Vascular Hypertrophy and Increased P70S6 Kinase in Mice Lacking the Angiotensin II AT$_2$ Receptor

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

Methods and Results—In vivo pressure responses to angiotensin II or the $\alpha_1$-adrenergic receptor agonist phenylephrine were greatly enhanced in AT$_2$-KO mice. Deletion of the angiotensin 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 AT$_2$-KO mice. Cardiac contractility as assessed by LV catheterization and by rapid MRI also did not differ between AT$_2$-KO and WT mice. Isolated femoral arteries from 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 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 AT$_2$-KO mice.

Conclusions—These results indicate that vascular 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 $\square$ receptors $\square$ hypertrophy $\square$ vasculature $\square$ magnetic resonance imaging

Angiotensin II (Ang II) is a major regulator of cardiovascular homeostasis. Its biological actions are mediated via 2 different types of G protein–coupled receptors, called AT$_1$ and AT$_2$. The AT$_1$ subtype mediates most of the cardiovascular actions, including vasoconstriction and aldosterone release. Transgenic animals have been invaluable models to dissect the physiological role of individual components of the renin-angiotensin system. The contribution of the AT$_2$ subtype in cardiovascular regulation has remained elusive, although transgenic mouse models lacking or overexpressing this receptor have been generated. Deletion of the gene encoding the AT$_2$ receptor in mice (AT$_2$-KO mice) led to a phenotype with enhanced blood pressure sensitivity to Ang II, reduced exploratory behavior, and impaired drinking response after water deprivation.

A growing body of evidence suggests that AT$_1$ and AT$_2$ elicit countervailing influences on cell growth (for review, see Reference 1). The AT$_2$ receptor may exert an antigrowth effect on cells of the cardiovascular system and thus counteract the growth-promoting action of the AT$_1$ receptor. AT$_2$ receptors cause apoptosis in vascular smooth muscle cells (VSMCs). Part of the growth-inhibitory effect of AT$_2$ receptor stimulation may be mediated by activation of phosphatases, which could attenuate several intracellular growth pathways (for review, see Nouet and Nahmias[13]). On the basis of studies using pharmacological ligands, the role of AT$_2$ receptors in vascular remodeling in vivo is controversial. The regression of vascular hypertrophy is a potential therapeutic target for the reduction of complications associated with hypertension.

Here, we investigate whether deletion of the AT$_2$ receptor gene in mice leads to long-term functional or structural alterations in the cardiovascular system that might explain the increased pressure sensitivity of AT$_2$-KO mice to Ang II infusion. Our studies show that hypertrophy of VSMCs is the structural basis for increased in vivo and in vitro responses to several vasoconstrictors, including Ang II and norepinephrine. Vascular hypertrophy in AT$_2$-KO mice was associated with...
with increased abundance of phosphorylated P70S6 kinase, which is a key regulator of protein synthesis and cell growth.

Methods

Generation and Genotyping of AT₂ Receptor–Deficient Mice

The generation of mice lacking functional AT₂ receptors has been described previously.15 Germline-transmitting chimeric mice were crossed back onto an FVB/N background for 8 generations. This study, only wild-type (WT) and hemizygous male littermates derived from crosses of male WT FVB/N mice and heterozygous AT₂-KO mice were used (3 to 4 or 10 to 12 months old). One group of 4-week-old WT and AT₂-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.16 For acute blockade of AT₁ receptors, 30 mg/kg PD123319 was injected intravenously 15 minutes before infusion of Ang II, which was sufficient to block >98% of the AT₁ 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.17

Isolated Blood Vessels

Vessels were placed in a physiological salt solution consisting of (mmol/L) NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.18, NaHCO₃ 24.9, glucose 10, and EDTA 0.03 (37°C and 5% CO₂/95% O₂). Two tungsten wires (40-μm diameter) were threaded through the lumen of the vessel and mounted in a vessel myograph (Myo500, JPTrading).18 Pre-tension of the vessels was set to 90% of the ID, which corresponded to an intraluminal pressure of 100 mm Hg.18

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.19 Ang II concentrations were measured by radioimmunoassay, and ACE activity was determined by use of 10 mmol/L Z-Phe-His-Leu as substrate.20

Western Blotting

Frozen aortas from WT and AT₂-KO mice were homogenized in 500 μL lysis buffer (50 mmol/L Tris-HCl, 2% SDS, 1 mmol/L Na₂VO₃, pH 6.7). After addition of 10 μL mercaptoethanol and 100 μL benzotriazole (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 AT₂ Receptor–Knockout Mice

To assess the role of the AT₁ 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 AT₂-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/dtmax; c) did not differ between AT₂-KO and WT mice (n=8). ECG-triggered series of MR images of 10- to 12-month-old male mice were recorded as described to determine ejection fractions (d). Insets show representative images in maximal diastole or systole. LV ejection fractions did not differ between AT₂-KO and WT mice (n=5).

