Inhibitory Effect of Angiotensin II Type 2 Receptor on Coronary Arterial Remodeling After Aortic Banding in Mice

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Background—The renin-angiotensin system is thought to be critical for the development of cardiac hypertrophy, whereas the role of the angiotensin II type 2 (AT₂) receptor in the process is not defined. Using the AT₂ receptor-null (Agtr2⁻) mouse, we tested the hypothesis that the AT₂ receptor could exert an antigrowth effect in cardiac hypertrophy.

Methods and Results—Cardiac hypertrophy was induced by suprarenal abdominal aortic banding in 10- to 12-week-old Agtr2⁻ and wild-type (Agtr2⁺) mice for 6 or 12 weeks. Carotid arterial pressure was not different between the strains, although aortic banding increased arterial pressure by ∼40 mm Hg. Aortic banding increased the heart-weight/body-weight ratio and the cross-sectional area of cardiomyocytes by 15%, resulting in comparable cardiomyocyte hypertrophy in the 2 strains. In contrast, coronary arterial thickening and perivascular fibrosis, determined by the media/lumen-area ratio and the collagen/vessel-area ratio, respectively, were 50% greater in Agtr2⁻ than in Agtr2⁺ mice after banding, whereas these parameters were similar in sham-operated mice. Radioligand binding studies using the whole heart and immunohistochemistry showed that AT₂ receptor expression was limited and localized in the coronary artery and perivascular region.

Conclusions—These results suggest that the AT₁ receptor mediates an inhibitory effect on coronary arterial remodeling, such as medial hypertrophy and perivascular fibrosis in response to pressure overload, and an activation of the renin-angiotensin system. (Circulation. 2000;102:1684-1689.)

Key Words: angiotensin receptors; muscle, smooth; myocytes; collagen

Cardiac hypertrophy is an adaptive response to hemodynamic or nonhemodynamic stimuli, such as hypertension and myocardial infarction, and is a major risk factor for heart failure. ACE inhibitors prevent progression and induce regression of cardiac hypertrophy in hypertensive patients. As well as in experimental animal models. Components of the renin-angiotensin-aldosterone system (RAAS), including angiotensinogen, renin, and ACE, are upregulated in the hypertrophied heart. These findings suggest that the RAAS plays a critical role in the development of cardiac hypertrophy.

Most of the known actions of angiotensin II, an effector peptide in the renin-angiotensin system, are mediated by the well-characterized angiotensin II type 1 (AT₁) receptor. The functions of the recently cloned type 2 (AT₂) receptor, however, are still unclear. The recent increase in the clinical application of AT₁ receptor blockers versus ACE inhibitors for the treatment of hypertension and heart failure has raised a question concerning the role of the AT₂ receptor in cardiac hypertrophy. Several studies have shown that AT₂ receptor expression is upregulated in the hypertrophied or failing heart, although this upregulation may be species-dependent. In vitro studies have demonstrated that AT₂ receptor stimulation inhibits the growth of various cell types, including vascular smooth muscle cells, cardiomyocytes, and cardiac fibroblasts, by counteracting AT₁ receptor signaling. These findings have led us to hypothesize that the AT₂ receptor could exert antigrowth effects on the development of cardiac hypertrophy.

Thus, to test this hypothesis, we developed cardiac hypertrophy by abdominal aortic banding in the AT₂ receptor-deficient (Agtr2⁻) mouse. By comparing it with the littermate wild-type (Agtr2⁺) mouse, we demonstrated that coronary arterial thickening and coronary perivascular fibrosis were exaggerated in the Agtr2⁻ mouse, whereas hypertrophy of cardiomyocytes was similar in the 2 strains.

Methods

Animals

Pairs of adult male Agtr2⁻ and Agtr2⁺ littersmates were used in this study. Because the AT₂ receptor gene is located on the X chromo-

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some, heterozygous females were mated with Agtr2+ males to obtain hemizygous and Agtr2+ males. Animal genotyping was performed as previously described.15 These mice, back-crossed for 6 generations into the FVB/N background, had 98% FVB/N and 2% 129/SV background on average. All mice used in this study were 10 to 12 weeks of age and weighed 25 to 30 g. The mice were housed in a room where lighting was controlled (12 hours on, 12 hours off) and room temperature was kept at 22°C. They were given a standard diet and water ad libitum. All experimental procedures were approved and carried out in accordance with the guidelines of the Harvard Medical Area Standing Committee on Animals.

