Effect of Angiotensin II Type 1 Receptor Blockade on Cardiac Remodeling in Angiotensin II Type 2 Receptor Null Mice

Lan Wu, Masaru Iwai, Hironori Nakagami, Rui Chen, Jun Suzuki, Masahiro Akishita, Marc de Gasparo, Masatsugu Horiuchi

Abstract—To clarify the possible involvement of uninhibited angiotensin II (Ang II) type 2 (AT₂) receptor stimulation in the effects of an Ang II type 1 (AT₁) receptor blocker, valsartan, we examined the cardiovascular remodeling induced by aortic banding with the use of wild-type (Agtr2⁺⁺ and AT₂ receptor null (Agtr2⁻⁻) mice. Aortic banding caused cardiac hypertrophy in Agtr2⁺⁺ and Agtr2⁻⁻ mice to a similar degree 6 weeks after surgery, whereas coronary arterial thickening and perivascular fibrosis were more exaggerated in Agtr2⁻⁻ mice. The AT₂ receptor was observed predominantly in the coronary arteries and perivascular region of Agtr2⁺⁺ mice. Valsartan at a dose of 1 mg/kg per day, which did not influence systolic blood pressure, suppressed cardiac hypertrophy similarly in both strains. Valsartan inhibited coronary arterial thickening and perivascular fibrosis in both groups; however, the inhibitory effects of valsartan were significantly weaker in Agtr2⁻⁻ mice. The inhibitory effects of a nonselective Ang II receptor antagonist, [Sar¹, Ile⁸]⁻⁻ Ang II, on cardiac hypertrophy, coronary artery thickening, and perivascular fibrosis were not significantly different in Agtr2⁺⁺ and Agtr2⁻⁻ mice. These results suggest that the improvement by valsartan of coronary arterial thickening and perivascular fibrosis after pressure overload is caused by uninhibited AT₂ receptor stimulation in addition to AT₁ receptor blockade. (Arterioscler Thromb Vasc Biol. 2002;22:49-54.)

Key Words: angiotensin II receptors • cardiomyocytes • fibrosis • coronary arteries

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. Accumulating evidence suggests that angiotensin II (Ang II) plays a critical role in the development of cardiac hypertrophy and cardiovascular remodeling. In addition to the classic Ang II type 1 (AT₁) receptor, another distinct receptor subtype of Ang II has been defined on the basis of differential pharmacological and biochemical properties and has been designated the Ang II type 2 (AT₂) receptor.⁰ Recent evidence suggests that AT₂ receptors may exert antagonistic effects against the AT₁ receptor and that the balance of AT₁ and AT₂ receptors may determine cardiovascular remodeling.²⁻⁵

However, the functions of the recently cloned AT₂ receptor are still an enigma. In vitro studies have demonstrated that AT₂ receptor stimulation inhibits the growth of various cell types, including vascular smooth muscle cells (VSMCs), endothelial cells, cardiomyocytes, and cardiac fibroblasts, by counteracting the AT₁ receptor signaling. These findings have led us to hypothesize that the AT₂ receptor could exert antigrowth effects on cardiovascular remodeling. To examine the in vivo role of the AT₂ receptor in cardiac hypertrophy and cardiovascular remodeling, we developed a pressure overload–induced cardiac hypertrophy model in wild-type (Agtr2⁺⁺) and AT₂ receptor null (Agtr2⁻⁻) mice. Arterial pressure and cardiomyocyte hypertrophy were similarly increased in the 2 strains after abdominal aortic banding.¹¹ In contrast, coronary arterial thickening and perivascular fibrosis were 50% greater in Agtr2⁻⁻ mice than in Agtr2⁺⁺ mice. These results suggest that the AT₂ receptor exerts an inhibitory effect on coronary arterial remodeling.

