Vascular AT2 Receptors and Diabetes

Angiotensin Type 2 Receptor in Resistance Arteries of Type 2 Diabetic Hypertensive Patients

Carmine Savoia, Rhian M. Touyz, Massimo Volpe, Ernesto L. Schiffrin

Abstract—The role of angiotensin type 2 receptor (AT2R) on vascular responses to angiotensin II in humans remains unclear. In this study we explored whether AT2R is expressed and functionally active on peripheral resistance arteries of hypertensive diabetic patients treated for 1 year with either the angiotensin receptor blocker valsartan or the β-blocker atenolol. Twenty-six hypertensive type 2 diabetic patients treated with oral hypoglycemic and antihypertensive agents (not receiving angiotensin receptor blockers or β-blockers) were randomly assigned to double-blind treatment for 1 year with valsartan or atenolol once daily added to their previous therapy in a clinical trial that we reported recently and compared with 10 normal subjects. Resistance arteries dissected from gluteal subcutaneous tissues were assessed on a pressurized myograph. Vasomotor response curves to angiotensin II (1 nmol/L to 1 μmol/L) were performed on norepinephrine precontracted vessels in the presence of valsartan (10 μmol/L) with or without the AT2R inhibitor PD123319 (1 μmol/L). AT2R expression was evaluated by confocal microscopy. After 1 year of treatment, systolic and diastolic blood pressure was controlled and comparable in the valsartan and atenolol groups. Angiotensin II evoked a significant vasodilatory response only on resistance arteries from patients treated with valsartan, effect blocked by PD123319. AT2R expression was 4-fold higher in small arteries of valsartan-treated patients. In conclusion, AT2Rs are upregulated and contribute to angiotensin II–induced vasodilation in resistance arteries of hypertensive diabetic patients treated with angiotensin type 1 receptor blockers and may mediate, in part, vascular actions of these drugs in high cardiovascular risk patients. (Hypertension. 2007;49:341-346.)

Key Words: AT2 receptor ▪ angiotensin receptors ▪ human hypertension ▪ type 2 diabetes ▪ resistance arteries

Angiotensin II (Ang II) is the main biological effector of the renin–angiotensin system, which plays a major role in cardiovascular and renal homeostasis. Most of the physiological and pathophysiological effects of Ang II are mediated by the angiotensin type 1 receptor (AT1R), which is widely expressed by most cell types. Expression of the angiotensin type 2 receptor (AT2R) is more limited and occurs predominantly in fetal tissues where it may play a role during development.1-3 Recent studies, mostly in cellular and adult animal models, mapped the distribution of AT2R in certain areas of the brain,4 kidneys,4 coronary arteries, cardiomyocytes, ventricular myocardium,5 and the vasculature.6,7 Increased AT2R expression has been observed under pathological conditions, such as vascular injury,8 hypertension,6,7 myocardial infarction,9,10 congestive heart failure,11 renal failure,12 and brain ischemia.13 Few studies have investigated the functional expression of AT2R in humans. AT2Rs are expressed in the human skin (throughout the epidermal and dermal structures)14 and are markedly upregulated in incisional cutaneous wounds.15 AT2Rs have been demonstrated in the human coronary microcirculation, where they may contribute to vasodilation.16 Studies in cellular and animal models suggest that AT2R counteracts many AT1R actions by inducing vasodilation, antiproliferation, and apoptosis.2 We showed recently in animal models of hypertension that AT2R is upregulated and mediates vasodilation only when AT1Rs are blocked,6,7 suggesting that AT2R participates in the mechanisms whereby Ang II receptor antagonism lowers blood pressure (BP).7 Nevertheless, the role of AT2R remains unclear in human pathophysiology (eg, hypertension, diabetes, and other cardiovascular diseases), where Ang II–induced AT1R stimulation plays a major role.

In primary hypertensive17 and high-risk hypertensive type 2 diabetic patients,18 we reported that selective AT1R antagonism improved remodeling of resistance arteries beyond BP control, which could result in improved cardiovascular outcomes. Clinical studies in diabetic patients have shown that angiotensin receptor blockers (ARBs) exert renal protective effects better than other antihypertensive drugs,19-21 Thus, in humans, selective AT1R blockade may improve the structure of resistance arteries and tissue perfusion, effects that may involve an action mediated by AT2R. There is, however, no
convincing evidence of AT₂R functional expression in human peripheral resistance arteries in normal or pathological conditions. In this study, we investigated the hypothesis that AT₂R is expressed and functionally active in peripheral resistance arteries of hypertensive type 2 diabetic patients treated with an ARB compared with similar subjects treated with a β-blocker.

