Cardiac Hypertrophy Is Associated With Decreased eNOS Expression in Angiotensin AT2 Receptor–Deficient Mice

Marc Brede, Wilhelm Roell, Oliver Ritter, Frank Wiesmann, Roland Jahns, Axel Haase, Bernd K. Fleischmann, Lutz Hein

Abstract—Angiotensin II receptors play an essential role in cardiovascular physiology and disease. The significance of angiotensin type II (AT2) receptors in cardiac disease still remains elusive. Thus, we tested in gene-targeted mice whether AT2 receptors modulate cardiac function and remodeling after experimental myocardial injury. To generate myocardial infarcts of reproducible size, a cryolesion was generated at the free wall of the left ventricle of wild-type mice (Agtr2+/Y) and mice carrying a deletion of the AT2 receptor gene (Agtr2-/Y). Postinjury remodeling was followed up for 4 weeks after cryoinjury. The cryoprocess led to an increased heart weight/body weight ratio and heart weight/tibia length ratio in AT2-deficient mice compared with control mice. Morphometric analysis revealed a significant increase in myocyte cross-sectional area after cardiac injury (infection vs sham Agtr2+/Y, +53%; vs Agtr2-/Y, +95%). Expression of endothelial nitric oxide synthase (eNOS) was significantly lower in hearts from Agtr2-/Y than from Agtr2+/Y mice. eNOS downregulation was accompanied by a decrease in cardiac cGMP levels in Agtr2-/Y mice. In isolated murine cardiomyocytes, angiotensin II induced eNOS expression through AT2 receptors, and inhibition of NO production by N ω -nitro-L-arginine methyl ester abolished the antihypertrophic effect of AT2 on cardiac myocytes. Our results demonstrate in a genetic mouse model that angiotensin II AT2 receptors exert an antihypertrophic effect in cardiac remodeling after myocardial cryoinjury and link the expression of cardiac eNOS to AT2 receptor activation. (Hypertension. 2003;42:1177-1182.)

Key Words: myocardial infarction • cryoinjury • animals, transgenic • mice • nitric oxide synthase

A growing body of experimental evidence suggests that the angiotensin II receptor subtypes, AT1 and AT2, exert opposing effects in the cardiovascular system. Activation of AT2 receptors might cause antigrowth effects on cells of the cardiovascular system and thus, counteract the growth-promoting action of AT1 receptors.1–3 However, the pathophysiologic relevance of AT2 receptors for cardiac remodeling has not yet been firmly established. Recently, transgenic and gene-targeted mouse models with overexpression or deficiency in angiotensin II receptor subtypes have significantly advanced our understanding of the renin-angiotensin system. Unfortunately, data obtained from AT2 receptor–deficient mice have been contradictory with respect to the specific function of this receptor subtype for cardiac hypertrophy in vivo. In mouse models of left ventricular pressure overload, either AT2 receptors were required for hypertrophic remodeling4 or they did not affect cardiac hypertrophy.5 After experimental coronary ligation in mice, one report concluded that AT2 receptor activation enhanced cardiac hypertrophy.6 However, the majority of the myocardial infarction studies performed with AT2-deficient mice document that AT2 has antihypertrophic effects7,8 or mediates part of the protective effects of AT1 receptor antagonists.9 In contrast, transgenic overexpression of the AT2 receptor in cardiac myocytes in vivo resulted in enhanced hypertrophy10 or dilated cardiomyopathy.11

Several signaling pathways have been proposed to mediate the intracellular actions of AT2 receptor activation in the heart.12–14 We have recently identified that AT2 receptors in rat cardiac myocytes induce expression of the endothelial nitric oxide (eNOS) isoform through a calcineurin-dependent pathway.15 Thus, the aim of this study was to determine whether AT2 receptor activation and eNOS expression are also linked in the murine heart in vivo and whether AT2-dependent eNOS regulation affects cardiac myocyte hypertrophy. Because pressure-overload and coronary artery ligation models have yielded divergent results, part of which might be due to variability of the initial insults, we chose a cryoinfarction model with precisely defined cardiac injury to assess the effects of AT2 gene deletion on postinjury cardiac remodeling in vivo. We also investigated expression of NOS isoforms in this model in vivo and in vitro. Our data show that
AT₂-deficient mice display an enhanced hypertrophic response to myocardial cryoinjury, which was associated with lower cardiac eNOS and cGMP levels. In isolated myocytes, AT₂ activation was sufficient to induce eNOS expression, and inhibition of NO production abolished the antihypertrophic effects of AT₂.

