Vascular Hypertrophy and Increased P70S6 Kinase in Mice Lacking the Angiotensin II \( \text{AT}_2 \) Receptor

Marc Brede; Kerstin Hadamek; Lorenz Meinel; Frank Wiesmann, MD; Jörg Peters, MD; Stefan Engelhardt, MD; Andreas Simm, MD; Axel Haase, PhD; Martin J. Lohse, MD; Lutz Hein, MD

**Background**—Angiotensin II activates 2 distinct G protein–coupled receptors, the \( \text{AT}_1 \) and \( \text{AT}_2 \) receptors. Most of the known cardiovascular effects of angiotensin II are mediated by the \( \text{AT}_1 \) receptor subtype. The aim of the present study was to test whether deletion of the \( \text{AT}_2 \) receptor gene in mice (\( \text{AT}_2 \)-KO mice) leads to long-term functional or structural alterations in the cardiovascular system.

**Methods and Results**—In vivo pressure responses to angiotensin II or the \( \beta_1 \)-adrenergic receptor agonist phenylephrine were greatly enhanced in \( \text{AT}_2 \)-KO mice. Deletion of the angiotensin \( \text{AT}_2 \) receptor did not lead to a compensatory increase of the activity of the circulating renin-angiotensin system, and arterial blood pressure was identical in wild-type control mice (WT) and \( \text{AT}_2 \)-KO mice. Cardiac contractility as assessed by LV catheterization and by rapid MRI also did not differ between \( \text{AT}_2 \)-KO and WT mice. Isolated femoral arteries from \( \text{AT}_2 \)-KO mice, however, showed enhanced vasoconstriction to angiotensin II, norepinephrine, and \( K^+ \) depolarization compared with WT. Morphometric analysis of large and small femoral arteries revealed a significant hypertrophy of media smooth muscle cells. Phospho-P70S6 kinase levels were significantly increased in aortas from \( \text{AT}_2 \)-KO mice compared with WT mice. Treatment of mice with an ACE inhibitor for 8 weeks abolished the increased pressure responsiveness, vascular hypertrophy, and enhanced P70S6 kinase phosphorylation in \( \text{AT}_2 \)-KO mice.

**Conclusions**—These results indicate that vascular \( \text{AT}_2 \) receptors inhibit the activity and, hence, hypertrophic signaling by the P70S6 kinase in vivo and thus are important regulators of vascular structure and function. (Circulation. 2001;104:2602-2607.)

**Key Words:** angiotensin \( \text{ receptors} \) hypertrophy \( \text{vasculature} \) magnetic resonance imaging
with increased abundance of phosphorylated P70S6 kinase, which is a key regulator of protein synthesis and cell growth.

Methods

Generation and Genotyping of AT_2 Receptor–Deficient Mice

The generation of mice lacking functional AT_2 receptors has been described previously. Germline-transmitting chimeric mice were crossed back onto an FVB/N background for 8 generations. For this study, only wild-type (WT) and hemizygous male littermates derived from crosses of male WT FVB/N mice and heterozygous AT_2-KO mice were used (3 to 4 or 10 to 12 months old). One group of 4-week-old WT and AT_2-KO mice was treated with the ACE inhibitor captopril for 8 weeks (1 mg/mL drinking water). All animal procedures were approved by the responsible university and government authorities (protocol No. 621-2531.01-10/98).

Cardiac Catheterization and Rapid MR Imaging

Under tribromoethanol anesthesia, a 1.8F high-fidelity catheter-tip micromanometer (Millar Instruments) was inserted via the right carotid artery into the aorta for arterial pressure measurements or into the left ventricle (LV) to assess cardiac contractility. For acute blockade of AT_2 receptors, 30 mg/kg PD123319 was injected intravenously 15 minutes before infusion of Ang II, which was sufficient to block >98% of the AT_2 receptors in an ex vivo receptor-binding assay. For MRI of the heart, mice were anesthetized with isoflurane (2.0% isoflurane [vol/vol] in 1 L/min oxygen flow). Images of the heart were taken with a 7.05-T BIOSPEC 70/20 scanner.