Methods

Patients and Trial Design
The protocol was approved by the Ethics Committee of the Clinical Research Institute of Montreal. Ten normotensive nondiabetic subjects and 26 hypertensive patients with type 2 diabetes (aged 30 to 70 years) were included in this study from a population of subjects who were part of a clinical trial that we reported recently. In 2 of the hypertensive diabetic subjects from that study, the number of vessels isolated was not enough to perform the protocol described here. The details of the inclusion criteria and protocol of the study were reported previously.

Vascular Studies
The study of resistance arteries was performed by individuals unaware of the groups to which samples belonged. Small arteries (lumen diameter: 150 to 300 μm) were isolated from subcutaneous tissue immediately after the biopsy and mounted on a pressurized myograph as described previously. Endothelium-dependent and -independent relaxations were assessed by the dilatory responses to acetylcholine hydrochloride (1 nmol/L to 100 nmol/L), respectively, in vessels precontracted with norepinephrine (1 μmol/L). Dose–response curves to Ang II (1 nmol/L to 1 μmol/L) in the presence or not of excess specific AT₂R blocking antibody (Santa Cruz Biotechnology, lot E0203) were assayed by the dilatory responses to nitroprusside (10 nmol/L to 1 mmol/L), respectively, in vessels precontracted with norepinephrine (1 μmol/L). Dose–response curves to Ang II–induced vasodilatory responses were assessed in norepinephrine-precontracted vessels.

Laser Confocal Microscopy
Resistance arteries were fixed for 30 minutes under a constant intraluminal pressure (60 mm Hg) with a solution containing 3.5% formaldehyde and 0.75% glutaraldehyde in 50 mmol/L PBS (pH 7.4). The fixative was washed from the organ bath by repeated changes with PBS (pH 7.4) and finally washed with PBS containing 0.1% Triton X-100 (pH 8.0). Arteries were incubated with 0.5% BSA/PBS for 5 minutes at 42°C. Subsequently, tissues were incubated with or without AT₂R antibody (Santa Cruz Biotechnology, lot E0203) in the presence or not of excess specific AT₂R blocking peptide (Santa Cruz Biotechnology, lot 1028) for 16 hours at 4°C. Tissues were washed with PBS and incubated with 200 μg/mL of Alexa Fluor 647 anti-goat IgG (Molecular Probes Inc) for 30 minutes at 37°C. For the final 30 minutes of incubation, rhodamine/phalloidin (Sigma, 10 μmol/L) was added to stain α-actin. Arteries were mounted in 1:1 glycerol/PBS (pH 7.4) on glass coverslips and studied by confocal immunofluorescence microscopy with a Zeiss LSM 510 system. The amount of AT₂R present in the vessel wall was quantified by imaging (Northern Eclipse program, EMPIX Imaging Inc) and expressed as a percentage of the AT₂R per total surface area.

Materials
Acetylcholine, sodium nitroprusside, and norepinephrine were obtained from Sigma Chemicals. Ang II was from Peptide Internationale. The selective AT₁R antagonist valsartan was a gift from Novartis, and PD123319 was a kind gift from Pfizer Canada. Except for valsartan, which was dissolved in 0.1 N KOH (pH 8), all of the other agents were dissolved in saline.

Data Analysis
Results are presented as mean±SEM and were analyzed by 2-way ANOVA and 1-way ANOVA followed by Newman–Keuls test, as appropriate. P<0.05 was considered statistically significant.

Results
The demographics of normotensive controls and patients were already reported and are summarized in the Table for the subjects who were included in the present study. Ang II–induced vasodilatory responses were assessed in norepinephrine precontracted vessels. To ensure complete AT₂R blockade, valsartan was added to vessels ex vivo in the vessel chamber. Norepinephrine-induced contractions did not differ

Demographics of the Subjects Studied

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Atenolol Basal</th>
<th>Atenolol After 1 Year</th>
<th>Valsartan Basal</th>
<th>Valsartan After 1 Year</th>
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<tr>
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<td>13</td>
<td>13</td>
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<td>Sex, male/female</td>
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<td>8/5</td>
<td>8/5</td>
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<tr>
<td>Age, y</td>
<td>51.2±1.7</td>
<td>59.7±1.2*</td>
<td>+1</td>
<td>55.1±2.3</td>
<td>+1</td>
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<td>Body weight, kg</td>
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<td>81.4±4</td>
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<td>BMI, kg/m²</td>
<td>26.8±1</td>
<td>28.4±1</td>
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<tr>
<td>SBP, mm Hg</td>
<td>109.4±3</td>
<td>143.6±2.1*</td>
<td>127.9±2.6†</td>
<td>143.5±3.2*</td>
<td>122.6±2.7†</td>
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<td>DBP, mm Hg</td>
<td>76±1.8</td>
<td>83.3±2.3*</td>
<td>75±2.1†</td>
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<td>73.4±2†</td>
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<td>No antihypertensive drugs (before randomization)</td>
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<td>1.7±0.2</td>
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<td>2.2±0.3</td>
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</tr>
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<td>No patients on ACEIs</td>
<td>—</td>
<td>8 (61%)</td>
<td>—</td>
<td>9 (69%)</td>
<td>—</td>
</tr>
<tr>
<td>No patients on CCBs</td>
<td>—</td>
<td>6 (46%)</td>
<td>—</td>
<td>7 (54%)</td>
<td>—</td>
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<td>Fasting glucose, mmol/L</td>
<td>4.7±0.1</td>
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<td>Glycated hemoglobin, %</td>
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<td>4.4±0.3</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.4±0.1</td>
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<td>1.3±0.1</td>
<td>1.4±0.1</td>
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<td>1.5±0.2</td>
<td>1.9±0.2</td>
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</tbody>
</table>