Methods

AT₂ Receptor–Deficient Mice and Myocardial Cryoinjury

Since the initial generation of AT₂-deficient mice,¹⁶ the Agtr2⁻/⁻ deletion was crossed back onto an FVB/N background for 10 generations. For the present study, male littermates with intact (Agtr2⁺/⁺) or deleted (Agtr2⁻/⁻) AT₂ genes were used, which were derived from crosses of heterozygous female (Agtr2⁺/+), and wild-type male FVB/N mice.¹⁷ All animal procedures were approved by the responsible authorities of the University of Würzburg and the Government of Unterfranken (protocol No. 621-2531.01-28/01). Left ventricular infarcts were induced at the free wall of the left ventricle after thoracotomy under isoflurane anesthesia by a copper rod (4-mm diameter), which was precooled in liquid N₂.¹⁸ Altogether, 23 Agtr2⁺/⁺ and 20 Agtr2⁻/⁻ mice were subjected to the cryoinjury. Ninety-three percent of the animals survived the operation and the first 48 hours of the postoperative period. Sixteen mice (8 Agtr2⁺/⁺, 8 Agtr2⁻/⁻) were sham-operated. Mice that died during hemodynamic analysis (2 Agtr2⁺/⁺ and 1 Agtr2⁻/⁻) were not included for further analysis.

Hemodynamic Measurements

For magnetic resonance imaging (MRI) of the heart, mice were anesthetized with 2% isoflurane in 1 L/min oxygen flow through a nose cone. MRI was performed 4 weeks after myocardial cryo-injury in 6 Agtr2⁺/⁺ and 6 Agtr2⁻/⁻ mice, and 5 sham-operated mice per genotype were used for comparison. Cardiac images were taken with a 7.05-T BIOSPEC 70/20 scanner.¹⁹ Left ventricular dimensions, ejection fraction, and cardiac output were derived from 3D reconstructions of sequential short-axis slices as described.¹⁹ For direct cardiac catheterization with a 1.4F high-fidelity pressure-volume catheter (Millar Instruments), mice were anesthetized with trichloroethanol (13 µL of 2.5% solution per g body weight).¹⁷ For evaluation of cardiac contractility, hemodynamic parameters were determined 10 to 12 minutes after induction of anesthesia in all mice.²⁰

Histology

Hearts were fixed in 4% paraformaldehyde in phosphate-buffered saline, embedded in paraffin, stained with Sirius red, and analyzed by computer-assisted morphometry.²¹ Midequatorial sections from a total of 51 hearts (sham, 8 Agtr2⁺/⁺ and 8 Agtr2⁻/⁻; infarct, 19 Agtr2⁺/⁺ and 16 Agtr2⁻/⁻) were used for determination of interstitial fibrosis and myocyte cross-sectional area (16 to 24 nucleated myocytes per section).

NOS Expression

To analyze protein expression by Western blotting, cardiac tissue was prepared and analyzed as described.¹⁷ Monoclonal antibodies against eNOS, neuronal NOS, inducible NOS (all from Transduction Laboratories), β-actin, or histone H1 (Santa Cruz Biotechnology) were used. Left ventricular cGMP levels were determined with a cGMP immunoassay (R&D Systems).

Isolated Cardiac Myocytes

Neonatal cardiac myocytes were isolated from Agtr2⁺/⁺ and Agtr2⁻/⁻ mice as described previously.²² To assess eNOS expression in cardiac myocytes, cells were stimulated for 48 hours with 100 nmol/L angiotensin II in minimal essential medium-1. For determination of protein synthesis, [³H]leucine (2.5 µCi/mL) was added to isolated myocytes, and cells were incubated for 4 hours at 37°C. For some experiments, NO production was inhibited by addition of 200 µmol/L Nω-nitro-L-arginine methyl ester (L-NAME), and cells were stimulated with for 4 hours with 100 nmol/L angiotensin II. Proteins were precipitated in 10% trichloroacetic acid and resuspended in 1% sodium dodecyl sulfate. Radioactivity was measured in a β-counter and normalized to DNA content of the samples.

Statistical Analysis

For all experiments, 1- or 2-way ANOVA followed by appropriate post hoc tests or t tests was used to determine statistical significance (P<0.05) with the use of Prism 3.0cx software (GraphPad).

