, 3 mmol/L hippuryl-L-histidyl-L-leucine (HHL), and the respective buffer. The reaction was allowed to proceed for 1 hour at 37°C. After the incubation, the tubes were sealed, and the reaction was allowed to proceed for 24 hours at 25°C. When the reaction was completed, the digest was centrifuged at 5000 g for 20 minutes. The supernatant was dialyzed against 1% acetic acid, which was used for the measurement of the hydroxyproline content and Western blot analysis of collagen type 1. Because the percentage of recovery of collagen was more than 95% in terms of hydroxyproline, the aortic collagen content was measured by the hydroxyproline concentration of the tissue, as described by Bergman and Losley. The sample was hydrolyzed in 6 N hydrochloric acid solution at 100°C. Then, p-dimethylaminobenzaldehyde (Ehrlich’s reagent) dissolved in buffer at pH 7.0 was added to form a complex with hydroxyproline. The concentration of hydroxyproline was measured by a spectrophotometric analysis at a wave length of 558 nm. The concentration of hydroxyproline was expressed as micrograms of hydroxyproline per milligram of protein, which was then measured by the Lowry method. For the measurement of collagen type 1, a Western blot analysis was performed using anti-collagen type 1 rabbit IgG (Chemicon International Inc) and anti-rabbit IgG from goat (Kirkegaard and Perry Laboratories Inc), and the blots were visualized by the ECL Western blot detection system (Amersham Corp). The density of each band was analyzed with a densitometer (model 620, Japan Bio-Rad). The amount of collagen type 1 was described as the ratio to standard rat collagen (Biomedical Technologies Inc).

Histomorphometric Study
Three successive 25-μm cross-sectional sections were stained with hematoxylin and eosin. The size of the aorta was evaluated at a low magnification (×40). The distance between the internal and external elastic laminae, which contained smooth muscle cell layers, was defined as the media. The media cross-sectional area of the aorta was measured by planimetry from an enlarged color photograph.

Isolation of Total RNA and Quantification of mRNAs
Total RNA was isolated from the middle portion of the thoracic aorta with RNeasy Lipid Tissue Kit (Qiagen) according to a slight modification of the manufacturer’s procedure. Each mRNA (ACE, AT1, AT2, and GAPDH) was measured by reverse transcription–polymerase chain reaction (RT-PCR) as described previously. Briefly, 1 μg of total RNA was reverse transcribed into cDNA and then amplified using an RT-PCR kit (Gibco Life Technologies). A nonradiolabeled system (Gibco Life Technologies) was used to label the PCR product with biotin-14-dCTP. The amplification profile involved denaturation at 95°C for 1 minute, annealing at 64°C for 1 minute, and extension at 72°C for 1 minute. After we determined what ranges of the cycles were exponential (after determining the efficiency of amplification) in each mRNA, the PCR cycles for determining the amount of each mRNA were established. For AT1 receptor, the sense primer was 5'-GGGAGTCCGGCAATG-3'; the anti-sense primer was 5'-TTCTTTGACGTCACT-3'; and the PCR product size was 494 bp, 26 cycles. For the AT2 receptor, the sense primer was 5'-TGGTCCGATTTAACGTC-3'; the anti-sense primer was 5'-TGAGTCCGGATTTAACGTC-3'; and the PCR product size was 494 bp, 26 cycles.

Figure 1. Collagen type 1 concentration measured by Western blot analysis for WKY, untreated SHR, and SHR treated with each drug from 10 to 20 weeks. Marker indicates standard rat collagen.

| Table 1. Systolic Blood Pressure and Plasma ACE Activity |
|-----------------|--------------------|-----------------|-----------------|
|                  | WKY                | SHR             | Losartan        |
| Systolic blood pressure, mm Hg | 143±5              | 201±8*          | 151±7†          |
| ACE activity, nmol·min⁻¹·mL⁻¹ | 49±3               | 94±5*           | 88±4*           |
|                  | Enalapril          | PD123319        |                 |
| Systolic blood pressure, mm Hg | 149±6†             | 191±7§          |                 |
| ACE activity, nmol·min⁻¹·mL⁻¹ | 60±4§              |                 |                 |