SBP and DBP indicate systolic and diastolic blood pressure; BMI, body mass index; ACEI, angiotensin-converting enzyme inhibitor; CCb, calcium channels blocker; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

*P<0.05 vs control; †P<0.05 pretreatment vs posttreatment.
between the groups as already reported. Norepinephrine-mediated contraction was unaltered by the addition of either valsartan or PD123319 to the organ chamber (data not shown). We already reported that endothelium-dependent and -independent relaxations of these vessels were similar in patients before randomization and the control group and did not change significantly in the treated groups at the end of the study.

Ang II did not evoke dilation in norepinephrine-precontracted vessels from normotensive subjects and patients before the randomization (Figure 1a). Ang II did not elicit significant vasodilatory responses in arteries obtained from patients treated for 1 year with atenolol compared with those from patients before treatment (Figure 1b). In contrast, Ang II induced a dose-dependent vasodilation in precontracted resistance arteries from patients treated for 1 year with valsartan \( (P<0.05 \text{ versus before treatment, Figure 1c}) \); \( P<0.05 \text{ versus patients treated 1 year with atenolol, Figure 1d} \). The AT$_2$R antagonist PD123319 did not exert any effect on vessels from atenolol-treated patients (Figure 2a), whereas Ang II–elicited vasodilation was significantly blunted by PD123319 in the vessels from patients treated for 1 year with valsartan (Figure 2b).

AT$_2$Rs were expressed in resistance arteries from normotensive and hypertensive subjects at a very low density, as assessed by confocal microscopy (Figure 3a and 3b). AT$_2$R expression increased significantly in hypertensive type 2 diabetic patient arteries only after 1-year treatment with valsartan (Figure 3a and 3b). Expression of AT$_2$R was observed in the entire vascular wall, albeit mainly in the media (Figure 4a). Specificity of labeling of AT$_2$R by the antibody was demonstrated by preventing antibody binding with AT$_2$R blocking peptide (Figure 4b).

**Discussion**

Major findings from the present study demonstrate for the first time that small peripheral resistance arteries from hypertensive diabetic patients treated with an AT$_2$R antagonist exhibit enhanced AT$_2$R expression and associated AT$_2$R-mediated vasodilation in response to Ang II. In adult animals, AT$_2$R expression is variable in different tissues in normal and pathological conditions. In the vasculature of normotensive and hypertensive rats, low levels of AT$_2$R mRNA and protein have been detected. AT$_2$Rs are upregulated and participate in vasodilation after in vivo chronic AT$_1$R blockade only in the vasculature of hypertensive animals. Moreover, in normotensive and hypertensive rats, evidence suggests that pharmacological stimulation of AT$_2$R contributes to BP reduction in the presence of AT$_1$R blockade. In endothelial cells transfected with the AT$_2$R promoter, Ang II–induced expression of AT$_2$R mRNA was abrogated when AT$_1$R was active and was restored after AT$_1$R blockade. Thus, in vitro and in vivo experiments indicate that AT$_2$R expression is downregulated by AT$_1$R stimulation and is restored after AT$_1$R inhibition, mostly in pathophysiological conditions. Our results in humans are consistent with these findings, because AT$_2$R expression was present in very low density in vessels from normotensive and hypertensive subjects before the randomization and rose 4-fold only in those patients who had received treatment with the ARB valsartan. This effect was independent from BP reduction, because similar BP control with atenolol did not induce significant AT$_2$R expression.
To test the functional significance of AT2R upregulation in resistance arteries in hypertensive diabetic patients, Ang II–induced vasodilatory responses of precontracted vessels were assessed. The isolated vessels were further exposed to valsartan ex vivo to ensuring complete AT1R blockade, allowing for examination of functional AT2R-mediated effects by Ang II. The results highlight the countervailing roles of AT1R and AT2R on Ang II–induced vasomotor responses in humans.