Results

Cardiac Remodeling After Cryoinjury

To obtain left ventricular infarcts of reproducible size, we chose a cryoinjury model that has been used previously for cellular cardiomyoplasty.¹⁸,²³ In brief, the hearts of anesthetized mice were exposed through a small thoracotomy, and a copper rod (4-mm diameter) that had been precooled in LN₂ was placed on the free wall of the left ventricle to injure a defined area of myocardium. The overall mortality of mice during this procedure was 7%: 2 of 23 wild-type mice (Agtr2⁺/⁺) and 1 of 20 AT₂-deficient mice (Agtr2⁻/⁻) died within 48 hours after the operation. During the following

Figure 1. Cardiac hypertrophy after myocardial infarction. a and b, Sirius red staining of midventricular tissue sections from sham-operated (a) or cryolesioned (b) Agtr2⁻/⁻ mice 4 weeks after operation. Area of the cryolesion is visible as a fibrotic scar (b, arrows), which covers 40% of the left ventricular internal circumference. LV indicates left ventricle; RV, right ventricle. Bars=1 mm. Inserts show myocyte cross sections with hypertrophy in operated Agtr2⁻/⁻ hearts (bars=20 µm). c, Heart weight–body weight ratios were significantly increased after cryoinjury. In AT₂-deficient mice, the magnitude of hypertrophy after cryoinjury was greater than in Agtr2⁺/⁺ mice. d, Morphometric analysis of left ventricular tissue sections revealed enhanced cardiac myocyte hypertrophy, ie, cross sectional areas, in Agtr2⁻/⁻ mice 4 weeks after the cryolesion compared with wild-type mice. Data are mean±SEM, n=8 to 19 mice per group. *P<0.05, Agtr2⁻/⁻ vs Agtr2⁺/⁺. †P<0.05, infarct vs sham.
Cardiovascular Parameters of Wild-Type and Agtr2-/Y Mice After Cryoinjury

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Agtr2+/Y Sham (n=8)</th>
<th>Agtr2+/Y Sham (n=8)</th>
<th>Agtr2+/Y Infarct (n=19)</th>
<th>Agtr2-/Y Infarct (n=16)</th>
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</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>31.8±1.9</td>
<td>30.5±1.1</td>
<td>32.4±0.7</td>
<td>32.6±0.5</td>
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<td>Heart weight, mg</td>
<td>116.1±5.4</td>
<td>115.5±3.6</td>
<td>132.0±4.2†</td>
<td>145.7±4.4†</td>
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<tr>
<td>Heart weight/tibia length, mg/mm</td>
<td>6.25±0.21</td>
<td>6.34±0.19</td>
<td>7.14±0.23†</td>
<td>8.07±0.25†</td>
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<td>LV collagen volume, %</td>
<td>1.5±0.3</td>
<td>2.1±0.3</td>
<td>2.8±0.4†</td>
<td>4.5±0.8†</td>
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<tr>
<td>Infarct area, mm²</td>
<td>...</td>
<td>...</td>
<td>8.0±0.3</td>
<td>8.3±0.4</td>
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<tr>
<td>Infarct scar thickness, μm</td>
<td>...</td>
<td>...</td>
<td>238±18</td>
<td>254±7</td>
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<tr>
<td>LV catheterization</td>
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<td>n=16</td>
<td>n=15</td>
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<td>Heart rate, min⁻¹</td>
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<td>462±18</td>
<td>517±16†</td>
<td>529±15†</td>
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<td>Systolic pressure, mm Hg</td>
<td>96.5±2.3</td>
<td>99.7±3.9</td>
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<td>dP/dt max, mm Hg/s</td>
<td>8468±605</td>
<td>8296±501</td>
<td>6373±377†</td>
<td>7874±555*</td>
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<td>dP/dt min, mm Hg/s</td>
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<td>−6617±837</td>
<td>−6080±350</td>
<td>−6172±961</td>
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<tr>
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<td>n=5</td>
<td>n=6</td>
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<tr>
<td>Stroke volume, μL</td>
<td>56.4±4.4</td>
<td>59.5±6.0</td>
<td>36.2±3.6†</td>
<td>37.4±1.3†</td>
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<tr>
<td>Cardiac output, mL/min</td>
<td>28.4±3.1</td>
<td>27.7±2.6</td>
<td>19.1±2.1†</td>
<td>19.9±1.6†</td>
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<td>End-diastolic volume, μL</td>
<td>82.9±5.3</td>
<td>87.5±4.1</td>
<td>101.2±9.1†</td>
<td>97.4±5.9†</td>
</tr>
</tbody>
</table>

LV indicates left ventricle.

*P<0.05, Agtr2+/Y vs Agtr2+/Y; †P<0.05, infarct vs sham-operated.

4-week observation period, none of the infarcted or sham-operated Agtr2+/Y and Agtr2-/Y mice died. Thus, there was no selection bias between genotypes caused by differences in survival during or after cryoinjury.