Isolated Blood Vessels

Vessels were placed in a physiological salt solution consisting of (mmol/L) NaCl 118, KCl 4.7, CaCl_2 2.5, MgSO_4 1.18, KH_2PO_4 1.18, NaHCO_3 24.9, glucose 10, and EDTA 0.03 (37°C and 5% CO_2 /95% O_2). Two tungsten wires (40-mm diameter) were threaded through the lumen of the vessel and mounted in a vessel myograph (Myo500, JP Trading). Pre-tension of the vessels was set to 90% of the ID, which corresponded to an intraluminal pressure of 100 mm Hg.

Histological Analysis

For morphometric analysis of the arterial vessels, mice were anesthetized with tribromoethanol and perfused with 4% glutaraldehyde in PBS at a pressure of 100 mm Hg through the apex of the LV. For histological investigation, the heart, aorta, kidney, and femoral and mesenteric arteries were embedded in paraffin or in epoxy resin. Cross sections and longitudinal sections were digitized with a Zeiss IM35 microscope, and morphometric analyses were performed with NIH Image and Adobe Photoshop software.

Plasma Renin-Angiotensin System

Plasma renin concentration and activity were determined as described previously. Ang II concentrations were measured by radioimmunoassay, and ACE activity was determined by use of 10 mmol/L Z-Phe-His-Leu as substrate.

Western Blotting

Frozen aortas from WT and AT_2-KO mice were homogenized in 500 μL lysis buffer (50 mmol/L Tris-HCl, 2% SDS, 1 mmol/L Na_2VO_3, pH 6.7). After addition of 10 μL mercaptoethanol and 100 μL benzonase (6%; Merck), samples were electrophoresed on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Millipore). Polyclonal antibodies were used to detect total levels of the respective kinase (ERK1/2, PKB/Akt, P70S6 kinase), and their phosphorylated forms were detected with phosphorylation-specific antibodies (Cell Signaling Technology). For controls, the expression of β-actin was determined (Sigma).

Results

Cardiac Function of AT_2 Receptor–Knockout Mice

To assess the role of the AT_2 receptor in cardiovascular physiology, blood pressure, cardiac contractility, and vascular function were determined. In anesthetized mice, heart rate and maximal LV systolic pressure did not differ between AT_2-KO and WT control mice (Figure 1a and 1b). Cardiac contractility was assessed by LV catheterization with a high-fidelity microtip catheter and by rapid MRI. Maximal LV contractility (dP/dt_{max}) and LV ejection fractions were not altered in AT_2-KO mice compared with WT controls (Figure 1c and 1d). Thus, the deletion of the AT_2 receptor gene did not result in any detectable alteration of cardiac function in these mice.

Blood Pressure Regulation

To test the effects of vasoconstrictors on blood pressure regulation in AT_2-KO mice, arterial pressure was measured in anesthetized mice. Under these conditions, baseline mean arterial pressure was similar in AT_2-KO and WT mice (Figure 2). This finding is consistent with the fact that no adaptive change in the activity of the circulating renin-angiotensin system could be detected in AT_2-KO mice (Table 1). We also could not detect alterations in AT_2 receptor mRNA or protein levels in the aorta from AT_2-KO mice compared with WT specimens (data not shown). Small doses of Ang II led to a
significantly higher increase in blood pressure of AT 2-KO mice than of WT controls (Figure 2a). This finding is similar to the increased pressure sensitivity that has previously been reported in awake AT 2-KO mice. The increased sensitivity to Ang II required genetic deletion of the AT 2 receptor gene, however, whereas acute pharmacological inhibition of the AT 2 receptor by the antagonist PD123319 in vivo did not alter the pressure effect of Ang II in WT mice (Figure 2b). Taken together, these data suggest that the deletion of the AT 2 receptor gene exerted a long-term effect on the vascular system rather than an acute effect. This hypothesis could be further supported by the fact that acute infusion of the \( \alpha 1 \)-receptor agonist phenylephrine led to a significantly greater increase in diastolic and systolic blood pressure in AT 2-KO mice than in WT mice (Figure 2c).