Although it is clearly established that Ang II induces vasoconstriction and increased BP by stimulating AT1R,1,2 the role of AT2R on vascular responses to Ang II and on systemic BP has remained unclear in humans. It was reported recently that AT2R-mediated vasodilation occurs in the coronary microcirculation of nondiseased hearts in a cohort of subjects with a wide age range.16 As well, in healthy young subjects treated for 1 week with an AT1R antagonist, intra-brachial arterial infusion of the AT2R antagonist PD123319 resulted in increased BP, with no changes in forearm vascular responses.27

Ang II elicited dose-dependent AT2R-mediated vasodilation only in resistance arteries from hypertensive diabetic patients treated for 1 year with valsartan. This effect was associated with reduced BP in treated patients. These observations support and extend the existence of a biological “crosstalk” between Ang II receptor subtypes6,7,26,28,29 in human peripheral resistance vessels. They suggest that, in the presence of AT1R blockade, Ang II stimulates functionally active, unblocked AT2R in resistance vessels of hypertensive diabetic subjects and induces vasodilation. This effect could contribute to the mechanisms whereby ARBs lower BP.

It has been reported that Ang II induces vasodilation through AT2R-stimulated NO–cGMP-dependent pathways.30,31 This occurs either directly32 or indirectly through enhanced bradykinin formation6,33 or increased endothelial NO synthase activity/expression.34 Thus, AT2R may also favorably affect endothelial function. However, in the present study, patients exhibited preserved endothelium-dependent vasodilation already at the time of randomization. This may

Figure 2. Concentration–response curves to Ang II of norepinephrine-precontracted resistance arteries from hypertensive diabetic patients after 1 year treatment with (a) atenolol or (b) valsartan, all in the presence ex vivo of AT1R blockade with valsartan (Val)±AT2R blockade with PD123319. Ang II–induced dilation occurred and was blunted by PD123319 only in patients treated for 1 year with valsartan. *P<0.05, 2-way ANOVA.

Figure 3. a, Confocal imaging of AT2R expression in resistance arteries from normotensive subjects and hypertensive diabetic patients before and after 1-year treatment with either atenolol or valsartan (red fluorescence: rhodamin/phalloidin staining α-actin; green fluorescence: AT2R). b, The abundance of AT2R is expressed as AT2R/total surface area in a percentage. *P<0.05, 1-way ANOVA.
be attributed to the fact that BP was well controlled, and \( \approx 65\% \) of the patients were being treated with an angiotensin-converting enzyme inhibitor and \( \approx 50\% \) with a calcium channels blocker,\(^{18} \) both of which may already have improved endothelial function.\(^{17,35} \)

AT\(_2\)R expression was increased mainly in the media of the arteries from hypertensive diabetic patients treated with valsartan. Therefore, it is also possible that Ang II can induce vasodilation by direct stimulation of AT\(_2\)R on vascular smooth muscle cells. Indeed, we demonstrated recently that Ang II, through its binding with AT\(_2\)R, negatively regulates the Rho/Rho kinase pathway (which is involved in vascular contraction and cell growth) in vascular smooth muscle cells and in the vasculature of hypertensive rats chronically treated with AT\(_1\)R blockers.\(^7 \) This effect was associated with Ang II–induced vasodilation.

We reported recently that tight BP control with the AT\(_1\)R antagonist valsartan added to other antihypertensive medications improved structural changes in resistance vessels in this cohort of hypertensive diabetic patients in whom we have now studied AT\(_2\)R regulation. Equally effective BP control, ARBs may exert beneficial actions on vascular remodeling acting as a vasodilator by blocking AT\(_1\)R. Furthermore, the functional expression of AT\(_2\)R on resistance arteries of hypertensive diabetic patients treated with an ARB may also participate in these beneficial effects. In presence of AT\(_1\)R antagonism, Ang II may stimulate unblocked AT\(_2\)R, which could also have participated in the effects of the ARB on vascular remodeling in that study.

**Perspectives**

Our study highlights the functional contribution of AT\(_2\)R-mediated vasodilation to antihypertensive effects of selective AT\(_1\)R blockade with ARBs in high cardiovascular risk patients. Another potentially important consequence of the role of AT\(_2\)R shown in this study in hypertensive diabetic patients relates to therapeutic implications. Consistent with results of large clinical trials,\(^{19–21} \) our findings indirectly support possible advantages of AT\(_1\)R blockers compared with other antihypertensive drugs, including angiotensin-converting enzyme inhibitors (which would not provide the benefit of AT\(_2\) stimulation), in hypertensive diabetic subjects.

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**Disclosures**

R.M.T. has received honoraria <$10,000 from Bristol-Myers Squibb. E.L.S. has received honoraria <$10,000 from Bristol-Myers Squibb, Boehringer-Ingelheim, Merck, Novartis, and Speedel. The remaining authors report no conflicts.

**References**


34. Lother H, Abdel-tawab AM, Quittener U. The angiotensin II AT1 receptor is an AT receptor, antagonist. J Biol Chem. 2001;276:39721–39726.
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