High Blood Pressure Reduction Reverses Angiotensin II Type 2 Receptor–Mediated Vasoconstriction Into Vasodilation in Spontaneously Hypertensive Rats

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Background—We have previously shown that angiotensin II type 2 receptor (AT2R) stimulation causes endothelium-dependent vasodilation that does not desensitize after chronic angiotensin II type 1 receptor (AT1R) blockade, suggesting a role for AT2R in antihypertensive treatment.

Methods and Results—We recorded mean arterial pressure (MAP) and investigated AT2R by Western blot analysis, immunohistochemistry, and function in isolated mesenteric resistance arteries (205 µm in diameter) from Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) receiving the following for 4 weeks in drinking water: placebo, AT1R blockade (candesartan; 2 mg/kg per day), ACE inhibitor (perindopril; 3 mg/kg per day), nonselective vasodilator (hydralazine; 16 or 24 mg/kg per day), or candesartan plus hydralazine (16 mg/kg per day). In precontracted isolated arteries, AT2R stimulation (angiotensin II in the presence of candesartan) caused vasodilation in WKY rats (MAP = 118 mm Hg) and vasoconstriction in SHR (MAP = 183 mm Hg). In SHR treated with candesartan (MAP = 146 mm Hg) or hydralazine (16 mg/kg per day; MAP = 145 mm Hg), AT2R-induced contraction was reduced by 50%. In SHR treated with perindopril (MAP = 125 mm Hg), AT2R stimulation induced vasodilation. In SHR treated with hydralazine (24 mg/kg per day; MAP = 105 mm Hg) and in SHR treated with hydralazine (16 mg/kg per day) plus candesartan (MAP = 102 mm Hg), an AT2R-mediated vasodilation was restored. Immunochemistry and Western blot analysis showed that AT2R expression, lower in SHR than in WKY rats, was restored to normal levels by treatments reducing arterial pressure in SHR.

Conclusions—Our results suggest that in resistance arteries of SHR, (1) AT2R is downregulated by hypertension, and (2) specific and nonspecific antihypertensive treatments restore AT2R expression and vasodilator functions. (Circulation. 2005;111:1006-1011.)

Key Words: angiotensin receptors | vasculature | vasodilation | hypertension

Angiotensin II (Ang II), the key effector of the renin-angiotensin-aldosterone system, has an important role in the control of blood pressure and blood volume. Ang II activates at least 2 receptor types: the Ang II type 1 receptors (AT1R) and the Ang II type 2 receptors (AT2R). Stimulation of AT2R undoubtedly induces relaxation in several vascular territories. In most blood vessels, AT2R-dependent relaxation is associated with activation of the bradykinin or NO/cGMP pathway. In vitro, the vasodilator role of AT2R is supported by evidence based on enhanced Ang II–mediated vasoconstriction in the presence of AT2R blockade or in AT2R-knockout mice. AT2R mRNA and protein expression were demonstrated in resistance arteries from normotensive rats. We have previously shown in mesenteric resistance arteries from Wistar-Kyoto (WKY) rats that AT2R is involved in NO-dependent flow-mediated dilation, whereas in spontaneously hypertensive rats (SHR), flow (shear stress) stimulation of the endothelium is associated with an AT2R and endothelin-1 type A receptor activation, thus counteracting endothelium-dependent dilation. Furthermore, acute administration of an AT2R inhibitor reversed both the acute antihypertensive effects and elevated level of bradykinin, NO, and cGMP in renal interstitial fluid caused by AT1R blockade in renal wrap and salt-restricted rats. Finally, we have recently shown the reproducibility of the vasodilator effect of AT2R stimulation under acute and chronic AT1R blockade, further supporting the assumption that AT2R stimulation might play a role in the antihypertensive effect of AT2R-blocking drugs. Thus, we speculate that AT2R stimulation, and hence vasodilation, might play a role...
in antihypertensive treatments, especially when AT,R-blocking agents are used. Indeed, AT,R antagonists induce an important rise in circulating Ang II. AT,R might then be chronically overstimulated, participating in the maintenance of vasodilation, particularly because this effect was not easily desensitized.20

Thus, in the present study we evaluated the vasomotor role of AT,R in resistance arteries isolated from SHR (hypertensive conditions) and after various chronic antihypertensive treatments, including ACE inhibition, AT,R blockade or a nonselective treatment, and AT,R expression and immunolocalization. We hypothesized that AT,R-mediated dilation, which was impaired in untreated SHR, would be reestablished in mesenteric arteries taken from treated SHR in parallel with reductions in blood pressure.

