Angiotensin II AT$_1$ receptors regulate ACE2 and angiotensin-(1–7) expression in the aorta of spontaneously hypertensive rats

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ANGIOTENSIN-CONVERTING ENZYME (ACE)2 is a newly recognized ACE homolog within the renin-angiotensin system (RAS) that is produced and secreted from a variety of cell types (19). Although ACE2 may act on several substrates, it exhibits high catalytic efficiency specifically for the hydrolysis of ANG II into the vasodilator and growth inhibitor heptapeptide angiotensin-(1–7) [(ANG-(1–7))] (7, 13, 15). In previous experiments, we showed that ANG-(1–7) mediates the vasodilator effects of combined ACE inhibition and angiotensin type 1 (AT$_1$) receptor blockade (22). Furthermore, a continuous intravenous infusion of ANG-(1–7) reduced neointimal growth in carotid arteries subjected to balloon-catheter injury (29). Taken together, these studies suggest that the regulation of ANG-(1–7) production by ACE2 may be an important component not only of blood pressure control but also of vascular remodeling because ANG-(1–7) opposes the actions of ANG II in both cases (13).

Angiotensin II AT$_1$ receptors regulate ACE2 and angiotensin-(1–7) expression in the aorta of spontaneously hypertensive rats. Am J Physiol Heart Circ Physiol 289: H1013–H1019, 2005. First published April 15, 2005; doi:10.1152/ajpheart.00068.2005.—When increased in vascular tissues, angiotensin-converting enzyme 2 (ACE2), a carboxypeptidase that hydrolyzes angiotensin II to angiotensin-(1–7), may augment the growth inhibitory and vasodilatory effects of the heptapeptide. We investigated the regulation of ACE2 and angiotensin-(1–7) expression in aortas and carotid arteries of 12-wk-old male spontaneously hypertensive rats (SHR) by determining the effect of sustained angiotensin type 1 (AT1) receptor blockade with olmesartan (10 mg·kg$^{-1}$·day$^{-1}$, $n = 13$) compared with those that received atenolol (30 mg·kg$^{-1}$·day$^{-1}$, $n = 13$), hydralazine (10 mg·kg$^{-1}$·day$^{-1}$, $n = 13$), or vehicle ($n = 21$). Systolic blood pressures were ~30% lower ($P < 0.05$) in rats treated for 2 wk with olmesartan compared with vehicle-treated rats. Both atenolol and hydralazine produced similar decreases in systolic blood pressure. ACE2 mRNA in the thoracic aorta of olmesartan-treated rats ($n = 8$) was fivefold greater ($P < 0.05$) than that in vehicle-treated rats ($n = 16$), whereas atenolol ($n = 8$) or hydralazine ($n = 8$) had no effect. Immunostaining intensities in rats treated with olmesartan ($n = 5$) were also associated with increased ($P < 0.05$) ACE2 and angiotensin-(1–7) in thoracic aorta media compared with vehicle-treated rats. In contrast, immunostaining intensities for both ACE2 and angiotensin-(1–7) were not different from vehicle ($n = 5$) in carotid arteries of SHR medicated with either atenolol ($n = 5$) or hydralazine ($n = 5$). A comparison of vessel wall dimensions showed that olmesartan selectively reduced the thoracic aorta media-to-lumen ratio ($P < 0.05$) and media thickness ($P < 0.05$) without an effect on carotid artery morphology. Compared with vehicle-treated SHR, vascular hypertrophy determined from media and lumen measurements was not changed in SHR given either atenolol or hydralazine. These data represent the first report of ACE2 and angiotensin-(1–7) expression in the aorta and carotid arteries of SHR. Increased ACE2 and angiotensin-(1–7) in association with altered dimensions of the thoracic aorta but not carotid arteries in response to olmesartan treatment provides evidence that this pathway is regulated by AT$_1$ receptors and may be important in mediating the pressure-independent vascular remodeling effects of angiotensin peptides.

angiotensin-converting enzyme 2; angiotensin type 1 receptor; vascular remodeling

