Angiotensin II Type 2 Receptor Is Essential for Left Ventricular Hypertrophy and Cardiac Fibrosis in Chronic Angiotensin II–Induced Hypertension

Sahoko Ichihara, MD; Takaaki Senbonmatsu, MD, PhD; Edward Price, Jr, BS; Toshihiro Ichiki, MD, PhD; F. Andrew Gaffney, MD; Tadashi Inagami, PhD

Background—The roles of angiotensin II (Ang II) in the regulation of heart function under normal and pathological conditions have been well documented. Although 2 types of Ang II receptor (AT₁ and AT₂) are found in various proportions, most studies have focused on AT₁-coupled events. In the present study, we examined the hypothesis that signaling by AT₂ is important to the development of left ventricular hypertrophy and cardiac fibrosis by Ang II infusion in mice lacking the AT₂ gene (Agtr₂⁻/⁻).

Methods and Results—Male Agtr₂⁻/⁻ and age-matched wild-type (WT) mice were treated long-term with Ang II, infused at a rate of 4.2 ng · kg⁻¹ · min⁻¹ for 3 weeks. Ang II elevated systolic blood pressure to comparable levels in Agtr₂⁻/⁻ and WT mice. WT mice developed prominent concentric cardiac hypertrophy, prominent fibrosis, and impaired diastolic relaxation after Ang II infusion. In contrast, there was no cardiac hypertrophy in Agtr₂⁻/⁻ mice. Agtr₂⁻/⁻ mice, however, did not show signs of heart failure or impairment of ventricular relaxation and only negligible fibrosis after Ang II infusion. The absence of fibrosis may be a clue to the absence of impairment in ventricular relaxation and account for the normal left ventricular systolic and diastolic performances in Agtr₂⁻/⁻ mice.

Conclusions—Chronic loss of AT₂ by gene targeting abolished left ventricular hypertrophy and cardiac fibrosis in mice with Ang II–induced hypertension. (Circulation. 2001;104:346-351.)

Key Words: angiotensin ■ collagen ■ heart failure ■ hypertrophy

Myocardial fibrosis is a pathological feature associated with hypertrophy and cardiac hypertrophy, and circulating angiotensin II (Ang II) and aldosterone are involved in the increase in fibrosis and resultant heterogeneity in tissue structure. The progressive interstitial fibrosis and perivascular fibrosis contribute to an increase in cardiac muscle stiffness and development of diastolic dysfunction. Many studies have demonstrated important roles of Ang II in the cardiovascular system under various basal or pathological conditions, such as hypertension and heart failure. Indeed, Ang II was implicated in the development of cardiomyocyte hypertrophy and cardiac fibrosis and modulation of cardiac fibroblast growth and collagen synthesis in humans as well as in animal models.

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The biological effects of Ang II are exerted through specific Ang II receptors. Of the 2 major Ang II receptor isoforms, AT₁ and AT₂, it is generally accepted that most of the traditional Ang II functions in the cardiovascular system are attributable to AT₁. Several recent reports of in vivo as well as in vitro studies using receptor subtype–specific blockers and antisense DNA suggested possible roles of AT₂ in medical hypertrophy and fibrosis in aorta and cultured cells. Synthetic AT₂ antagonists, however, have very short half-lives. Thus, we resorted to mice lacking the AT₂ gene (Agtr₂⁻/⁻). In our recent studies, pressure overload failed to induce left ventricular hypertrophy (LVH) in Agtr₂⁻/⁻ mice, suggesting that AT₂ may be responsible for cardiac hypertrophy. These pressure-overload effects, however, may not have been the direct effects of Ang II/AT₂. To gain insight into the effect of AT₂, we tested the hypothesis that signaling by AT₂ is essential for the development of cardiomyocyte hypertrophy and cardiac fibrosis in Ang II–induced hypertension. For this purpose, we measured the LVH and cardiac fibrosis and their effects on diastolic and systolic functions by M-mode and Doppler echocardiography in long-term Ang II–infused Agtr₂⁻/⁻ and wild-type (WT) mice.

