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/H9262 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/H11005 118 mm Hg) and vasoconstriction in SHR (MAP/H11005 183 mm Hg). In SHR treated with candesartan (MAP/H11005 146 mm Hg) or hydralazine (16 mg/kg per day; MAP/H11005 145 mm Hg), AT2R-induced contraction was reduced by 50%. In SHR treated with perindopril (MAP/H11005 125 mm Hg), AT2R stimulation induced vasodilation. In SHR treated with hydralazine (24 mg/kg per day; MAP/H11005 105 mm Hg) and in SHR treated with hydralazine (16 mg/kg per day) plus candesartan (MAP/H11005 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 types1,2: the Ang II type 1 receptors (AT1R) and the Ang II type 2 receptors (AT2R).3–5 Stimulation of AT1R undoubtedly induces relaxation in several vascular territories.6,7 In most blood vessels, AT2R-dependent relaxation is associated with activation of the bradykinin or NO/cGMP pathway.8–11 In vitro, the vasodilator role of AT1R is supported by evidence based on enhanced Ang II–mediated vasoconstriction in the presence of AT1R blockade or in AT1R-knockout mice.12–14 AT1R mRNA and protein expression were demonstrated in resistance arteries from normotensive rats.15,16 We have previously shown in mesenteric resistance arteries from Wistar-Kyoto (WKY) rats that AT1R is involved in NO-dependent flow-mediated dilation,16 whereas in spontaneously hypertensive rats (SHR), flow (shear stress) stimulation of the endothelium is associated with an AT1R and endothelin-1 type A receptor activation, thus counteracting endothelium-dependent dilation.17 Furthermore, acute administration of an AT1R 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.18,19 Finally, we have recently shown the reproducibility of the vasodilator effect of AT1R stimulation under acute and chronic AT1R blockade,20 further supporting the assumption that AT1R stimulation might play a role in the antihypertensive effect of AT1R-blocking drugs. Thus, we speculate that AT1R stimulation, and hence vasodilation, might play a role...
in antihypertensive treatments, especially when AT\(_2\)R-blocking agents are used. Indeed, AT\(_2\)R antagonists induce an important rise in circulating Ang II. AT\(_1\)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\(_1\)R in resistance arteries isolated from SHR (hypertensive conditions) and after various chronic antihypertensive treatments, including ACE inhibition, AT\(_1\)R blockade or a nonselective treatment, and AT\(_1\)R expression and immunolocalization. We hypothesized that AT\(_2\)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\(_1\)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; 2 groups: 16 or 24 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 \(\mu\)m, 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.\(^{6,17,20}\) Briefly, arteries were bathed in a physiological salt solution (PSS) maintained at 37°C, pH 7.4. The PO\(_3\) was 160 mm Hg, the PCO\(_2\) 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 \(\mu\)L/min, with intraluminal pressure set at 75 mm Hg.\(^{20}\) Arterial diameter was measured (LIVING Systems Instruments) 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 the relaxing effect of acetylcholine (1 \(\mu\)mol/L) after precontraction with phenylephrine (1 \(\mu\)mol/L). The arteries were then exposed to candesartan (100 nmol/L) for at least 30 minutes before exposure to Ang II while they were precontracted with phenylephrine (1 \(\mu\)mol/L) and serotonin (0.1 \(\mu\)mol/L) to induce a stable reduction in diameter. When this response had reached a plateau, a concentration-response curve to Ang II at 0.1 to 100 nmol/L or Ang II at 100 nmol/L was performed, and changes in diameter were measured. Acetylcholine (1 \(\mu\)mol/L) was used to completely dilate the vessels. Eight rats were used in each group.

In separate series of experiments (n=5 per group), AT\(_2\)R stimulation was repeated before and after application of one of the following drugs: the cyclooxygenase inhibitor indomethacin (10 \(\mu\)mol/L), the thromboxane \(A_2\) receptor blocker SQ29548 (10 \(\mu\)mol/L), the bradykinin B\(_2\) receptor blocker HOE140 (0.1 \(\mu\)mol/L), and the endothelin receptor blocker bosentan (10 \(\mu\)mol/L).

Stimulation of AT\(_2\)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\(_2\)R**

Western blot analysis of AT\(_2\)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 mg/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 \(\mu\)g) 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\(_2\)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\(_2\)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 \(\mu\)m 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\(_2\)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±4 \(\mu\)m 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 AT2R 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 AT2R stimulation (dilation in WKY rats or contraction in SHR) were suppressed by the AT2R 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 B2 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 Nω-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 AT1R 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 AT1R (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 AT1R stimulation and MAP determined in the different groups are shown in Figure 2.

There was no significant difference in MAP and AT1R-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 AT1R-mediated contraction, although this did not reach significance (8±3 versus 3±2 μm diameter decrease).