The recent increase in the clinical application of AT₁ receptor blockers for the treatment of hypertension and heart failure has raised the question of the role of the simultaneous stimulation of the AT₁ receptor and the use of AT₂ receptor blockade in cardiovascular remodeling. Thus, to test this hypothesis in the present study, we used a pressure overload–induced model of cardiac hypertrophy in the Agtr2⁻⁻ mouse, which provided us with a unique opportunity to address the pathophysiological role of AT₂ receptor combination with an AT₁ receptor blockade.

Methods

Animals
Ten-week-old male Agtr2⁻⁻ mice and Agtr2⁺⁺ mice weighing 25 to 30 g were used in the present study. Animal genotyping was performed as previously described.¹² The mice were kept in a room in which lighting was controlled (12 hours on, 12 hours off) and in...
which the temperature was kept at 25°C. They were given a standard diet (MF, Oriental Yeast Co Ltd) and water ad libitum. The Animal Studies Committee of Ehime University approved the experimental protocol.

**Surgical Procedures**

The surgical procedure of abdominal aortic banding was performed according to the method described previously. Briefly, the mice were anesthetized with ketamine (70 mg/kg) and xylazine (4 mg/kg) by intraperitoneal injection. The abdominal aorta was constricted at the suprarenal level with 7-0 nylon sutures with the use of a blunted 30-gauge needle, which was then pulled out. Sham operation was performed by isolation of the aorta without ligation. After the experimental period, the mice were killed by an overdose of anesthesia and perfused with PBS via an 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. Some Agtr2⁺ and Agtr2⁻ mice were treated with valsartan, an AT₁ receptor blocker, or [Sar₁,Ile₈]-Ang II, a nonselective Ang II receptor blocker, by use of an osmotic minipump (model 1002, Alza) implanted intraperitoneally at the time of surgery. The pump delivered valsartan (1 mg/kg per day, provided by Novartis Pharma AG) or [Sar₁,Ile₈]-Ang II (1000 ng/kg per minute) continuously for 6 weeks at a rate of ~0.25 μL/h.

**Morphometric Analysis**

Fixed hearts were dehydrated and embedded in paraffin. The middle segment of the heart was cut into 5 subserial cross sections with a thickness of 5 μm at intervals of 0.3 mm. The sections were stained with elastic van Gieson’s stain for measurement of coronary arterial thickness, perivascular fibrosis area, and myocyte cross-sectional area. Regions in the left ventricular free wall were used for analysis. Suitable cross sections for measurement of cross-sectional area were defined as having nearly circular capillary profiles and nuclei, as previously reported. To evaluate coronary arterial thickening and perivascular fibrosis, circular arteries with a long-axis–to–short-axis ratio <1.3 were chosen as suitable cross sections. The wall–to–luminal area ratio, an index of arterial thickening, was defined as the ratio of cross-sectional medial area to luminal area. Perivascular fibrosis was assessed by calculating the ratio of the area of collagen-stained material to total vessel area, which was defined as medial area plus luminal area. Each field was analyzed with image-analyzing software (NIH Image). The average of >20 regions for myocytes and >10 regions for coronary arteries was taken as the value for each animal.

**Immunohistochemistry**

Frozen sections (5 μm thick) were immunohistochemically stained by the streptavidin-biotin-peroxidase method, as described previously. 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 α-smooth muscle actin (clone 1A4, Sigma Chemical Co), platelet and endothelial cell adhesion molecule-1 (PECAM-1, an antibody to endothelial cells; clone MEC 13.3, BD Biosciences), proliferating cell nuclear antigen (PCNA) antibody (clone PC10, Novocastra Laboratories Ltd), 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 conjugate were applied. Positive staining was visualized with diaminobenzidine, and counterstaining with hematoxylin was performed.

**Data Analysis**

Values are expressed as mean±SEM in the text and figures. Data were analyzed by 2-way ANOVA. If a statistically significant effect was found, the Newman-Keuls test was performed to detect the difference between the groups. A value of P<0.05 was considered to be statistically significant.