Methods

Animal Preparation

Twelve- to 16-week-old Agtr₂⁻/⁻ (n=38) and WT (n=35) mice were used after 8 backcrosses to C57BL/6. In a pentobarbital-anesthetized (10 mg/kg IP) mouse, an Ang II–impregnated pellet (Inno-
Physiological Studies

Mouse tail-cuff systemic blood pressure (BP) was measured before and 3 weeks after Ang II treatment. Transthoracic echocardiography was performed for measurements of LV internal diameter at end diastole and end systole, interventricular septal wall thickness, posterior wall thickness, and LV mass as previously reported. Pulsed Doppler tracings of the estimated mitral inflow velocity were obtained in a modified parasternal long-axis view. The peaks of the E and A waves of the mitral inflow velocity tracing were recorded as the average of 3 beats.

Morphometric Measurements

Fixed tissues were dehydrated, embedded in paraffin, sectioned at 4 μm thick, and stained with hematoxylin-eosin and van Gieson image system. These parameters were slightly but insignificantly decreased.

Western Blot Analysis

Western blot analysis was performed for ventricular extracts with rabbit polyclonal antibody to collagen type I (Calbiochem-Novabiochem Corp) as previously reported. ECL (Amersham Pharmacia Biotech) was used for detection of immunoreactive bands.

Northern Blot Analysis

Total RNA was extracted from hearts of mice at 3 days and 1 week after implantation. Northern blot analyses of collagen I and III, was performed as previously reported. The outer borders of the bands were traced and areas and densities were determined with the NIH image system.

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT Placebo</th>
<th>WT Ang II</th>
<th>Agtr2−/Y Placebo</th>
<th>Agtr2−/Y Ang II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>29.70±1.11</td>
<td>30.58±0.97</td>
<td>28.62±0.66</td>
<td>29.21±1.06</td>
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<tr>
<td>Blood pressure, mm Hg</td>
<td>114.8±4.9</td>
<td>145.6±6.7</td>
<td>115.3±2.3</td>
<td>147.0±5.31</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>492.9±40.3</td>
<td>527.0±36.5</td>
<td>517.3±37.1</td>
<td>534.7±36.6</td>
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<tr>
<td>RV weight, mg</td>
<td>35.92±2.09</td>
<td>37.00±2.02</td>
<td>35.20±2.66</td>
<td>38.45±2.51</td>
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<tr>
<td>RV/body weight, mg/g</td>
<td>1.28±0.07</td>
<td>1.38±0.06</td>
<td>1.26±0.06</td>
<td>1.33±0.08</td>
</tr>
</tbody>
</table>

*P<0.05 vs WT placebo; †P<0.05 vs Agtr2−/Y placebo.

Statistical Analysis

Data are expressed as mean±SEM. Paired data were compared by Student’s t test. Differences in multiple parameters between 2 groups were compared by 2-way ANOVA, and when interaction was significant, differences were compared by 1-way ANOVA followed by Dunnett’s test. A level of P<0.05 was considered statistically significant.

Results

Arterial Pressure

The systolic arterial pressure increased significantly after Ang II infusion in both strains, as shown in Table 1. In preliminary experiments, we examined Ang II dose dependency at 2.8, 4.2, and 7.0 ng · kg⁻¹ · min⁻¹ (data not shown). There was no significant difference in the pressor response between the 2 groups.

Cardiomyocyte Hypertrophy

Right ventricular (RV) weight in each of the Ang II–treated groups was comparable to those of the placebo groups. This parameter was not different between Ang II– and placebo-treated mice in both strains (Table 1).

At 3 weeks, interventricular septal wall thickness, posterior wall thickness, and the ratio of LV mass to body mass were clearly increased in WT mice over their respective baseline values, whereas no significant change was detectable in Agtr2−/Y mice (Table 2). Cross-sectional areas after Ang II treatment significantly increased in WT mice, whereas there was no significant change in Agtr2−/Y mice (data not shown). Both LV internal diameters at end diastole and end systole were increased after Ang II infusion in WT mice. These parameters were slightly but insignificantly decreased.

Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT Placebo</th>
<th>WT Ang II</th>
<th>Agtr2−/Y Placebo</th>
<th>Agtr2−/Y Ang II</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS, mm</td>
<td>0.87±0.01</td>
<td>0.94±0.01</td>
<td>0.70±0.01</td>
<td>0.73±0.01</td>
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<tr>
<td>PW, mm</td>
<td>0.69±0.01</td>
<td>0.75±0.01</td>
<td>0.63±0.01</td>
<td>0.66±0.01</td>
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<tr>
<td>LVDd, mm</td>
<td>3.59±0.06</td>
<td>3.64±0.05</td>
<td>3.74±0.03</td>
<td>3.68±0.04</td>
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<tr>
<td>LVDs, mm</td>
<td>2.52±0.05</td>
<td>2.65±0.04</td>
<td>2.62±0.04</td>
<td>2.60±0.05</td>
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<tr>
<td>LVM, g</td>
<td>98.21±3.23</td>
<td>107.27±3.08*</td>
<td>83.68±2.07</td>
<td>85.85±2.21</td>
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<tr>
<td>LVM/BW, g/kg</td>
<td>3.28±0.09</td>
<td>3.56±0.09</td>
<td>2.87±0.06</td>
<td>2.95±0.07</td>
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</tbody>
</table>

*P<0.05 vs WT placebo.

IVS indicates interventricular septal wall thickness; PW, posterior wall thickness; LVDd and LVDs, LV internal diameter at end diastole and end systole; LVM, left ventricular mass; and BW, body weight.
in Agtr2−/Y mice. LV fractional shortening remained unchanged in both groups (WT, 27.9±1.0% to 25.8±0.8% versus Agtr2−/Y, 27.7±0.9% to 27.5±1.2%).

Effects of Ang II on LV Function
To characterize the diastolic function of the LV, we compared the Doppler echocardiographic data of each group (Figure 1). To obtain a clear separation of the early diastolic filling wave (E wave) and late filling wave (A wave) of transmitral flow, we reduced the heart rate to ≈350 bpm with pentobarbital (10 mg/kg). Heart rate was not significantly different between the 2 strains used. E/A in WT mice with Ang II treatment was significantly lower than in those with placebo pellet implants (1.55±0.19 versus 2.39±0.42, P<0.05), whereas in Agtr2−/Y mice, this parameter (2.51±0.40 versus 2.82±0.15) remained unchanged after Ang II infusion.

Collagen Deposition
Both interstitial fibrosis and perivascular fibrosis were significantly increased after 3 weeks of Ang II infusion in WT mice (Figure 2, A and B). No significant increase was seen, however, in Agtr2−/Y mice after the identical Ang II infusion. At 3 weeks, LV type I collagen content in Agtr2−/Y mice was significantly less than that of WT mice (Figure 2C). The interstitial collagen fraction in the RV was not affected by Ang II treatment in WT and Agtr2−/Y mice (data not shown).

Expression of Collagen I and III, Fibronectin, TGF-β1, and AT1 mRNAs
To test whether the infusion of Ang II results in the stimulation of the cascade of fibrotic signals, we determined the levels of mRNAs of collagen I and III, fibronectin, TGF-β1, and AT1 in Agtr2−/Y mice. Collagen I and III, fibronectin, and TGF-β1 mRNAs were significantly elevated at 3 days in WT mice (Figure 3, A and B). In Agtr2−/Y mice, however, there were no significant changes compared with WT mice. The level of AT1 mRNA was increased to similar extents at 3 days in WT and Agtr2−/Y mice.

Discussion
We demonstrated that in Agtr2−/Y mice, hypertension produced by chronic administration of Ang II did not induce typical hypertrophy-associated changes in the heart, such as cardiomyocyte hypertrophy, induction of extracellular matrix gene expression, and cardiac and perivascular fibrosis, whereas in WT mice, the same treatment induced marked increases in these responses. These results suggest that the absence of the AT1 gene practically abolished LVH and cardiac fibrosis in mice with Ang II-induced hypertension.