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Vascular ACE2 mRNA detection. RNA was isolated from the thoracic aorta and carotid arteries using Trizol reagent (Invitrogen; Carlsbad, CA) as directed by the manufacturer. The RNA concentration and integrity were assessed using an Agilent 2100 Bioanalyzer with a RNA 6000 nano LabChip (Agilent Technologies; Palo Alto, CA). Approximately 1 µg of total RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase in a mixture containing deoxyribonucleotides, random hexamers, and RNase inhibitor in reverse transcriptase buffer. Heating the reverse transcriptase reaction product to 95°C terminated the reaction. For real-time RT-PCR, the resultant cDNA was added to TaqMan Universal PCR Master Mix (Applied Biosystems; Foster City, CA) with an ACE2 primer-probe set (forward primer 5'–CCCGAGAAGACGCTGAGCCAAA–3', reverse primer 5'-GCTCCACACACACAGCAGT-3', and probe 5'-FAM-CTCGCGTCTATCC-TAMRA-3'), and amplification was performed on an ABI 7000 Sequence detection system (Applied Biosystems). The mixtures were heated for 1 cycle at 50°C for 2 min and 1 cycle at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. All reactions were performed in triplicate, and 18S rRNA, amplifying using the TaqMan Ribosomal RNA detection Kit (Applied Biosystems), served as a control. The results were quantified as threshold cycle (Ct) values, where Ct was defined as the threshold cycle of PCR at which the amplification product was first detected, and expressed as the target control ratio.

Vascular immunohistochemistry and morphometry. Analysis was performed on four sequential 5-µm cross sections of the proximal thoracic aorta and carotid arteries from each rat. ACE2 and ANG-(1–7) immunohistochemistry were performed as described elsewhere (2, 32). Briefly, cross sections adhered to glass slides were washed in PBS and incubated overnight at 4°C with affinity-purified rabbit polyclonal antibodies to ACE2 and ANG-(1–7) produced in our laboratory at a dilution of 1:1,250 in 1% BSA and 1:25 in 1% BSA, respectively. The specificity and applicability of the antibodies for immunohistochemistry have been previously reported (2, 32). After being washed, sections were incubated for 3 h at 4°C with biotinylated goat anti-rabbit antibody diluted 1:400 in 1% BSA. Sections were rinsed, and a peroxidase-conjugated avidin-biotin method (VectorStain, Vector Laboratories; Burlingame, CA) in combination with 3,3’-diaminobenzidine (DAB; Sigma; St. Louis, MO) in Tris-buffered saline (0.05 mol/L, pH 7.6–7.65) was used according to the manufacturer’s instructions to visualize the location of the primary antibody. Sections were counterstained with hematoxylin (Sigma). Analysis of immunostaining controls was performed on tissue sections from which the primary antibodies were excluded from the immunoreactions. To further illustrate the specificity of the antibodies, immunohistochemical staining was also performed after antibody preadsorption with excess antigen as previously described (2, 32). Tissue sections were examined using light microscopy, photographed with a MicroPublisher 3.3 RTV camera (QImaging; Burnaby, British Columbia, Canada) and analyzed using QCapture Pro software (QImaging). The digitized images at ×20 and ×40 magnification were saved as JPEG files (2,048 × 1,536). Calculation of the immunostaining intensities expressed in arbitrary units was based on Photoshop (version 7.0, Adobe Systems; San Jose, CA) analysis of the luminosity of selected pixels as previously described (2).

Lumen and media cross-sectional areas were determined by measurement of internal (IEL) and external elastic lamina (EEL) lengths traced manually on digitized images using Adobe Photoshop 7.0 (Adobe Systems). Pixel values between the laminae were converted to area (in mm²) by dividing the pixel value by mm². Media area was obtained by subtracting the area encompassed by the IEL from the area encompassed by the EEL. Lumen area represented the area enclosed by the IEL. The media-to-lumen ratios were calculated by dividing the media cross-sectional area by the lumen cross-sectional area. The average medial thickness (in µm) was calculated using the vessel outer diameter at the EEL and the inner diameter at the IEL [(EEL diameter – IEL diameter)/2].

Statistical analysis. All data are expressed as means ± SE. Statistical analyses for differences between rats medicated with the various agents were performed by one-way ANOVA with significance evaluated with the Bonferroni post hoc analysis (GraphPad Software; San Diego, CA). A probability value of <0.05 was established to achieve statistical significance. Averages for density and morphometric values were calculated from numerical data obtained in four sequential tissue sections from each rat.