Cryoinjury of the left ventricle led to a transmural necrosis, which was replaced by a solid fibrotic scar (Figure 1a and 1b). The infarct covered ≈40% of the internal circumference of the left ventricle. Total infarct area and scar thickness were identical between wild-type and AT2-deficient mice (Table). Cryoinjury caused cardiac remodeling, which was apparent as left ventricular hypertrophy and mild interstitial fibrosis (Figure 1 and the Table). Several parameters indicated that postinjury remodeling was enhanced in Agtr2-/Y mice compared with that in wild-type control animals. Heart weight normalized to body weight or tibia length was significantly greater in AT2-deficient mice than in wild-type mice after injury (Figure 1c and the Table). At the microscopic level, cardiac myocyte cross-sectional area was increased by 53% in Agtr2+/Y mice and by 95% in Agtr2-/Y mice after cryoinjury (Figure 1d). Whereas myocytes were completely replaced by fibrous tissue in the area of cryoinjury, interstitial collagen content had increased only mildly in the noninfarcted area of the septum (Table). The degree of fibrosis did not differ significantly between AT2-deficient and wild-type mice (Table). In sham-operated mice, no differences in cardiac weight and myocyte structure were detected between wild-type and AT2-deficient mice. Thus, genetic deletion of the AT2 receptor gene was associated with increased cardiac hypertrophy after myocardial injury.

Cardiac Function After Cryoinjury

Four weeks after infarction, left ventricular function was assessed by rapid MRI and by direct cardiac catheterization of anesthetized mice. MRI revealed significant dilation of Agtr2+/Y and Agtr2-/Y left ventricles after infarction (Figure 2a). End-systolic and end-diastolic volumes were elevated after cryoinjury, resulting in a decrease of left ventricular ejection fraction from nearly 70% in sham-operated mice to 35% to 38% in infarcted mice (Figures 2b through 2d). Left ventricular volumes, ejection fractions, and cardiac output as determined by MRI did not differ between genotypes after cryoinjury. In contrast, the maximal rate of left ventricular contraction (dP/dt max) was significantly lower in wild-type mice than in AT2-deficient animals after cryolesioning (Figure 2e). Left ventricular systolic and end-diastolic pressures and heart rate were similar in Agtr2+/Y and Agtr2-/Y mice after the infarction (Table), indicating that hemodynamic load did not differ between genotypes.

Cardiac eNOS Expression

Because activation of NO signaling has been suggested to mediate part of the biologic actions of AT2 receptors,24 we next determined the levels of expression of NOS isoforms in hearts of wild-type and AT2 receptor–deficient mice. In sham-operated animals, left ventricular eNOS expression was reduced by 35% in Agtr2-/Y mice compared with Agtr2+/Y mice (Figure 3a). After cryoinjury, hearts from Agtr2+/Y mice showed an increase in cardiac eNOS expression (+24%), which was not observed in Agtr2-/Y mice (Figure 3a). Similar results were obtained when eNOS expression levels were normalized to histone H1 (sham Agtr2+Y, 100±5%; sham Agtr2-/Y, 77±8%; infarcted Agtr2+Y, 124±8%; and infarcted Agtr2-/Y, 83±4%). No significant differences were detectable in cardiac expression levels of neuronal NOS or inducible NOS isoforms (Figures 3b and 3c). Altered eNOS expression in hearts from AT2-deficient mice was also correlated with decreased cardiac cGMP levels (Figure 3d). After cryoinjury, cGMP concentration in the noninfarcted remote myocardium was decreased by 80% in Agtr2-/Y hearts compared with Agtr2+/Y ventricles.
We have previously shown that AT2 receptor activation in isolated rat cardiac myocytes regulates eNOS expression through a calcineurin-dependent pathway. To test whether AT2 receptor activation in mice is also directly linked to eNOS expression, cardiac myocytes were isolated from newborn Agtr2+/H11001 Y and Agtr2-/Y mice. Stimulation of these cardiac myocytes with angiotensin II for 48 hours increased eNOS protein levels in Agtr2+/H11001 Y but not in Agtr2-/Y myocytes (Figure 4), indicating that AT2 receptors participate in the control of cardiac myocyte eNOS levels. To assess whether decreased eNOS expression is causally linked to cardiac myocyte hypertrophy after Agtr2 gene deletion, we determined protein synthesis in cardiac myocytes in vitro. In unstimulated myocytes, incorporation of [3H]leucine did not differ between myocytes isolated from AT2-deficient or wild-type hearts (Figure 5). After stimulation with angiotensin II, protein synthesis increased by 56% in wild-type myocytes and by 113% in AT2-deficient cells. On addition of angiotensin II and the NO synthesis inhibitor L-NAME, protein synthesis was further increased, but the difference between genotypes was abolished (Figure 5), indicating that altered NO signaling after disruption of the Agtr2 gene and myocyte hypertrophy are directly linked.