Previously, we had reported a complete loss of hypertrophic response to pressure overload by aortic constriction in Agtr2−/Y mice. In the present studies, again the hypertrophic response was not seen in Agtr2−/Y mice despite elevated BP. The absence of the compensatory hypertrophy under sustained hypertension would be expected to promote early cardiac dilatation and ultimately heart failure. In both the aortic constriction and Ang II infusion models of hypertension, however, cardiac function remained normal. They did not show signs of heart failure or presumed “compensatory” cardiac hypertrophy.

Chronic infusion of Ang II causes proliferation of cardiac fibroblasts and an increase in collagen deposition, which contributes to an increase in cardiac muscle stiffness and development of diastolic dysfunction. Indeed, Doppler echocardiographic study revealed the development of diastolic dysfunction in WT mice. Conversely, Agtr2−/Y mice maintained normal function, presumably because they did not have an increased fibrosis and collagen deposition. The absence of impaired LV relaxation may account for the maintenance of normal LV systolic and diastolic performances. It is postulated that the concentric geometric remodeling with a reduction in LV chamber size relative to wall thickness is an adaptation to preserve LV pump function. In Agtr2−/Y mice, we observed a very slight decrease (statistically insignificant) in LV dimension after Ang II treatment. This adaptation may be another model of the compensatory mechanisms in Nω-nitro-L-arginine methyl ester–induced hypertension, as previously reported by Matsubara et al and...
Figure 2. Collagen deposition. A, Light micrographs of cardiac fibrosis on LV wall sections. All sections were stained with van Gieson stain; magnification ×200. B, Interstitial collagen fraction and perivascular fibrosis, and C, Collagen type I in WT and Agtr2−/− mice. Values are mean±SEM. *P<0.05 vs WT placebo.

Figure 3. Effect of Ang II treatment on LV mRNA levels for selected genes. A, Representative Northern blot showing placebo (p) and effect of 3 days (3d) and 7 days (7d) of treatment with Ang II in WT and Agtr2−/− mice. B, Summary of densitometric analysis. Data are expressed as ratio of designated mRNA to GAPDH mRNA relative to placebo-implanted mice, which was given arbitrary value of 1. Values are mean±SEM. *P<0.05.
Bartunek et al. The latter models showed a decrease in LV volume, absence of LVH, and preserved LV function presumably due to inhibition of nitric oxide (NO) synthesis. AT2 was shown to stimulate the production of bradykinin, which stimulates the NO/cGMP system by a paracrine mechanism to promote vasodilation in aortic vascular smooth muscle cells. Furthermore, Siragy et al reported that Agrt2−/− mice had decreased basal levels of renal interstitial fluid bradykinin and cGMP. These results suggest that the absence of hypertrophy by pressure overload in the NG-nitro-L-arginine methyl ester infusion model may involve a mechanism similar to that in Agrt2−/− mice. Thus, the similarity in the phenotype may explain that AT2 stimulates the signal pathway of NO due to pressure overload in heart.

In vitro studies have shown that Ang II directly stimulates proliferation of cardiac fibroblasts and production of extracellular matrix proteins. It has also been widely recognized that TGF-β1 is involved in Ang II–induced synthesis of collagens. These studies demonstrated that the responses were mediated by AT1. In the present studies, we investigated expression of connective tissues and TGF-β1 mRNAs. As in a number of previous studies, the expression of collagen I and III, fibronectin, and TGF-β1 was increased in WT mice. In contrast, expression of these mRNAs in Agrt2−/− mice was markedly suppressed compared with those of WT mice. There was no significant difference between WT and Agrt2−/− mice in the level of AT1 mRNA at 3 days after Ang II infusion. These results suggest that loss of AT2 signaling suppresses Ang II–induced reprogramming of gene expression, which is known to induce synthesis of extracellular matrix and thus is responsible for tissue fibrosis.