**Results**

**Effects of AT₁ Receptor Blockade on Cardiac Hypertrophy Induced by Aortic Banding**

As we previously reported, suprarenal aortic banding for 6 weeks produced a systolic pressure gradient of nearly 40 mm Hg between the carotid and femoral arteries. No significant difference was found in mean arterial pressure in the carotid artery after aortic banding between Agtr2⁺ and Agtr2⁻ mice. The degree of cardiomyocyte hypertrophy was evaluated by calculating the heart weight–to–body weight ratio (HW/BW ratio) and by measurement of the cross-sectional area of cardiomyocytes. Consistent with previous results, these parameters did not differ between Agtr2⁺ and Agtr2⁻ mice in the sham-operated group (Figures 1 and 2). The HW/BW ratio and cross-sectional area of cardiomyo-
cytes were increased to the same extent in Agtr2− mice and Agtr2+ mice 6 weeks after aortic banding. Continuous administration of an AT1 receptor blocker, valsartan, at 1 mg/kg per day, which did not affect systolic blood pressure (data not shown), inhibited the increase in the HW/BW ratio significantly in both strains (P<0.05; Figure 1, Table). Valsartan also significantly suppressed the increase in cross-sectional area of cardiomyocytes similarly in both strains (data not shown), inhibited the increase in the HW/BW ratio and cross-sectional area of cardiomyocytes in Agtr2− mice 6 weeks after aortic banding. Treatment of mice with valsartan decreased coronary arterial thickening in Agtr2+ mice 6 weeks after banding. Valsartan significantly reduced perivascular fibrosis in Agtr2+ and Agtr2− mice (P<0.05; Figure 4, bottom; Table). However, in the large as well as small coronary arteries, this inhibition was weaker in Agtr2− mice than in Agtr2+ mice (Figure 4, bottom; Table). In contrast, the inhibitory effects of [Sar1,Ile8]-Ang II on coronary artery

Effects of Valsartan and [Sar1,Ile8]-Ang II on HW/BW Ratio, Cross-Sectional Area, Coronary Artery Thickening, and Perivascular Fibrosis Area

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Values are expressed as percentage of the value with aortic banding (n=8 to 15 for each group). Hearts were taken 6 weeks after aortic banding with or without valsartan or [Sar1,Ile8]-Ang II administration. Morphological analysis was performed as described in Methods.

*p<0.05 vs Agtr2+.

Effects of AT1 Receptor Blockade on Coronary Arterial Thickening and Perivascular Fibrosis After Aortic Banding

After aortic banding, coronary arterial thickening and perivascular fibrosis were observed. As shown in Figure 3, these changes were exaggerated in Agtr2− mice. To analyze the histological changes quantitatively, we measured the medial area of the coronary arteries and fibrosis and then calculated each index in large (100≤diameter<200 μm) and small (50≤diameter<100 μm) coronary arteries separately, because the index-diameter relationship differed between large and small arteries. As shown in Figure 4, top, the wall area-to-luminal area ratio, an index of coronary arterial thickening, in the left ventricle was 1.6-fold greater in Agtr2− mice than in Agtr2+ mice 6 weeks after aortic banding. We examined the cell populations in the thickened coronary arteries and observed that the major cell type was the VSMC (Figure 5A through 5F) and that the number of PCNA-positive cells was higher in Agtr2− mice than in Agtr2+ mice (Figure 5G). These results suggest that enhanced proliferation of VSMCs contributed to the exaggerated coronary artery thickening in Agtr2− mice after aortic banding. Treatment of mice with valsartan decreased coronary arterial thickening in Agtr2− as well as Agtr2+ mice, in large and small coronary arteries. However, this inhibitory action of valsartan was 1.7-fold weaker in the coronary arteries of Agtr2− mice than in those of Agtr2+ mice (Figure 4, top; Table). The ratio of the collagen area to the total vessel area, an index of perivascular fibrosis, in the left ventricle was also higher in Agtr2− mice than in Agtr2+ mice 6 weeks after banding. Valsartan significantly reduced perivascular fibrosis in Agtr2+ and Agtr2− mice (P<0.05; Figure 4, bottom; Table). In contrast, the inhibitory effects of [Sar1,Ile8]-Ang II on coronary artery