**Methods**

**Animal Model**

Male WKY rats and SHR (aged 7 to 8 weeks) were separated into 8 groups receiving the following for 4 weeks in drinking water: WKY rats, placebo or AT,R antagonist (candesartan cilexetil; 2 mg/kg per day); SHR, placebo, ACE inhibitor (perindopril; 3 mg/kg per day), candesartan (2 mg/kg per day), nonselective antihypertensive drug (hydralazine; 16 mg/kg per day), or candesartan plus hydralazine (16 mg/kg per day).

The protocol used was in accordance with the European Community standards on the care and use of laboratory animals (authorization No. 00577).

**Mean Arterial Pressure Measurement**

After 4 weeks of treatment, rats were anesthetized with sodium pentobarbital (50 mg/kg IP). Mean arterial pressure (MAP) was measured in the right carotid artery with a catheter connected to a Gould transducer and an analog-digital signal recording system (Biopac).16,17

**Isolated Mesenteric Artery**

A 3- to 4-mm-long segment of mesenteric artery (205±11 μm, internal diameter measured at 75 mm Hg in the absence of tone) was dissected, cannulated at both ends, and mounted in a video-monitored perfusion system,21 as we have previously described.6,15,17,20 Briefly, arteries were bathed in a physiological salt solution (PSS) maintained at 37°C, pH 7.4. The PO<sub>2</sub> was 160 mm Hg and the PCO<sub>2</sub> 37 mm Hg.16,17,20 The artery was superfused (4 mL/min), and flow through the vessel was maintained at a rate of 100 μL/min, with intraluminal pressure set at 75 mm Hg.20 Arterial diameter was measured (Living Systems Instrumentation) and recorded continuously (Biopac). Vessels were allowed to stabilize for at least 30 minutes before drugs were added to the PSS superfusion. The integrity of the endothelium was assessed by testing for at least 30 minutes before drugs were added to the PSS superfusion. The integrity of the endothelium was assessed by testing for the relaxing effect of acetylcholine (1 μmol/L) after precontraction with Ang II at 100 nmol/L. The arteries were then exposed to relaxation was repeated before and after application of one of the following drugs: the cyclooxygenase inhibitor indomethacin (10 μmol/L), the thromboxane A<sub>2</sub>-PGH<sub>2</sub> receptor blocker SQ29548 (10 μmol/L), the bradykinin B<sub>2</sub> receptor blocker HOE140 (0.1 μmol/L), and the endothelin receptor blocker bosantan (10 μmol/L).

Stimulation of AT,R was also performed before and after endothelium removal (5 seconds of air perfusion; n=4 rats per group) in WKY rats, untreated SHR, and SHR treated with candesartan plus hydralazine (16 mg/kg per day).

**Western Blot Analysis of AT,R**

Western blot analysis of AT,R was performed in mesenteric resistance arteries of WKY rats and SHR (n=8 per group). Mesenteric arteries were also isolated from SHR treated with hydralazine (24 mg/kg per day for 18, 23, or 48 days; n=5 per group) or with candesartan plus hydralazine (16 mg/kg per day; n=5 per group).

Mesenteric arteries were homogenized with a lysis buffer (1% sodium dodecyl sulfate, 10 mmol/L Tris-HCl [pH 7.4], 1 mmol/L sodium orthovanadate, 2.5 mmol/L leupeptin, and 5 mg/L aprotinin). Extracts were incubated at 25°C for 30 minutes and then centrifuged (1000g, 15 minutes, 14°C). Protein concentration was determined with the use of the Micro BCA Protein Assay Kit (Pierce). After denaturation at 100°C for 5 minutes, equal amounts of proteins (15 μg) were loaded on a 9% polyacrylamide gel and transferred to nitrocellulose membranes for 12 hours (40 V, 4°C). Membranes were blocked with 10% BSA in TBST (20 mmol/L Tris pH 8.0, 150 mmol/L NaCl, and 0.1% Tween-20) for 1 hour and were then incubated with AT,R rabbit polyclonal antibody (dilution 1:100, Santa Cruz) in washing solution at room temperature for 20 hours. The membranes were then washed and incubated with the anti-rabbit horseradish peroxidase antibody (dilution 1:5000; Amersham Pharmacia Biotech) for 1 hour at room temperature. After 3 washes with TBST, immunocomplexes were detected by chemiluminescent reaction (ECL kit; Amersham Pharmacia Biotech) with a computer-based imaging system (Fuji LAS 1000 Plus; Fuji Medical Systems). Quantification was performed by densitometric analysis.