Surgical Procedures

All surgical procedures were performed under anesthesia with ketamine (70 mg/kg) and xylazine (4 mg/kg) administered by IP injection. Abdominal aortic banding was performed as described previously,16 with some modification. Briefly, the abdominal aorta was constricted at the suprarenal level with 7-0 nylon sutures together with a blunted 30-gauge needle, which was pulled out thereafter. Sham operation was performed by isolation of the aorta without ligature. After the experimental period, the hemodynamic effects of aortic constriction were monitored. A polyethylene catheter (PE10; Becton Dickinson) was inserted into the left common carotid artery, tunneled under the skin, and exteriorized at the back of the neck. In some mice, another catheter (PE10) was inserted into the left femoral artery to monitor the pressure gradient between the carotid and femoral arteries. The mice were allowed to recover overnight, then, with the mice in a conscious and unrestrained condition, arterial pressure and heart rate were recorded over 60 minutes through the catheter connected to a Statham pressure transducer. The mice were killed by an overdose of anesthesia and perfused with PBS via the arterial catheter. Subsequently, the heart was perfusion-fixed at 100 mm Hg with 10% neutral buffered formalin. The hearts were excised, weighed, and postfixed in 10% neutral buffered formalin for histological analysis. For receptor binding assay and immunohistochemistry, the hearts were dissected after perfusion with PBS, immediately frozen in liquid nitrogen, and stored at −80°C.

Morphometric Analysis

Fixed hearts were dehydrated and embedded in paraffin. The middle segment of the heart was cut into 5 subserial cross sections 5 μm thick at intervals of 0.3 mm. The sections were stained with hematoxylin and eosin for examination of overall morphology or with van Gieson’s stain for examination of coronary arterial thickness and collagen.16 The regions in the left and right ventricular free wall were used for analysis. Myocyte cross-sectional area was measured in sections stained with hematoxylin and eosin, and suitable cross sections were defined as having nearly circular capillary profiles and nuclei.16 To evaluate coronary arterial thickening and perivascular fibrosis, circular coronary arteries with a long-axis-to-short-axis ratio <1.3 were chosen as suitable cross sections. Wall-area-to-lumen-area ratio, an index of arterial thickening, was defined as the ratio of cross-sectional medial area to lumenal area. Perivascular fibrosis was assessed by calculating the ratio of the area of collagen-stained fibrosis to total vessel area, which was defined as medial area plus lumenal area. Each field was scanned together with a microscope by a CCD camera connected to a Macintosh computer and analyzed with image-analyzing software (NIH Image Ver. 1.61) by an observer blinded to the animal genotype and treatment. The average of >20 regions for myocytes and >10 regions for coronary arteries was taken as the value for each animal.

Radioligand Binding Assay

Binding assays were performed with crude membranes isolated from the hearts (n=3 for each group) as previously described.17 Membrane fractions (100 μg protein) were incubated for 2 hours at room temperature in 100 μL of 20 mmol/L Tris-HCl (pH 7.4) containing 0.25% BSA and 0.2 nmol/L [125I]-labeled [Sar1,Ile8]angiotensin II (NEN Life Science Products) in the absence (for the total count) or presence of 1 μmol/L losartan (Merck & Co, Inc) or 1 μmol/L PD123319 (Research Biochemicals International). Bound and free ligands were separated with GF/C filters (Whatman). AT1 receptor binding was calculated as the difference between the total count and the count from samples incubated with losartan. AT2 receptor binding was determined by subtracting the count of samples incubated with PD123319 from the total count. The net radioactivity count was converted to molar values by use of specific activity of the ligand.

Immunohistochemistry

Frozen sections (5 μm thick) were immunohistochemically stained with the streptavidin-biotin-peroxidase method as described previously.18 Briefly, endogenous peroxidase and the nonspecific binding of the antibody were blocked with 0.3% hydrogen peroxide in methanol and 2% goat serum in PBS, respectively. The antibody to the AT2 receptor (gift from Dr Robert M. Carey, University of Virginia Health Sciences Center; diluted 1:500) or normal rabbit serum diluted in 1% BSA in PBS was applied to the sections and incubated for 16 to 24 hours at 4°C. Subsequently, biotinylated secondary antibody and then streptavidin-peroxidase conjugate were applied. Positive staining was visualized with diaminobenzidine and counterstained with hematoxylin.

Data Analysis

Values are expressed as mean±SEM in the text, table, and figures. Data were analyzed by 1-factor ANOVA. If a statistically significant effect was found, the Newman-Keuls test was performed to isolate the difference between the groups. A value of P<0.05 was considered to be significant.