RESULTS

Table 1 compares the effects of the three treatment regimens versus vehicle on tail-cuff systolic blood pressure, heart rate, body weight, and heart weight-to-body weight ratio. All treated

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Body Weight, g</th>
<th>Systolic Blood Pressure, mm Hg</th>
<th>Heart Rate, beats/min</th>
<th>Heart Weight, g</th>
<th>Heart Weight-to-Body Weight Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>292 ± 4</td>
<td>202 ± 3</td>
<td>312 ± 7</td>
<td>1.31 ± 0.01</td>
<td>4.27 ± 0.09</td>
</tr>
<tr>
<td>After</td>
<td>307 ± 4</td>
<td>207 ± 2</td>
<td>311 ± 5</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>Before</td>
<td>290 ± 4</td>
<td>208 ± 2</td>
<td>308 ± 6</td>
<td>1.19 ± 0.06†</td>
</tr>
<tr>
<td>After</td>
<td>319 ± 4</td>
<td>152 ± 3†</td>
<td>306 ± 9</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Atenolol</td>
<td>Before</td>
<td>285 ± 3</td>
<td>199 ± 4</td>
<td>306 ± 3</td>
<td>1.34 ± 0.03*</td>
</tr>
<tr>
<td>After</td>
<td>320 ± 3</td>
<td>158 ± 3†</td>
<td>258 ± 4†</td>
<td>&lt;0.05</td>
<td>4.19 ± 0.11*</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.05</td>
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<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Hydralazine</td>
<td>Before</td>
<td>284 ± 2</td>
<td>200 ± 3</td>
<td>300 ± 6</td>
<td>1.25 ± 0.07</td>
</tr>
<tr>
<td>After</td>
<td>317 ± 2</td>
<td>146 ± 4†</td>
<td>350 ± 6†</td>
<td>&lt;0.05</td>
<td>3.86 ± 0.20</td>
</tr>
<tr>
<td>P value</td>
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<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SE. P values compare values before and after each treatment period. NS, not significant. *P < 0.05 compared with the olmesartan-treated group; †P value vs. vehicle control; ‡P < 0.05 compared with the hydralazine-treated group.
groups gained weight during the 14 days of the treatment regimen. Vehicle and treatment groups had equivalent systolic blood pressures and heart rates before starting treatment, and all three treatments reduced the elevated blood pressure of SHR compared with vehicle-treated rats. On the other hand, the decrease in systolic blood pressure attained by animals medicated with atenolol was the same as in the olmesartan-treated group but less than that determined in the hydralazine-treated group (Table 1). However, the blood pressure reductions found in SHR assigned to either olmesartan or hydralazine were equivalent. Olmesartan had no effect on heart rate, whereas atenolol induced bradycardia and hydralazine increased heart rate (Table 1). The fall in blood pressure associated with the medications was accompanied by reduced cardiac hypertrophy in SHR assigned to olmesartan but not those medicated with either atenolol or hydralazine.

Figure 1 shows the effects of the treatment regimens on plasma concentrations of ANG II and ANG-(1–7). Olmesartan treatment was associated with increased ($P < 0.05$) plasma ANG II and ANG-(1–7) compared with all other groups, whereas none of the treatments had an effect on plasma ACE activity (data not shown).

Figure 2 shows that only the treatment with olmesartan was associated with a fivefold increase in ACE2 mRNA in the thoracic aorta compared with animals given the vehicle or alternate medications. Although there was a tendency for atenolol to reduce ACE2 mRNA expression in the thoracic aorta, the difference was not statistically significant [mean difference change: 0.34 relative gene expression units; 95% confidence interval (CI): −2.50 to 3.18, $P > 0.05$]. In contrast, ACE2 mRNA in the common carotid artery was significantly reduced in SHR medicated with atenolol but not different than vehicle in both olmesartan- and hydralazine-treated rats. Values are means ± SE. *$P < 0.05$ vs vehicle; **$P < 0.01$ compared with the atenolol-treated group; ***$P < 0.01$ compared with the hydralazine-treated group, respectively.