Values are mean±SEM. *P<0.05 vs WKY; †P<0.05 vs SHR; ‡P<0.05 vs losartan; §P<0.05 vs enalapril.
5'-ACCAGCTATATTCTCGG-3'; and the PCR product size was 476 bp, 28 cycles. For ACE,23 the sense primer was 5'-GACCTGCTCAACATCTATG-3'; the anti-sense primer was 5'-GACTGGTCCAACATCTATG-3'; the product size was 739 bp, 28 cycles. GAPDH (349 bp) was chosen as an internal control from the rat cDNA24,27 (sense, 5'-GGCTAAGCAGTTGGTGGTGC-3'; 12 cycles). After PCR was completed, 2 μL of each PCR product from the same animal was applied to the same well of a 12.5% polyacrylamide gel (Daiichi Pure Chemicals). After electrophoresis, the amplified DNA was transferred to a nylon membrane, and the blots were analyzed and visualized with a biotin detection kit (Millipore) as described previously.19,21 The density of each

### Results

Systolic blood pressure in the 20-week-old SHR was significantly higher than that in the WKY. The administration of losartan or enalapril to SHR significantly reduced the systolic blood pressure to the level of WKY. However, the administration of PD123319 had no effect on systolic blood pressure (Table 1). The plasma ACE activity in the experimental animals at 20 weeks of age is also shown in Table 1. The ACE activity in the untreated SHR was significantly increased in comparison to WKY. This activity was not significantly different among the untreated SHR or the SHR treated with losartan or PD123319. However, the ACE activity in the SHR treated with enalapril decreased significantly to below the level of WKY. Figure 1 shows a typical example of collagen type 1 by Western blotting. The amounts of collagen type 1, described as the ratio to standard collagen, are shown in Table 2. The aortic hydroxyproline and collagen type 1 concentrations in the untreated control SHR were almost double those observed in the WKY, and they were prevented by enalapril and losartan almost entirely through AT1 receptors (Table 2). The extent of media hypertrophy, expressed as the media cross-sectional area, was an increase of ~50% in untreated SHR, which was virtually unaffected by AT1 block but was approximately halved by enalapril and PD123319 (Table 2). The internal radius in the untreated SHR was significantly smaller than that in WKY, which was virtually unchanged by AT2 block but was enlarged to the same size as that of WKY by enalapril and losartan (Table 2). Figures 2 and 3 show typical examples of the PCR products for the animals in all 5 groups. The amounts of aortic ACE and AT1 and AT2 mRNA were measured by scanning and expressed as a ratio to GAPDH mRNA; the findings are shown in Figure 4. Figure 4 shows that only enalapril inhibits local aortic ACE, which is upregulated considerably in both untreated SHR and in SHR treated with the 2 antagonists in comparison to WKY. The expression of AT1 and AT2 mRNA was also reduced by enalapril in comparison to the untreated SHR because of the inhibition of both receptors. Each specific antagonist lowered its own

### Statistical Analysis

The data are given as the mean±SEM. Comparisons among 3 or more groups were made using a 1-way ANOVA followed by Dunnett’s modified t test. A value of P<0.05 was considered to be statistically significant.

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR</th>
<th>Losartan</th>
<th>Enalapril</th>
<th>PD123319</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyproline, μg/mg protein</td>
<td>3.20±0.71</td>
<td>6.37±0.87*</td>
<td>3.40±0.49†</td>
<td>3.51±0.56†</td>
<td>5.99±0.91*‡§</td>
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<tr>
<td>Collagen type 1, ratio to standard collagen</td>
<td>0.515±0.095</td>
<td>1.121±0.160*</td>
<td>0.521±0.070†</td>
<td>0.554±0.076†</td>
<td>0.995±0.155*‡§</td>
</tr>
<tr>
<td>Cross-sectional area, mm²</td>
<td>0.892±0.035</td>
<td>1.327±0.046*</td>
<td>1.247±0.099*</td>
<td>1.116±0.068†</td>
<td>1.098±0.056†</td>
</tr>
<tr>
<td>Internal radius, mm</td>
<td>1.163±0.048</td>
<td>0.947±0.057*</td>
<td>1.122±0.061†</td>
<td>1.145±0.064†</td>
<td>1.002±0.068*</td>
</tr>
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</table>

Values are mean±SEM. Values in parentheses are percentage of WKY.