Results

Hemodynamic Effects of Aortic Banding

Suprarenal aortic banding for 6 weeks produced a systolic pressure gradient of nearly 40 mm Hg between the carotid and femoral arteries (Agtr2+, 38±5 mm Hg; Agtr2−, 39±3 mm Hg, n=6). Carotid mean arterial pressure (MAP), heart rate, and body weight after aortic banding (Banding) or sham operation (Sham) for 6 or 12 weeks are shown in the Table. MAP in the Sham groups was comparable in Agtr2+ and Agtr2− mice. Aortic banding for 6 or 12 weeks increased MAP by 35 mm Hg in the 2 strains compared with that in the corresponding Sham groups. Accordingly, no significant difference was found in MAP after aortic banding between Agtr2+ and Agtr2− mice. Heart rate and body weight were similar in the groups.

Cardiomyocyte Hypertrophy

The degree of cardiomyocyte hypertrophy was evaluated by calculating the ratio of heart weight to body weight (HW/BW ratio) and by measurement of the cross-sectional area of cardiomyocytes. As shown in Figure 1A, HW/BW ratio was not different between sham-operated Agtr2+ and Agtr2− mice at 6 and 12 weeks. Aortic banding increased HW/BW ratio by 15% in the 2 strains, resulting in similar HW/BW ratio in Agtr2+ and Agtr2− mice at 6 and 12 weeks after banding. Consistent with this result, there was no significant difference in the cross-sectional area of cardiomyocytes in the left ventricle between Agtr2+ and Agtr2− mice in the Sham and Banding groups at 6 and 12 weeks (Figure 1B). The cross-sectional area of cardiomyocytes in the right ventricle was not increased by aortic banding or did not differ between Agtr2+ and Agtr2− mice (data not shown).
Body Weight, MAP, and Heart Rate in Agtr2+ and Agtr2− Mice After Aortic Banding (Banding) or Sham Operation (Sham)

<table>
<thead>
<tr>
<th></th>
<th>Body Weight, g</th>
<th>MAP, mm Hg</th>
<th>Heart Rate, bpm</th>
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</thead>
<tbody>
<tr>
<td>6 weeks</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Agtr2+</td>
<td>28.8±0.9</td>
<td>109±2</td>
<td>585±30</td>
</tr>
<tr>
<td>Sham</td>
<td>26.1±0.3</td>
<td>145±5*</td>
<td>597±21</td>
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<tr>
<td>Banding</td>
<td></td>
<td></td>
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<tr>
<td>Agtr2−</td>
<td>27.6±0.7</td>
<td>109±1</td>
<td>596±25</td>
</tr>
<tr>
<td>Sham</td>
<td>26.9±0.4</td>
<td>143±5*</td>
<td>611±19</td>
</tr>
<tr>
<td>Banding</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>12 weeks</td>
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<tr>
<td>Agtr2+</td>
<td>27.3±0.6</td>
<td>106±2</td>
<td>590±20</td>
</tr>
<tr>
<td>Sham</td>
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<td>141±3*</td>
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<tr>
<td>Banding</td>
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<tr>
<td>Agtr2−</td>
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<td>106±2</td>
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<td>Sham</td>
<td>28.2±0.6</td>
<td>140±4*</td>
<td>610±14</td>
</tr>
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</table>

n=6 to 8 for each group. Values are mean±SEM. *P<0.05 vs Sham.

Coronary Arterial Thickening and Perivascular Fibrosis

Coronary arterial thickening and perivascular fibrosis were observed in the heart after aortic banding, but interstitial fibrosis was not. As shown in Figure 2A, these histopathological changes were exaggerated in the Agtr2− mouse. To quantitatively analyze the histology, we measured the medial area of the coronary artery and the area of fibrosis, then calculated each index in the large (100≤ diameter <200 μm) and small (50≤ diameter <100 μm) coronary arteries separately, because the index-diameter relationship was different between large and small arteries.

As shown in Figure 2B, wall-area–to–lumen-area ratio, an index of coronary arterial thickening, in the left ventricle was greater in Agtr2− than in Agtr2+ mice at 6 and 12 weeks after banding, whereas it was similar in sham-operated Agtr2+ and Agtr2− mice.