**Immunofluorescence Analysis of AT,R**

Segments of mesenteric resistance arteries (n=6 rats per group) were mounted in embedding medium (Miles, Inc), frozen in isopentane precooled in liquid nitrogen, and stored at −80°C on transverse cross sections 7 μm thick. Sections were incubated with candesartan (30 minutes, 10 nmol/L, 25°C), then with fluorescent Ang II (FITC-bound Ang II, 30 minutes, 10 pmol/L, 25°C; Molecular Probes). Fluorescence staining was visualized by confocal microscopy (Bio-rad MRC-600). Control experiments were performed after incubation with nonfluorescent Ang II. Image analysis was performed with the use of Histolab (Microvision). Briefly, pixel quantification was performed after the media and the endothelial layer were separated. Data are given as percentage of control (with fluorescence in WKY rats taken as 100%).

**Drugs**

Candesartan cilexetil was kindly provided by AstraZeneca (Sweden). Other products were purchased from Sigma.

**Statistical Analysis**

Results are expressed as mean±SEM. The significance of the different treatments was determined by ANOVA or 2-tailed Student paired t test. Probability values <0.05 were considered significant. Number of rats was used for the analysis.

**Results**

MAP and AT,R-Mediated Dilation in Isolated Arteries

Figure 1 shows typical recordings obtained with mesenteric arteries isolated from WKY rats (Figure 1A) and SHR (Figure 1B). MAP was 118±8 mm Hg (n=8) in WKY rats and 183±11 mm Hg (n=8) in SHR. Isolated arteries were first incubated with candesartan (100 nmol/L, 30 minutes) and precontracted with phenylephrine (54±2 μm diameter decrease). Addition of Ang II (100 nmol/L) induced a
significant dilation (24±3 μm diameter increase) in control WKY rats. By contrast, in SHR the stimulation of AT_{2}R led to a significant contraction (8±3 μm diameter decrease; Figure 1B). In both WKY rats and SHR, the arteries were able to fully dilate when acetylcholine was added after Ang II. Diameter changes in response to AT_{2}R stimulation (dilation in WKY rats or contraction in SHR) were suppressed by the AT_{2}R antagonist PD123319 (1 μmol/L). In the presence of candesartan and PD123319, Ang II produced no significant change in diameter (2±3 μm, n=5 in WKY rats and −1±2 μm, n=4 in SHR).

The contraction induced by Ang II (100 nmol/L) in arteries isolated from SHR was significantly reduced by indomethacin (10 μmol/L; 6±2 versus 12±3 μm reduction in diameter; n=5), SQ29548 (10 μmol/L; 6±3 versus 14±3 μm reduction in diameter; n=5) and by bosentan (10 μmol/L; 7±3 versus 16±3 μm reduction in diameter; n=5). The combination of indomethacin (10 μmol/L) and bosentan (10 μmol/L) suppressed Ang II–induced contraction in SHR (2±3 versus 14±3 μm reduction in diameter; n=5). The bradykinin B_{2} receptor blocker HOE140 (0.1 μmol/L) did not significantly affect Ang II–induced contraction in SHR (13±3 versus 15±4 μm reduction in diameter). In WKY rats, Ang II–induced dilation was significantly reduced by N\textsuperscript{5}-nitro-L-arginine methyl ester (L-NAME) (4±2 versus 24±4 μm) and by HOE140 (6±3 versus 26±5 μm).

The stimulation of AT_{1}R produced a concentration-dependent dilation in arteries isolated from WKY rats and a concentration-dependent contraction in arteries isolated from SHR (Figure 1C; n=8 per group).

Concentration-dependent stimulation of AT_{1}R (Ang II 0.01 to 100 nmol/L) was repeated in arteries isolated from WKY rats and SHR submitted to various treatments. Maximal responses to AT_{1}R stimulation and MAP determined in the different groups are shown in Figure 2.