Representative cross sections of the thoracic aorta from olmesartan-treated SHR stained for ACE2 and ANG-(1–7) are shown in Fig. 3. Figure 3, A and B, shows immunostaining controls demonstrating the absence of staining when the primary ACE2 or ANG-(1–7) antibody, respectively, was excluded from the immunoreactions. The specificity of the antibodies in these tissues was confirmed with preadsorption controls, where the typical lack of staining is illustrated in Fig. 3, C and D. Figure 3, E and F, shows positive staining for both ACE2 and ANG-(1–7) throughout the media region of the thoracic aorta. Staining density for either ACE2 or ANG-(1–7) was comparable to that in the aorta in the carotid arteries of vehicle-treated SHR. Quantitative analysis of the ACE2 and ANG-(1–7) immunostaining intensities in aortas of SHR are shown in Fig. 4. The ACE2 staining intensity was fourfold greater ($P < 0.05$) in the thoracic aorta of olmesartan-treated SHR compared with the staining measured in vehicle-treated animals. Compared with vehicle-treated SHR, ACE2 immuno-
staining tended to increase in thoracic aortas of SHR medicated with either atenolol or hydralazine, but the changes were not statistically significant \((P > 0.05)\). The increased ACE2 staining found in the aorta of SHR treated with olmesartan was associated with a threefold increased intensity of ANG-(1–7) immunostaining (Fig. 4). In contrast, neither ACE2 nor ANG-(1–7) immunostaining intensities, respectively, were quantitatively different in carotid arteries of treated animals (Fig. 5).

Vascular dimensions after treatment of SHR with the various agents are displayed in Table 2. Compared with vehicle-treated SHR, media but not lumen areas were significantly reduced \((P < 0.05)\) in the thoracic aortas of olmesartan-treated rats. These changes were associated with a decrease in both medial thickness and media-to-lumen ratio \((P < 0.05)\). None of the other treatment regimens had an effect on the vascular dimensions in either the thoracic aorta or carotid arteries (Table 2).

**DISCUSSION**

The present results document for the first time the presence of ACE2 mRNA and protein associated with immunoreactive ANG-(1–7) in the thoracic aorta and carotid arteries of SHR. The study further demonstrates an increase in ACE2 mRNA as well as ACE2 and ANG-(1–7) immunoreactivity after exposure of SHR to systemic blockade of AT\(_1\) receptors. In addition, our experiments demonstrate a differential effect of the treatment on large conduit vessels in SHR, because the changes found in the thoracic aorta were not paralleled in the common carotid arteries from the same animals. In the thoracic aorta, increased ACE2 and ANG-(1–7) expression was associated with reduced medial thickness and area. The lower media-to-lumen ratio in aortas of olmesartan-treated SHR compared with vehicle-treated rats was the result of medial thinning, a finding
that documents reversal of hypertrophic remodeling. The demonstration of increased ACE2 gene expression associated with a greater intensity for ANG-(1–7) immunostaining in the thoracic aorta after AT1 receptor blockade suggests that locally generated ANG-(1–7) through increased ACE2 activity may contribute to vascular remodeling in the thoracic aorta of olmesartan-treated SHR.

Although direct wall content measures of vascular remodeling were not determined in the present study, the reduced media-to-lumen ratio was consistent with previous studies in SHR showing reduced large vessel hypertrophy with AT1 receptor blockade (26) and a reduction in aorta internal diameter (27). Our results also agree with studies of dimensional changes in the aorta of SHR after only 2 wk of antihypertensive therapy (18). The differential effect of AT1 receptor blockade on the aorta and carotid arteries may reflect the relatively short duration of olmesartan treatment and an immediate influence of increased aorta ACE2 and ANG-(1–7). Labat et al. (23) found that 10 wk of AT1 receptor blockade with valsartan was necessary to reduce carotid artery cross-sectional areas in SHR. Although we cannot exclude a temporal association between AT1 receptor blockade and regional changes in vascular dimensions, the selective effect of olmesartan on the aorta is also in agreement with studies of other drugs affecting hemodynamics in SHR that demonstrated a similarly contrasting vascular dimensional response in the aorta and carotid arteries (1). Taken together, our data provide further evidence for a differential regulation of both ACE2 and ANG-(1–7) in the vascular system of SHR that resulted in a remodeling event in the aorta but not the carotid arteries within a 2-wk period of exposure to blockade of ANG II receptors. Because the aorta and carotid arteries consist of heterogeneous structural components (5, 17), it is possible that regionally distinct wall restructuring accounts for the different vascular responses observed in this study in response to AT1 receptor blockade. Hence, the findings reported here are important in the further temporal evaluation of the effect of ANG II blockade on blood vessel responsiveness to a generalized stimulus.