The ratio of collagen area to total vessel area, an index of perivascular fibrosis, in the left ventricle was also greater in Agtr2− than in Agtr2+ mice at 6 and 12 weeks after banding, whereas they were similar in sham-operated Agtr2+ and Agtr2− mice (Figure 2C).

Morphological changes of coronary arteries in the right ventricle were also examined. Because the number of large coronary arteries was limited in the right ventricle, only the small coronary arteries were analyzed at 12 weeks. Sham-operated Agtr2+ and Agtr2− mice showed comparable wall-area–to–lumen-area ratio (Agtr2+, 0.200±0.016; Agtr2−, 0.206±0.011; n=8) and collagen-area–to–total-vessel-area ratio (Agtr2+, 0.084±0.010; Agtr2−, 0.094±0.008; n=8). After banding, however, wall-area–to–lumen-area ratio was greater in Agtr2− than in Agtr2+ mice, although the strain difference was smaller than that observed in the left ventricle (Agtr2+, 0.210±0.008; Agtr2−, 0.288±0.018; P<0.05, n=7). Similarly, the ratio of collagen area to total vessel area was greater in Agtr2− than in Agtr2+ mice (Agtr2+, 0.117±0.010; Agtr2−, 0.220±0.032; P<0.05, n=7).

AT1 Receptor Expression in the Heart

Receptor binding activity was examined by use of the membrane fraction of the whole heart. As shown in Figure 3A, most of the binding consisted of AT1-specific binding even in the Agtr2+ mouse after aortic banding. AT1-specific binding was not significantly greater in the Agtr2+ mouse in any treatment group than in the Agtr2− mouse. These results suggest that the AT1 receptor was not upregulated, at least in the heart overall, in this mouse model of cardiac hypertrophy. Furthermore, AT1 receptor binding was not different between the strains or the treatments.

To localize the AT1 receptor expression in the hypertrophied heart, we performed immunohistochemical studies. As shown in Figure 3, B through D, positive staining for the AT2 receptor was observed predominantly in the coronary artery and perivascular region of Agtr2+ mice.

Discussion

Histological changes in the hypertrophied heart include cardiomyocyte hypertrophy, coronary arterial thickening, and perivascular fibrosis. In the present study, suprarenal aortic banding induced these morphological changes in mice. Of interest, coronary arterial thickening and perivascular fibrosis were exaggerated in the Agtr2− mouse compared with the Agtr2+ mouse, whereas cardiomyocyte hypertrophy developed similarly in the 2 strains. Cardiac morphology and arterial pressure did not differ between Agtr2+ and Agtr2− mice without banding. In addition, arterial pressure after aortic banding was comparable in Agtr2+ and Agtr2− mice. Although another reported AT1 receptor–null mouse strain and our Agtr2− mouse strain have shown increased arterial pressure at baseline or in response to angiotensin II, the results of the present study indicate that different morphological changes were induced in the 2 strains at the same level of hypertension.

Suprarenal aortic banding creates renal ischemia as well as arterial hypertension, leading to RAS activation and subsequent coronary remodeling of the pressure-overloaded left ventricle and the nonoverloaded right ventricle. Thus, to...
study whether an activation of the RAAS would play an important role in the structural abnormalities in Agtr2− mice, we further examined coronary arterial thickening and perivascular fibrosis in the right ventricle. Importantly, Agtr2− mice showed exaggerated coronary remodeling of the right ventricle as well. This suggests that humoral factors, such as circulating angiotensin II, exerted trophic effects on the right ventricular coronary artery. The reason why the strain difference was smaller in the right ventricle than in the left ventricle may be attributable to the presence or absence of the pressure overload–induced RAAS activation in the myocardium.
In vitro cell culture studies suggest that the AT\(_2\) receptor exerts growth-inhibitory effects in AT\(_2\) receptor cDNA–transfected vascular smooth muscle cells,\(^{12,22}\) coronary endothelial cells,\(^{23}\) neonatal cardiomyocytes,\(^{13,14}\) and cardiac fibroblasts.\(^{14}\) Using in vivo transfer of the AT\(_2\) receptor gene into the balloon-injured rat carotid artery,\(^{12}\) we have also shown that the AT\(_2\) receptor can inhibit vascular smooth muscle cell growth in vivo. On the basis of these results, we investigated whether the AT\(_2\) receptor, which is expressed and/or upregulated in the hypertrophied heart, could mediate the antigrowth effect in cardiac hypertrophy.