Blood Pressure Regulation

To test the effects of vasoconstrictors on blood pressure regulation in AT₂-KO mice, arterial pressure was measured in anesthetized mice. Under these conditions, baseline mean arterial pressure was similar in AT₂-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 AT₂-KO mice (Table 1). We also could not detect alterations in AT, receptor mRNA or protein levels in the aorta from AT₂-KO mice compared with WT specimens (data not shown). Small doses of Ang II led to a
significantly higher increase in blood pressure of AT2-KO mice than of WT controls (Figure 2a). This finding is similar to the increased pressure sensitivity that has previously been reported in awake AT2-KO mice.5,6 The increased sensitivity to Ang II required genetic deletion of the AT2 receptor gene, however, whereas acute pharmacological inhibition of the AT2 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 AT2 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 AT2-KO mice than in WT mice (Figure 2c).

**Myograph Studies**

To test whether the increased blood pressure sensitivity in AT2-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 AT2-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 AT2-KO vessels than in WT vessels (Figure 3b). Moreover, the vasoconstrictive effect of 120 mmol/L \(K^+\) depolarization was also increased in AT2-KO mice (Figure 3c). Acetylcholine-induced vasorelaxation did not differ between WT and AT2-KO mice (E_max WT 92 ± 6% versus AT2-KO 97 ± 2%, n = 4), indicating unaltered endothelial function. These data suggest that the deletion of the AT2 receptor gene led to a structural alteration of the vasculature, because both receptor-mediated and depolarization-induced vasoconstriction were enhanced in AT2-KO vessels. We thus tested whether vascular morphology was altered in mice lacking AT2 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 AT2-KO vessels (Figure 4). In
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\%/11006 15% 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\%/11006 4%) and in renal resistance arteries (26\%/11006 3%) 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. b, Levels of ERK1/2, phospho-ERK1/2, and phospho-Akt kinase in aorta and heart of AT2-KO and WT mice. Insets show representative blots from 4 to 6 independent experiments with 6 to 8 lanes per genotype. For these experiments, male mice 3 to 4 months old were used. \( ^*P<0.05 \).

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\%/11006 5% 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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<th>TABLE 2. Morphometric Analysis of Small Femoral Resistance Arteries of AT2-KO (n=42) and WT (n=52) Mice (10–12 Months Old)</th>
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<tr>
<td><strong>Wild-Type</strong></td>
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<tr>
<td>External vessel diameter, ( \mu )m</td>
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<td>Lumen diameter, ( \mu )m</td>
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<td>Media thickness, ( \mu )m</td>
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<td>Media/lumen ratio, %</td>
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<td>Myocyte No./segment</td>
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<td>Myocyte cross-sectional area, ( \mu )m²</td>
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\(*P<0.01 \) 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.$^5$ 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.$^{21,22}$ Small amounts of AT$_2$ receptor could be detected in the aorta of WT mice by radioligand binding.$^7$ 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.$^{24}$

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.$^{25}$ This is the first report, however, to describe that AT$_1$ receptors may antagonize the effect of AT$_2$ receptors on P70S6 kinase activity in vivo. The mechanism by which AT$_1$ 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.$^{26,27}$ Neither phospho-ERK1/2 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.$^{23,28}$ 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.$^{29,30}$ 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$_2$.

Figure 6. Treatment with ACE inhibitor captopril (8 weeks) abolished vascular hypertrophy and increased P70S6 kinase phosphorylation in AT$_2$-KO mice. a and b, On intravenous infusion of $\alpha_1$-adrenergic receptor agonist phenylephrine, systolic and diastolic arterial pressure increased to significantly higher levels in untreated AT$_2$-KO mice than in WT mice. This difference between genotypes was abolished by ACE inhibitor treatment (n=6 to 8 mice per genotype). c, Maximal wall tension elicited between isolated femoral arteries from WT and AT$_2$-KO mice, whereas response of AT$_2$-KO vessels to 0.3 mol/L Ang II was increased (n=6 vessels per group). d, No difference was observed in levels of phospho-P70S6 kinase in aortic lysates (d) or in femoral artery structure (e) from WT and AT$_2$-KO mice after ACE inhibitor therapy (n=4 mice per group). $^*P<0.05$ AT$_2$-KO vs WT.
Background

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References

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