There was no significant difference in MAP and AT_{1}R-mediated dilation between control WKY rats (118±8 mm Hg; 24±3 μm diameter increase; n=8) and WKY rats treated with candesartan (108±9 mm Hg; 19±5 μm diameter increase; n=4).

In SHR, candesartan partly depressed MAP (146±8 mm Hg, n=4 versus 183±11 mm Hg, n=8; P<0.01 versus SHR and P<0.01 versus WKY rats) and tended to reduce AT_{1}R-mediated contraction, although this did not reach significance (8±3 versus 3±2 μm diameter decrease).

In SHR, treatment with perindopril reduced MAP (125±6 mm Hg; P<0.01), and AT_{1}R stimulation induced a significant vasodilation (6±2 μm diameter increase) that was significantly lower than AT_{2}R-induced dilation in WKY rats.

In SHR treated with hydralazine (16 mg/kg per day; n=4), MAP decreased to a level that was still higher than in WKY rats (145±11 mm Hg; n=6; P<0.01 versus SHR and P<0.05 versus WKY rats), and stimulation of AT_{2}R induced vasoconstriction (3±1 μm diameter decrease; n=6).

In SHR treated with a higher dose of hydralazine (24 mg/kg per day; n=4), MAP was reduced to a normal value (105±10 mm Hg; P=NS versus WKY rats), and AT_{2}R induced a significant vasodilation (27±7 μm diameter increase; P=NS versus WKY rats).

In SHR treated with candesartan plus hydralazine (16 mg/kg per day), MAP was reduced to a normal level (n=4; 102±9 mm Hg; P=NS versus WKY rats), and AT_{1}R induced a significant vasodilation (22±5 μm diameter increase; P=NS versus WKY rats).

Endothelium removal did not affect AT_{1}R-dependent contraction in untreated SHR (12±3 μm with endothelium versus 14±3 μm contraction without endothelium; n=4). On the other hand, AT_{1}R-dependent dilation was abolished by endothelium removal in both untreated WKY rats (24±4 versus 3±2 μm dilation; n=4) and SHR treated with candesartan plus hydralazine (16 mg/kg per day; 26±4 versus 4±2 μm increase in diameter; n=4).

Western Blot Analysis of AT_{2}R

In resistance arteries isolated from untreated WKY (n=8) rats and untreated SHR (n=8), as well as from SHR treated with hydralazine alone (24 mg/kg per day; n=5) or hydralazine (16 mg/kg per day; n=5) combined with candesartan, AT_{2}R expression was quantified. Western blot analysis showed that AT_{2}R was significantly less expressed in SHR than in WKY rats (46%) in isolated mesenteric resistance arteries (Figure 4). Hydralazine (24 mg/kg per day) gradually raised AT_{2}R expression in SHR treated for 18, 23, and 48 days. After 48...
days, AT2R expression was significantly higher than in SHR, but it remained significantly lower than in WKY rats (70%; Figure 3). However, after 44 days of treatment, candesartan plus hydralazine (16 mg/kg per day) restored AT2R expression in SHR to a level equivalent to that found in WKY rats (96.7% versus 100%; Figure 3).

Immunohistology Analysis of AT2R
In WKY rats, immunofluorescence analysis of mesenteric resistance arteries, with the use of confocal microscopy, indicated that AT2R was present in the endothelium, in the smooth muscle, and in the adventitia. In the endothelium, AT2R immunofluorescence was lower in SHR than in WKY rats (53% of WKY; \( P < 0.05 \); \( n = 6 \) per group). In the media a significant fluorescence could be detected, although it was lower than in WKY rats (32% of WKY; \( P < 0.01 \); \( n = 6 \)). In SHR treated with candesartan plus hydralazine (16 mg/kg per day), immunofluorescence of AT2R was restored to a level equivalent to that found in WKY rats (compared with WKY rats: 88% in the endothelium and 81% in the media) (Figure 4).

Discussion
The present study demonstrates that AT2R stimulation induced a vasoconstriction in untreated SHR resistance arteries associated with a decrease in AT2R expression. Specific or nonspecific antihypertensive treatments restored AT2R expression and its vasodilator function when the decrease in pressure was sufficient.