*P<0.05 vs WKY; †P<0.05 vs SHR; ‡P<0.05 vs losartan; §P<0.05 vs enalapril.
Discussion
The present study demonstrated that chronic treatment with losartan and enalapril normalized systolic blood pressure and collagen concentration in the aorta of SHR, whereas PD123319 had no effect on either. In contrast, the media cross-sectional area was reduced with PD123319 and enalapril, and it still remained well above the level in WKY. In the unpressurized aorta, the media cross-sectional area is known to be a valid measure of hypertrophy. These findings thus suggest that AT1 has important actions on the extracellular matrix, while AT2 has an important effect on the hypertrophy of the smooth muscle. Ang II has been reported to be involved in vascular hypertrophy and/or hyperplasia.6,7 Arterial hypertrophy and collagen content have been reported to be specifically related to aortic ACE activity.28 In our study, however, PD123319 suppressed both the aortic AT2 mRNA and the hypertrophy of the aortic smooth muscle to the same degree as enalapril without any reduction in the blood pressure. Regarding the reduction of hypertrophy in the smooth muscle with ACE inhibitor, it thus appears to be important to suppress AT2 receptor but not reduce the blood pressure in SHR. The aortic collagen content was reported to be specifically related to the aortic ACE activity independently of hemodynamic factors, since aortic collagen was reduced with ACE inhibitor but not with dihydralazine for the same reduction of the blood pressure.29 AT1 receptor blockades have been shown to prevent the development of the extracellular matrix30 and the collagen accumulation to the same extent as ACE inhibitor.11 In our study, the suppression of AT1 by ACE inhibitor or AT1 antagonist was also shown to be important for reducing the collagen content in the aorta because both losartan and enalapril normalized the AT1 mRNA in the aorta and the collagen concentration. Collagen type 1 increased the most in

Figure 3. Detection of AT2 mRNAs by RT-PCR in aorta from WKY, untreated SHR, and SHR treated with each drug from 10 to 20 weeks. GAPDH mRNA is an internal control. Marker indicates molecular weight marker.

Figure 4. Amounts of aortic ACE, AT1, and AT2 mRNA were measured by scanning and expressed as a ratio to GAPDH mRNA. a indicates P<0.05 vs WKY; b, P<0.05 vs SHR; c, P<0.05 vs losartan; and d, P<0.05 vs enalapril. Values are mean±SEM.
the aorta in SHR, but this was also reduced by either ACE inhibitor or AT\textsubscript{1} antagonist. The amount of collagen type 3 detected by Western blot analysis was small and did not differ in the 5 groups (data not shown). From the 2 main collagen types present, the synthesis of collagen type 1 exceeded that of type 3 in the hypertensive period at >10 weeks of age in the SHR.\textsuperscript{12} In our study, losartan did not reduce media hypertrophy as much as enalapril did. Previous treatment with ACE inhibitors showed an increase in the diameter of renal afferent arteriole\textsuperscript{33} or a large mesenteric artery.\textsuperscript{34} An increase in the lumen is known to increase the average circumferential wall stress, which may also provide nonspecific stimulus hypertrophy in SHR treated with losartan. In our study, however, the vascular internal lumen was not significantly different between the SHR treated with enalapril and losartan, although the radius did increase significantly in comparison to untreated SHR. The systolic blood pressure did not differ significantly either. We thus can speculate that AT\textsubscript{1} receptor shows little relation to media hypertrophy. It was recently reported that the chronic blockade of AT\textsubscript{2} receptor prevented vascular hypertrophy in rats receiving a subcutaneous infusion of Ang II.\textsuperscript{16,17} Levy et al\textsuperscript{18} concluded that PD123319 prevented aortic collagen accumulation but losartan had no effect. The discrepancy between their findings and ours may be due mainly to differences in the dosage and term of administration of the drugs used and the animal models, which received a subcutaneous infusion of Ang II for only 3 weeks. Furthermore, we also do not know how the drugs used by Levy et al changed the ACE and AT\textsubscript{1} and AT\textsubscript{2} mRNAs levels, since they did not examine these levels. Some previous studies have showed subtypes of angiotensin receptors to be downregulated by their selective subtype-specific antagonists in other organs.\textsuperscript{19,20} The present study for the first time showed the information regarding the interaction of mRNAs for AT\textsubscript{1} and AT\textsubscript{2} receptors and ACE to the extracellular matrix and vascular hypertrophy. In our study, all the treated SHR were given drugs between the ages of 10 to 20 weeks. By 10 weeks, changes in the vascular media had already been established, so that the treatment was not aimed at suppression but at reversing the established pathology, much as in human patients with hypertension. Our findings suggest that the reversal of smooth muscle hypertrophy, which is present at >10 weeks of age, is not as easy as many think, although the aortic hypertrophy is partially reversed by AT\textsubscript{2} inhibition. However, the increased aortic collagen that is present at >10 weeks of age can be reversed to normal levels by AT\textsubscript{1} inhibition. Because spironolactone decreased the aortic collagen and the collagen-to-elastic ratio\textsuperscript{17}, possibly through the inhibition of AT\textsubscript{1} receptor,\textsuperscript{20,28,30} the AT\textsubscript{2} antagonist losartan is expected to decrease the arterial stiffness in hypertension. Although a similar action may take place in human essential hypertension, further study is called for to investigate these kinds of drugs regarding the regression of vascular remodeling in the clinical treatment of hypertension.

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References


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