Studies of SHR remodeling in response to AT1 receptor blockade consistently show an interference with growth and reduced wall stiffness even without normalized blood pressures (20), but the mechanisms driving the interplay between wall structure and function are not well understood. Local vascular RAS activity is increasingly implicated in the pathogenesis of hypertension as a pathway mediating vascular endothelial and smooth muscle cell growth-related function (11). Benetos et al. (4) showed that chronic ACE inhibition prevented the increase in aortic collagen in SHR independent of blood pressure reduction. Divergent regional expression of the vascular RAS may be an explanation for the heterogeneity of the vascular tree response to pharmacological intervention with RAS inhibitors (3). In support of this hypothesis, Zhou et al. (32) reported that ANG II-induced contraction of carotid arteries was different from that in the thoracic aorta in isolated mouse vessels and was related to the lower AT1 mRNA level in the aorta compared with carotid arteries.

In the present study, we detected increased immunoreactive ANG-(1–7) in the thoracic aorta of SHR as well as increased plasma ANG-(1–7) concentrations with olmesartan treatment. Studies from our laboratory documented that ANG-(1–7) reverses neointimal growth induced by denudation of the vascular endothelium of a carotid artery (29) through a non-AT1/AT2 mechanism (30). Additionally, ANG-(1–7) was shown to block AT2 receptors in vascular smooth muscle cells (31). Divergent regional expression of the vascular RAS suggests that the effect of ANG II blockade on blood vessel responsiveness to a generalized stimulus.
because blockade of ANG-(1–7) receptors with a selective receptor antagonist or a selective ANG-(1–7) antibody reversed the antihypertensive effect mediated by chronic administration of lisinopril and losartan in rats (22). In keeping with these findings, Collister et al. (8) found a correlation between the antihypertensive effect of losartan in normotensive rats and increased plasma ANG (1–7). In recent studies with omapatrilat, one of the first generation of mixed vasopeptidase inhibitors, we showed stimulation of ACE2 expression and activity, leading to increased ANG-(1–7) (14). Moreover, in additional studies, we first reported that blockade of ANG II receptors with either olmesartan or losartan was associated with increased expression of cardiac ACE2 mRNA (21). Given that ANG II is hydrolyzed into ANG-(1–7) with the highest catalytic efficiency seen for any of the angiotensin peptides (28), the finding of increased ACE2 mRNA and protein together with increased ANG-(1–7) in the thoracic aorta of SHR suggests that altered ANG II metabolism by ACE2 may be a mechanism involved in the pressure-independent vascular structural processes associated with blockade of AT1 receptors.

The possibility that the effects of olmesartan on vascular ACE2 gene and protein expression were the result of reduced arterial pressure were ruled out by the comparative effect observed in SHR medicated with either atenolol or hydralazine. Although the administration of either agent had comparable effects in reducing the elevated arterial pressure of SHR, these drugs did not increase thoracic aorta ACE2 mRNA or protein and had no effect on plasma concentrations of ANG II and ANG-(1–7). These data demonstrate that the effects of olmesartan on these variables were mediated by blockade of AT1 receptors rather than a nonspecific effect of reduced blood pressure on vascular wall morphology. Interestingly, a decrease in ACE2 mRNA within the carotid arteries but not the thoracic aorta was found in SHR given atenolol. Because plasma levels of ANG II were not changed by atenolol, feedback inhibition of renin release with a subsequent decrease in ANG II formation cannot account for this change. Furthermore, our previous study (21) demonstrated that ACE2 gene expression is negatively regulated by ANG II because blockade of AT1 but not AT2 receptors resulted in increased ACE2 mRNA. Therefore, the reduced ACE2 mRNA mediated by atenolol suggests a potential influence of β1-adrenoreceptors in ACE2 gene expression within vessels such as the carotid artery. Further work will be required to elucidate the mechanism that is responsible for ACE2 mRNA in conduit vessels such as the carotid artery.

In summary, the association among increased ACE2 gene expression and protein, ANG-(1–7) staining in the thoracic aorta of SHR, and vascular remodeling during blockade of ANG II receptors posits the possibility that both the enzyme and ANG-(1–7) play a critical role in mediating the local effects of reversal of vascular hypertrophy in the thoracic aorta by a mechanism that is independent of arterial pressure.

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