Myograph Studies
To test whether the increased blood pressure sensitivity in AT 2-KO mice was due to altered structure or function of the vasculature, we studied contractile responses of isolated femoral arteries in a small-vessel myograph. Ang II–induced active wall tension was 41% higher in vessel segments from AT 2-KO mice than in those from WT mice (Figure 3a). Similarly, activation of \( \alpha 1 \)-adrenergic receptors by norepinephrine led to higher wall tension in AT 2-KO vessels than in WT vessels (Figure 3b). Moreover, the vasoconstrictive effect of 120 mmol/L K+ depolarization was also increased in AT 2-KO mice (Figure 3c). Acetylcholine-induced vasorelaxation did not differ between WT and AT 2-KO mice (\( E_{max} \) WT 92±6% versus AT 2-KO 97±2%, \( n=4 \)), indicating unaltered endothelial function. These data suggest that the deletion of the AT 2 receptor gene led to a structural alteration of the vasculature, because both receptor-mediated and depolarization-induced vasoconstriction were enhanced in AT 2-KO vessels. We thus tested whether vascular morphology was altered in mice lacking AT 2 receptors.

Vascular Morphology
In femoral arteries from the same location as that used for the myograph studies, a significant increase in media thickness and SMC size was apparent in AT 2-KO vessels (Figure 4).
contrast, intima and adventitia were not altered. A similar vascular phenotype was seen in small resistance arteries that supplied the thigh muscle (Table 2). In these femoral resistance arteries, media cell cross-sectional area was increased by \(82\%\) in AT2-KO vessels. This increase was due to an increase in the cell size rather than in SMC number (Table 2). Similarly, a significant enlargement of media myocytes was also observed in AT2-KO aortas (21\% to 26\%) and in renal resistance arteries (26\%) but not in femoral veins.

**Intracellular Signal Transduction**

Ang II has been shown to regulate diverse intracellular signaling pathways. Using conventional and phosphorylation-specific antibodies, we tested the abundance and level of the phosphorylated form of the P70S6 kinase (phospho-P70S6), which has been shown to be associated with cell hypertrophy. In cell lysates from the aortas of AT2-KO mice, the signal detected with a phosphorylation-specific antibody for the P70S6 kinase was 65\% greater in AT2-KO specimens than in WT control arteries (Figure 5a). The abundance of P70S6 protein and \(\beta\)-actin was unaltered. The enhanced phospho-P70S6 kinase level was specific for the arteries of AT2-KOs, because no alteration in phospho-P70S6 kinase could be detected in the heart (Figure 5b). In addition, expression and phosphorylated levels of ERK1/2 kinase and Akt/PKB, 2 upstream activators of P70S6, were unchanged in the vasculature (Figure 5b).

**ACE Inhibition**

To test whether vascular hypertrophy and increased P70S6 phosphorylation are causally linked in AT2-KO mice, animals were treated with the ACE inhibitor captopril for 8 weeks. ACE inhibition completely abolished the increased in vivo blood pressure responsiveness (Figure 6a and 6b), attenuated the enhanced in vitro vasoconstriction (Figure 6c) and phosphorylation of P70S6 kinase (Figure 6d), and prevented vascular hypertrophy in AT2-KO mice (Figure 6e, cross-sectional area of media SMCs 102\% AT2-KO versus WT).