**Discussion**

In the present study, we provide experimental evidence that angiotensin II AT2 receptors exert an antihypertrophic effect during cardiac remodeling after myocardial infarction and that AT2 receptor activation is directly linked to cardiac eNOS expression.

Previous experiments in mouse models of pressure overload–induced hypertrophy or angiotensin II–induced hypertension have yielded controversial results with respect to the...
cardiac hypertrophy. 31,32 In an ischemia/reperfusion model, cardiac cryoinjury was associated with a 50% decline in left ventricular ejection fraction and structural remodeling of the left ventricle. However, in contrast to coronary artery ligation 6–9 or aortic constriction models, 4,5 cardiac hypertrophy was still modest 4 weeks after cryoinjury (15% increase in heart weight and 50% increase in left ventricular cardiac myocyte cross section; see the Table).

Previous studies applying cardiac injury models to study the function of AT2 receptors in gene-targeted mice might also have yielded conflicting data, because mice used for these studies were maintained on different genetic backgrounds. When crossed onto a C57BL/6J background, AT2-deficient mice did not show cardiac hypertrophy after cardiac pressure overload 6 or angiotensin II–induced hypertension. 25 In contrast, our FVB/N mice did not display differences in cardiac hypertrophy after pressure overload between wild-type and AT2-deficient mice. 5 After coronary artery ligation, hypertrophic remodeling of Agtr2−/− mice was decreased in 1 report 6 or indistinguishable from the phenotype observed in wild-type mice in the second study. 9 However, in the presence of an AT1 receptor antagonist, postinfarction hypertrophy was significantly greater in AT2-deficient mice than in wild-type mice, 7 which corresponds well with our current results after cryoinjury. These data suggest that, at least in mice, AT2 receptors play an important role in the therapeutic effect of AT1 receptor antagonists. 9

NO is an important regulator of cardiac function, 26 and many studies have suggested multiple interactions between the renin-angiotensin system and NO signaling. Here we report that AT2-deficient mice show decreased cardiac eNOS expression resulting in diminished left ventricular cGMP levels. Most important, we provide a biochemical link between cardiac AT2 receptors and expression of eNOS (NOS3). Recently, we have shown that myocardial AT2 receptors induced eNOS expression by way of calcineurin. 15 Thus, AT2 receptors might stimulate NO production through 2 different pathways: (1) AT2 upregulates eNOS expression by calcineurin–nuclear factor-AT signaling 15 or (2) AT2 might increase NO production by a bradykinin B2 receptor–dependent mechanism. 24

Our findings suggest that reduced eNOS-mediated NO production might, at least in part, be responsible for the exacerbated hypertrophic response of AT2-deficient mice after cryoinjury. In isolated cardiomyocytes, inhibition of NO production abolished the difference in protein synthesis between wild-type and AT2-deficient cells. Owing to vascular hypertrophy in AT2-deficient mice, 17 we could not perform similar experiments in vivo without significantly altering the hemodynamic load of the heart. eNOS-knockout mice have systemic 27,28 and pulmonary 29 hypertension, but at baseline cardiac contractility was reported to be normal in eNOS-deficient mice. 30 However, with increasing age, both eNOS- and neuronal NOS–deficient mice develop cardiac hypertrophy. 31,32 In an ischemia/reperfusion model, eNOS-deficient mice had increased infarct sizes. 33 eNOS deficiency was associated with exacerbated left ventricular remodeling and dysfunction and increased mortality after myocardial infarction by ligation of the left anterior descending coronary
artery. Importantly, this phenotype was not affected by pharmacologic normalization of blood pressure. Thus, eNOS-derived NO limits deleterious effects of ischemia by an afterload-independent mechanism, possibly by increasing capillary density and/or by decreasing myocyte hypertrophy in the remote myocardium.

Perspectives
Cardiac angiotensin II AT2 receptors exert an antihypertrophic effect after myocardial injury and are linked to the regulation of cardiac eNOS expression. Thus, selective activation of AT2 receptors might provide further therapeutic benefit in the treatment of postmyocardial infarction remodeling. Future experiments are required to test whether activation of AT2 receptors in other tissues mediates biologic effects through upregulation of eNOS-NO signaling.

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