Figure 3. Representative cross sections of coronary artery after aortic banding in Agtr2− and Agtr2− mice with or without valsartan administration (1 mg/kg per day). Sections were examined after van Gieson’s staining. Coronary artery and cardiomyocytes were stained yellow, and collagen was stained red. A through C, Agtr2− mice. D through F, Agtr2− mice. A and D, Sham operation. B and E, Aortic banding. C and F, Aortic banding with valsartan. Original magnification ×50.
thickening and perivascular fibrosis were similar in both strains of mice (Figure 4, Table).

**Discussion**

Consistent with our previous results, we confirmed that pressure overload induced morphological changes of cardiomyocyte hypertrophy, coronary arterial thickening, and perivascular fibrosis in mice. Of interest, coronary arterial thickening and perivascular fibrosis were exaggerated in Agtr2/H11002 mice compared with Agtr2/H11001 mice, whereas cardiomyocyte hypertrophy developed similarly in the 2 strains. Using in vivo transfer of the AT2 receptor gene into the balloon-injured rat carotid artery and cuff placement around the femoral artery in Agtr2/H11001 and Agtr2/H11002 mice, we have also shown that the AT2 receptor can inhibit VSMC growth in vivo. In vitro cell culture studies also suggest that the AT2 receptor exerts growth-inhibitory effects on various cells. In contrast, Senbonmatsu et al recently observed that targeted deletion of the mouse AT2 receptor prevented left ventricular hypertrophy (assessed by echocardiography) resulting from pressure overload, implying that the AT2 receptor is a mediator of cardiac hypertrophy in response to increased blood pressure. Abdominal banding was also used in their study, and some difference in the severity of the increase in blood pressure was found in their study compared with the present study. They reported that compared with the features of Agtr2+ mice, the features of Agtr2− mice included (1) normal ventricular function and (2) thinner ventricular wall thickness, whereas we did not observe such apparent morphological differences between our Agtr2/H11001 and Agtr2/H11002 mice. They also observed that left ventricular and interstitial collagen type I was markedly reduced in aortic-banded Agtr2/H11002 mice compared with aortic-banded Agtr2/H11001 mice. Moreover, they reported that targeted deletion of the mouse AT2 receptor abolished left ventricular hypertrophy and cardiac fibrosis in mice with Ang II–induced hypertension. In contrast, AT2 receptor expression in the myocardium was not detectable before aortic banding, and we could not observe an apparent increase in AT2 receptors in the myocardium after aortic banding in our previous study, suggesting that it is difficult to clarify the role of the AT2 receptor in cardiac hypertrophy in this pressure-overload cardiac hypertrophy model. The difference in genetic back-
ground of Agtr2− mice may contribute to these apparent contradictory results, inasmuch as the genetic background of our Agtr2− mice is FVB/N and the genetic background of their mouse strain is C57BL/6. However, this possibility seems to be less important. Masaki et al17 have reported that cardiac-specific overexpression of the AT2 receptor gene with the use of α-myosin heavy chain promoter in the C57BL/6J mouse strain results in decreased sensitivity to AT1 receptor-mediated pressor and chronotropic actions, that no obvious morphological change was observed in the myocardium, and that there was no significant difference in cardiac development or the HW/BW ratio between wild-type and transgenic mice. Moreover, using the same mouse strain (C57BL/6J), Sugino et al18 reported that administration of a pressor dose of Ang II increased the HW/BW ratio to a similar degree in wide-type and AT2 receptor transgenic mice. Moreover, Bartunek et al19 reported that in adult Ang II–stimulated hypertrophied rat hearts, inhibition of cardiac AT2 receptors, which were upregulated in chronic left ventricular hypertrophy, amplified the immediate left ventricular growth response to Ang II. It has also been suggested that AT1 receptor stimulation may increase collagen synthesis in adult VSMCs and mesangial cells supplemented with retroviral gene transfer of the AT2 receptor but not in fibroblasts transfected with the AT2 receptor.20 Tsutsumi et al21 demonstrated that AT2 receptor expression is upregulated in failing human hearts, that fibroblasts present in the interstitial regions are the major cell type responsible for its expression, and that the AT2 receptor present in fibroblasts exerts an inhibitory effect on Ang II–induced fibrosis associated with heart failure. These apparently conflicting results may provide evidence for heterogeneity of the effects of AT2 receptor stimulation in different tissues, cells, and/or different experimental conditions. These issues and the roles of the AT2 receptor in the human myocardium should be addressed in the near future, because this new class of AT1 receptor blocker appears to possess cardiovascular protective effects, and most of the beneficial effects provided by AT1 receptor blockers appear to be related to more complete blockade of the AT1 receptor; furthermore, costimulation of the AT2 receptor appears to play some role in the improvement of cardiovascular remodeling.