A number of studies suggest that AT1 and AT2 induce phenotypically opposite responses. Little is known, however, about physiological roles of AT1 in heart. Recently, Levy et al demonstrated that chronic AT2 blockade in Ang II–induced hypertensive rats markedly suppressed arterial hypertrophy and fibrosis and proposed that the vasotrophic effects of Ang II may be at least partially mediated via the AT2 receptor. Brilla et al reported that Ang II stimulated collagen synthesis by both AT1 and AT2 receptors in cultured adult rat cardiac fibroblasts and that Ang II–induced inhibition of collagenase activity was specifically mediated by AT2. These findings with vascular and cardiac tissues are consonant with the present observation of abolition of LVH and cardiac fibrosis by chronic loss of AT2 under Ang II–induced hypertension. Conversely, Liu et al demonstrated that the effect of the AT1 antagonist is mediated in part by activation of AT2 in a model of heart failure induced by myocardial infarction in rats. Furthermore, they stated that AT2 might exert a cardioprotective effect only when AT1 was blocked. Results of the present studies suggest that the effects of AT1 may be suppressed without AT2 under the conditions of hypertension produced by administration of Ang II or aortic constriction. These results suggest that the marked suppression of p70^SGK, which may regulate ventricular protein synthesis, in Agrt2−/− mice may provide an explanation for the loss of LVH response.

The administration of Ang II in our study induced an elevation in arterial pressure. We confirmed that chronic loss of AT2 did not affect the hypertension-induced LVH and cardiac fibrosis. These results may suggest that cardiac hypertrophy under elevated BP resulted from growth processes mediated through AT2 upregulated by the elevation of BP. RV hypertrophy and cardiac fibrosis, which would accompany enhanced loading, have not been observed in this model. The reasons may be that AT2 is not expressed in RV when the elevation of BP by Ang II is not great enough or Ang II does not affect cardiac failure. Akishita et al reported different results on coronary artery remodeling in response to pressure overload. These mice were backcrossed for 6 generations into the FVB/N (related to the 129 strain) background, and ours were backcrossed for 8 generations into the C57BL/6 background. Akishita et al used the aortic banding model, whereas the present studies used the Ang II–infusion model. The Ang II–induced elevation of BP was lower than that after aortic banding in the former model. The level of circulating Ang II may also be different between each model. We cannot offer any obvious reasons for the discrepancy between 2 models at the present stage, however. Various studies suggest that AT2 is inhibitory to cell growth and perhaps even related to the induction of programmed cell death. Conversely, Xu et al recently demonstrated that the cardioprotective effect of acute treatment with an AT2 antagonist on LV functional recovery after ischemia/reperfusion in the isolated working rat heart is specifically due to AT2 blockade. Furthermore, Mifune et al reported that AT2 stimulation increased collagen synthesis in vascular smooth muscle via a Gαq-mediated mechanism. Our findings and those of Xu et al and Mifune et al raise the important clinical issue that Ang II may significantly influence patients receiving chronic treatment of AT1 blockers without AT2 inhibitor, because AT2 expression is upregulated in failing hearts and will determine the extent of interstitial fibrosis associated with heart failure.

In summary, we found that the hypertrophic responses to chronic Ang II infusion–induced hypertension were completely lost in Agrt2−/− mice, even if AT1 was expressed. These data indicate that simultaneous activation of both AT1 and AT2 may stimulate a signaling pathway that results in cardiomyocyte hypertrophy and cardiac fibrosis in parallel rather than in opposite directions. Several studies suggest that Ang II is directly implicated in the development of cardiomyocyte hypertrophy and collagen synthesis. Although it is not yet clear whether these effects are directly mediated by AT2, the present results clearly demonstrate that AT2 plays a significant role in cardiomyocyte hypertrophy and cardiac fibrosis in Ang II–induced hypertension.

Acknowledgments

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References


28. Ichihara et al. Role of AT2 in LVH and Fibrosis


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