In SHR, treatment with perindopril reduced MAP (n=4; 125±6 mm Hg; P<0.01), and AT1R stimulation induced a significant vasodilation (6±2 μm diameter increase) that was significantly lower than AT2R–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 AT1R 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 AT1R 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 AT1R induced a significant vasodilation (22±5 μm diameter increase; P=NS versus WKY rats).

Endothelium removal did not affect AT1R–dependent contraction in untreated SHR (12±3 μm with endothelium versus 14±3 μm contraction without endothelium; n=4). On the other hand, AT1R–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 AT2R

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, AT2R expression was quantified. Western blot analysis showed that AT2R was significantly less expressed in SHR than in WKY rats (46%) in isolated mesenteric resistance arteries (Figure 3). Hydralazine (24 mg/kg per day) gradually raised AT2R expression in SHR treated for 18, 23, and 48 days. After 48
days, AT$_2$R 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 AT$_2$R expression in SHR to a level equivalent to that found in WKY rats (96.7% versus 100%; Figure 3).

### Immunohistology Analysis of AT$_2$R

In WKY rats, immunofluorescence analysis of mesenteric resistance arteries, with the use of confocal microscopy, indicated that AT$_2$R was present in the endothelium, in the smooth muscle, and in the adventitia. In the endothelium, AT$_2$R immunofluorescence was lower in SHR than in WKY rats ($5\pm H10063\%$ 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 ($32\pm H10066\%$; $P<0.01$; $n=6$). In SHR treated with candesartan plus hydralazine (16 mg/kg per day), immunofluorescence of AT$_2$R was restored to a level equivalent to that found in WKY rats (compared with WKY rats: $88\pm H100611\%$ in the endothelium and $81\pm H100618\%$ in the media) (Figure 4).

### Discussion

The present study demonstrates that AT$_2$R stimulation induced a vasoconstriction in untreated SHR resistance arteries, associated with a decrease in AT$_2$R expression. Specific or nonspecific antihypertensive treatments restored AT$_2$R expression and its vasodilator function when the decrease in pressure was sufficient.

Recent evidence suggests that AT$_2$R 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 AT$_2$R 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 B$_2$ receptor activation is involved in AT$_2$R-dependent dilation.$^9,11,22$ Importantly, AT$_2$R-mediated dilation does not desensitize, in contrast to AT$_1$R-dependent contraction, supporting the assumption that AT$_2$R dilation might have a role in the...
In WKY rats, candesartan did not significantly change MAP, and AT$_2$R-mediated dilation was preserved, as we have previously reported. By contrast, candesartan partly decreased MAP and AT$_2$R-mediated vasoconstriction in SHR. Similarly, low-dose hydralazine, also partly reducing MAP, inhibited AT$_2$R-mediated vasoconstriction. Obviously, these treatments were not able to normalize MAP and to restore AT$_2$R vasodilator function. Indeed, it was only when MAP was sufficiently reduced in SHR that AT$_2$R-mediated vasodilation was observed. First, with the use of an ACE inhibitor, MAP was decreased to a level similar to that observed in WKY rats. In this case, stimulation of AT$_2$R induced vasodilation. A further decrease in MAP, with the use of a higher dose of hydralazine or candesartan plus hydralazine, was also associated with a vasodilator effect of AT$_2$R stimulation.

Interestingly, the different groups studied allowed a correlation to be drawn between MAP and the type and amplitude of the response to AT$_2$R stimulation (Figure 2). Thus, from high to low MAP, AT$_2$R stimulation moved progressively from constriction to dilation. In addition, this effect was associated with a different AT$_2$R expression. In SHR, AT$_2$R expression is low in the wall of mesenteric arteries compared with WKY. After MAP reduction in SHR, AT$_2$R expression was restored to the level of WKY rats. However, it is difficult to determine whether AT$_2$R-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 AT$_2$R expression, assessed by Western blots, was in parallel with the restoration of maximal AT$_2$R-mediated dilation and normotension, which may indicate a primary role for AT$_2$R. On the other hand, equivalent reductions in MAP caused by hydralazine were associated with maximal AT$_2$R-mediated dilation despite suppressed AT$_2$R expression. These discrepancies in AT$_2$R abundance between treatments may reflect distinct AT$_2$R locations within the vasculature. Indeed, more precise immunohistological analysis demonstrated that AT$_2$R expression was located in the endothelial and smooth muscle cells in WKY rats, whereas in SHR AT$_2$R was not detectable in the endothelium. On the other hand, in SHR treated with candesartan plus hydralazine, MAP was restored, AT$_2$R stimulation induced vasodilation, and AT$_2$R expression was equivalent to that found in WKY rats. In this group, immunohistology of AT$_2$R 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 dilator mechanism is likely to contribute to the maintenance of a vasodilator state during chronic treatment.

Acknowledgments
This work was supported in part by a grant from the French Foundation for Medical research (FRM, Paris, France).

References
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Circulation. 2005;111:1006-1011; originally published online February 14, 2005; doi: 10.1161/01.CIR.0000156503.62815.48
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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