In the present study, we demonstrated that treatment with valsartan effectively attenuated cardiac hypertrophy in Agtr2+ and Agtr2− mice, suggesting that valsartan inhibits the growth-promoting effects of Ang II via the AT1 receptor in cardiomyocytes. In contrast, the effects of valsartan on the inhibition of coronary artery thickening and perivascular fibrosis were weaker in Agtr2− mice. Consistent with these results, as we previously reported,11 AT1 receptor binding was not different between the strains or the treatments, whereas the AT2 receptor was observed predominantly in the coronary arteries and perivascular regions in Agtr2+ mice. Moreover, we observed that AT1 receptor expression assessed immuno-histochemically was comparable in the coronary arteries and perivascular regions of Agtr2+ and Agtr2− mice (data not shown). We also observed that the inhibitory effects of a nonselective Ang II receptor antagonist, [Sar1, Ile8]-Ang II, on cardiac hypertrophy, coronary artery thickening, and perivascular fibrosis were not different between Agtr2+ and Agtr2− mice. Therefore, we can speculate that stimulation of the AT2 receptor after the administration of an AT1 receptor blocker is proportional to the degree of receptor blockade. Indeed, Liu et al22 using a model of heart failure induced by myocardial infarction in rats, demonstrated that an AT1 receptor antagonist improved cardiac function and decreased interstitial collagen deposition and cardiomyocyte size and that these effects were blocked by the AT1 antagonist, suggesting that part of the effect of the AT1 receptor antagonist was due to stimulation of the AT2 receptor. Siragy et al23 also reported that AT1 receptor blockade by valsartan was associated with stimulation of the AT2 receptor, which mediates a renal bradykinin and NO cascade and decreases blood pressure. Our results clearly support the notion that AT2 receptor stimulation along with the use of valsartan contributes to the effect of valsartan in the improvement of cardiac remodeling and that the AT2 receptor plays important roles in cardiac remodeling, such as coronary artery thickening and fibrosis in pressure-overload cardiac hypertrophy.

Coronary arterial thickening as a result of VSMC hypertrophy or hyperplasia may lead to coronary narrowing and decreased coronary reserve, and cardiac fibrosis may lead to decreased compliance and increased myocardial stiffness, suggesting the pathophysiological and clinical importance of the AT2 receptor in cardiac hypertrophy. The specific blockade of the AT1 receptor by valsartan not only may abrogate AT1 receptor signaling but also may increase Ang II binding to uninhibited AT1 receptors, leading to the inhibition of coronary arterial remodeling, such as medial hypertrophy and perivascular fibrosis, resulting in the improvement of cardiovascular remodeling.

Acknowledgments

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References


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