Recent evidence suggests that AT2R stimulation causes vasodilation in small resistance arteries in normotensive rats.6,7,11,16,17,20,22 This vasodilation may play an important role in the regulation of arterial blood pressure by increasing the diameter of resistance arteries. Vasodilation induced by AT2R stimulation has been described in several vascular territories and is usually associated with NO production by endothelial cells and cGMP production by smooth muscle cells.23 In some but not all arteries investigated, bradykinin B2 receptor activation is involved in AT2R-dependent dilation.9–11,22 Importantly, AT2R-mediated dilation does not desensitize, in contrast to AT1R-dependent contraction, supporting the assumption that AT2R dilation might have a role in the
same arteries produces dilation. In addition, AT2R-dependent signaling from constrictor to dilator mechanisms due to increased endothelial AT2R expression. Indeed, our immunohistological analysis of AT2R in mesenteric arteries and skeletal muscle arterioles. We found that AT2R expression was lower in SHR resistance arteries than in WKY rats. However, a decreased expression cannot readily explain a reversal of dilation into contraction. The mechanism of this reversal remains to be discovered but may involve a switch in signaling from constrictor to dilator mechanisms due to increased endothelial AT2R expression. Indeed, our immunohistological analysis of AT2R in mesenteric arteries from SHR showed undetectable AT2R labeling in the endothelium. Thus, the difference in the type of response might reflect a change in AT2R expression between the endothelium and the smooth muscle. Nevertheless, because of the small size of the resistance arteries, AT2R expression and mRNA level were not significantly decreased by endothelium removal in either SHR or WKY rats (D. Henrion, unpublished data). In addition, cultured endothelial cells rapidly lose AT2R phenotype, thus preventing a study of AT2R expression in cultured endothelial cells from WKY rats or SHR.

Stimulation of the NO-cGMP pathway by AT2R has been shown initially in aortic cells. In the dog coronary circulation, NO production by Ang II–induced contraction was decreased by AT2R blockade. Interestingly, this effect involved stimulation of endothelin-1 and probably thromboxane A2. We found that AT2R expression was lower in SHR resistance arteries than in WKY rats. However, a decreased expression cannot readily explain a reversal of dilation into contraction. The mechanism of this reversal remains to be discovered but may involve a switch in signaling from constrictor to dilator mechanisms due to increased endothelial AT2R expression. Indeed, our immunohistological analysis of AT2R in mesenteric arteries from SHR showed undetectable AT2R labeling in the endothelium that was reestablished with antihypertensive treatment. AT2R expression was low in the wall of mesenteric arteries compared with WKY. After MAP reduction in SHR, AT2R expression was restored to the level of WKY rats. However, it is difficult to determine whether AT2R-mediated dilation is a cause or a consequence of the reduction in blood pressure. In the combined candesartan/hydralazine group of SHR, the time course for AT2R expression, assessed by Western blots, was in parallel with the restoration of AT2R-mediated dilation and normotension, which may indicate a primary role for AT2R. On the other hand, equivalent reductions in MAP caused by hydralazine were associated with maximal AT2R-mediated dilation despite suppressed AT2R expression. These discrepancies in AT2R abundance between treatments may reflect distinct AT2R locations within the vasculature. Indeed, more precise immunohistological analysis demonstrated that AT2R expression was located in the endothelial and smooth muscle cells in WKY rats, whereas in SHR AT2R was not detectable in the endothelium. On the other hand, in SHR treated with candesartan plus hydralazine, MAP was restored, AT2R stimulation induced vasodilation, and AT2R expression was equivalent to that found in WKY rats. In this group, immunohistology of AT2R showed the
presence of the receptor in both endothelia and smooth muscle cells. Thus, we can speculate that the presence or absence of AT2R on endothelial cells has a key role in determining the type of response, at least in part because the inhibition of the vasodilation in WKY rats by L-NAME does not uncover a vasoconstriction due to receptors located on the muscle, as revealed by immunohistology. We can also assume that MAP per se is the effector determining the type of response induced by AT2R stimulation (Figure 2C).

In conclusion, in resistance arteries of SHR, (1) AT2R is downregulated by hypertension, and (2) specific and nonspecific antihypertensive treatments restore AT2R expression and vasodilator functions. Whether or not this AT2R plasticity directly contributes to the blood pressure reduction, this AT2R response induced by AT2R stimulation (Figure 2C).

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**References**

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