In the present study, coronary arterial thickening and perivascular collagen deposition (fibrosis) were augmented in Agtr2\(^{-}\) mice, whereas cardiomyocyte hypertrophy was not. Localization and/or the expression level of the AT\(_2\) receptor may account for these apparently conflicting results in terms of the antigrowth action of the AT\(_2\) receptor. Indeed, ligand-binding studies revealed that AT\(_2\) receptor–binding activity was negligible even in the Agtr2\(^{+}\) mouse heart. Because the membrane fraction of the whole heart consisted predominantly of cardiomyocytes, this result suggests that the expression of the AT\(_2\) receptor in cardiomyocytes, with or without hypertrophy, was scanty in our experimental conditions, although some reports have shown baseline\(^{24,25}\) or hypertrophy-induced\(^{9,10}\) expression of the AT\(_2\) receptor in cardiomyocytes. Localized and limited expression of the AT\(_2\) receptor in other cell types might not be detected by the binding assay. If so, which cell type is responsible for the possible expression of the AT\(_2\) receptor and its effect observed in this study? Our immunohistochemical studies showed that positive staining for the AT\(_2\) receptor was localized in the coronary artery and perivascular region. The results suggest that fibroblasts, smooth muscle cells, and endothelial cells contain the AT\(_2\) receptor, although the expression level and cellular localization are still unclear. It has been reported that fibroblasts are the major cell type expressing the AT\(_2\) receptor in the diseased human heart.\(^{11,24}\) In addition, Tsutsumi et al.\(^{11}\) examined AT\(_2\) receptor signaling using the fibroblast compartment of the failing heart and confirmed the finding of the previous in vitro studies\(^{26,27}\) that the AT\(_2\) receptor exerted an inhibitory effect on p44/42 mitogen-activated protein kinase by counteracting the AT\(_1\) receptor. Coronary endothelial cells are also reported to contain the AT\(_2\) receptor.\(^{25}\) The AT\(_2\) receptor in fibroblasts and smooth muscle cells could inhibit fibrosis and medial thickening, respectively, by directly regulating cell growth. Alternatively, the AT\(_2\) receptor may modulate the production of vasoactive substances, such as nitric oxide\(^{28}\) and bradykinin.\(^{29}\) Thus, the AT\(_2\) receptors in endothelial cells, fibroblasts, and smooth muscle cells might influence each other by regulating growth and/or vascular tone in a paracrine fashion. Taken together, it can be hypothesized that the AT\(_2\) receptor expressed in perivascular fibroblasts, coronary endothelial cells, and smooth muscle cells exerted antigrowth effects directly or indirectly by interaction of the cell types in the process of cardiac hypertrophy. To address the exact localization and the level of AT\(_2\) receptor expression in the hypertrophied heart, which is still controversial,\(^{9–11,24}\) more detailed and fine time-course studies that use in situ hybridization, binding autoradiography, or immunohistochemistry are needed in the future. Because our binding assay and immunohistochemistry are too preliminary to exclude the possible AT\(_2\) receptor expression in cardiomyocytes, the role of the AT\(_2\) receptor in cardiomyocyte hypertrophy remains unclear. Other cardiac hypertrophy models or transgenic mice overexpressing the AT\(_2\) receptor specifically in cardiomyocytes\(^{30}\) may be useful to address this issue.
Coronary arterial thickening as a result of vascular smooth muscle cell hypertrophy or hyperplasia may lead to coronary artery narrowing and decreased coronary reserve. Indeed, medial thickening of the coronary arterioles >50 μm in diameter, which we defined as the small arteries, is reported to contribute to the increase in peripheral vascular resistance. Similarly, perivascular fibrosis alters vasomotor reactivity of intramural coronary arteries, and cardiac fibrosis may lead to decreased compliance and increased myocardial stiffness. Therefore, the finding of the present study concerning coronary arterial thickening and perivascular fibrosis suggests the pathophysiological and clinical importance of the AT$_2$ receptor in cardiac hypertrophy. Because activation of the RAAS is critical for these structural changes, blockade of the AT$_1$ receptor may not only abrogate AT$_1$ receptor signaling but also stimulate the AT$_2$ receptor, leading to inhibition of coronary arterial remodeling, such as medial hypertrophy and perivascular fibrosis.

In summary, coronary arterial thickening and perivascular fibrosis, but not cardiomyocyte hypertrophy, in response to pressure overload and RAAS activation were exaggerated in AT$_2$ receptor–null mice compared with wild-type mice. These results provide a new implication of the AT$_2$ receptor in cardiac hypertrophy.

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References
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