**Discussion**

Our studies demonstrate that Ang II AT2 receptors may play an important role in vascular development. Mice lacking AT2 receptors displayed a characteristic phenotype, with enhanced blood pressure sensitivity in vivo, increased vasoconstriction of isolated arteries, and hypertrophy of VSMCs. This phenotype was associated with an increased phosphorylation of the P70S6 kinase in the vascular wall and could be completely reversed by treatment with an ACE inhibitor. These data

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**TABLE 2. Morphometric Analysis of Small Femoral Resistance Arteries of AT2-KO (n=42) and WT (n=52) Mice (10–12 Months Old)**

<table>
<thead>
<tr>
<th></th>
<th>Wild-Type</th>
<th>AT2-KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>External vessel diam.</td>
<td>77.4 ± 4.7</td>
<td>77.9 ± 4.2</td>
</tr>
<tr>
<td>Lumen diameter, (\mu)m</td>
<td>53.1 ± 4.3</td>
<td>47.5 ± 3.6</td>
</tr>
<tr>
<td>Media thickness, (\mu)m</td>
<td>12.1 ± 0.4</td>
<td>15.2 ± 0.6*</td>
</tr>
<tr>
<td>Media/lumen ratio, %</td>
<td>22.8 ± 0.8</td>
<td>31.9 ± 1.3</td>
</tr>
<tr>
<td>Myocyte No./segment</td>
<td>6.32 ± 0.4</td>
<td>6.31 ± 0.3</td>
</tr>
<tr>
<td>Myocyte cross-sectional area, (\mu)m²</td>
<td>586 ± 41</td>
<td>1065 ± 91*</td>
</tr>
</tbody>
</table>

*P<0.01 AT2-KO vs WT.
AT_2 receptors are abundantly expressed in VSMCs of the murine fetal vasculature during late gestation, when the blood vessels undergo remodeling. Ang II–induced growth in embryonic VSMCs from WT mice was increased by the AT_2 receptor antagonist PD123319, indicating that vascular AT_2 receptors are functional and exert an antigrowth effect in the normal mouse vasculature. Further evidence suggests that the AT_2 receptor promotes vascular differentiation and contributes to vasculogenesis in mice. In the rat, AT_2 receptors were expressed more abundantly in SMCs and endothelial cells from microvessels than in large vessels, suggesting that AT_2 receptors may be situated in a position in which they could directly oppose the effects of vascular AT_1 receptor activation.

The SMC hypertrophy in AT_2-KO vessels was associated with an increased level of the phosphorylated form of P70S6 kinase (phospho-P70S6). It was previously demonstrated that Ang II can activate P70S6 kinase via AT_1 receptors in VSMCs and in cardiac myocytes. This is the first report, however, to describe that AT_2 receptors may antagonize the effect of AT_1 receptors on P70S6 kinase activity in vivo. The mechanism by which AT_2 receptors mediate inhibition of P70S6 kinase activity remains unclear. Both ERK and PI3 kinase/Akt pathways have been implicated in activation of P70S6 in VSMCs, but neither ERK/Akt nor phospho-Akt/PKB levels were altered in AT_2-KO aorta. AT_2 receptors can activate several intracellular phosphatases (for review see Reference 13), which might directly affect the phosphorylation status of P70S6 kinase. Identification of these phosphatases represents an important step to further identify the in vivo significance of the AT_2 receptor subtype.

It has been suggested that increased expression of AT_1 receptors alone may explain the AT_2-KO phenotype, such as increased blood pressure, higher sensitivity to Ang II, and altered renal function. In the present mouse model, it is unlikely that the vascular phenotype is due to increased Ang II production or upregulation of AT_1 receptors, because we did not observe an increase of blood pressure, increased activity of the renin-angiotensin system, or upregulation of vascular AT_1 receptors. Thus, the vascular hypertrophy and increased levels of phospho-P70S6 kinase are most likely due to an imbalance in AT_1 versus AT_2 receptor signaling for cardiovascular pathology are still unknown. These data suggest that inhibition of AT_2 receptors stimulates vascular hypertrophy and thus may be a prerequisite for development of overt hypertension. The role of AT_2 receptors may depend on the tissue, because in the heart, AT_2 is required for cardiac hypertrophy and inhibition of coronary artery remodeling after pressure overload by abdominal aortic constriction. These findings may have important clinical significance, because regression of hypertension-induced cardiac hypertrophy by AT_1 antagonists may be in part due to an unopposed antigrowth effect of Ang II mediated via AT_2.
Acknowledgment
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References
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