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. Recent studies, mostly in cellular and adult animal models, mapped the distribution of AT2R in certain areas of the brain, kidneys, coronary arteries, cardiomyocytes, ventricular myocardium, and the vasculature. Increased AT2R expression has been observed under pathological conditions, such as vascular injury, myocardial infarction, congestive heart failure, renal failure, and brain ischemia. Few studies have investigated the functional expression of AT2R in humans. AT2R are expressed in the human skin (throughout the epidermal and dermal structures) and are markedly upregulated in incisional cutaneous wounds. AT2R have been demonstrated in the human coronary microcirculation, where they may contribute to vasodilatation.

Studies in cellular and animal models suggest that AT2R counteracts many AT1R actions by inducing vasodilation, antiproliferation, and apoptosis. We showed recently in animal models of hypertension that AT2R are upregulated and mediate vasodilatation only when AT1R are blocked, suggesting that AT2R participates in the mechanisms whereby Ang II receptor antagonism lowers blood pressure (BP). 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 hypertensive and high-risk hypertensive type 2 diabetic patients, 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. 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 AT$_2$R functional expression in human peripheral resistance arteries in normal or pathological conditions. In this study, we investigated the hypothesis that AT$_2$R 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 μmol/L) in the presence of the AT1R blocker valsartan (1 μmol/L). Doce–response curves to Ang II (1 mmol/L) in the presence or not of excess specific AT2R blocking peptide 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 AT1R blockade, valsartan was added to vessels ex vivo in the vessel chamber. Norepinephrine-induced contractions did not differ significantly between the two groups.

**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 AT1R 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 AT1R present in the vessel wall was quantified by imaging (Northern Eclipse program, EMPIX Imaging Inc) and expressed as a percentage of the AT1R per total surface area.

**Materials**

Acetylcholine, sodium nitroprusside, and norepinephrine were obtained from Sigma Chemicals. Ang II was from Peptide International. 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 AT1R blockade, valsartan was added to vessels ex vivo in the vessel chamber. Norepinephrine-induced contractions did not differ significantly between the two groups.

<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>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>10</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>6/4</td>
<td>8/5</td>
<td>8/5</td>
<td>8/5</td>
<td>8/5</td>
</tr>
<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>
</tr>
<tr>
<td>Body weight, kg</td>
<td>75.1±4.3</td>
<td>79.8±3.4</td>
<td>81.4±4</td>
<td>89.5±5</td>
<td>90.3±5</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.8±1</td>
<td>28.4±1</td>
<td>28.8±1</td>
<td>32.3±2</td>
<td>32.6±2</td>
</tr>
<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>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>76.1±1.8</td>
<td>83.3±2.3*</td>
<td>75.2±1.1†</td>
<td>83.9±2.3*</td>
<td>73.4±2†</td>
</tr>
<tr>
<td>No antihypertensive drugs (before randomization)</td>
<td>–</td>
<td>1.7±0.2</td>
<td>–</td>
<td>2.2±0.3</td>
<td>–</td>
</tr>
<tr>
<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>
</tr>
<tr>
<td>Fasting glucose, mmol/L</td>
<td>4.7±0.1</td>
<td>7.1±0.5*</td>
<td>7.6±0.4*</td>
<td>8.5±0.5*</td>
<td>7.6±0.5*</td>
</tr>
<tr>
<td>Glycated hemoglobin, %</td>
<td>–</td>
<td>6.9±0.28</td>
<td>6.5±0.2</td>
<td>7.1±0.3</td>
<td>7.2±0.5</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.0±0.2</td>
<td>4.5±0.2</td>
<td>4.8±0.2</td>
<td>4.7±0.2</td>
<td>4.4±0.3</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.4±0.1</td>
<td>1.4±0.1</td>
<td>1.3±0.1</td>
<td>1.4±0.1</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.1±0.2</td>
<td>2.5±0.2</td>
<td>2.8±0.2</td>
<td>2.5±0.2</td>
<td>2.6±0.3</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.1±0.2</td>
<td>1.4±0.2</td>
<td>1.5±0.2</td>
<td>1.9±0.2</td>
<td>1.9±0.3</td>
</tr>
</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 versus before treatment, Figure 1c; P<0.05 versus patients treated 1 year with atenolol, Figure 1d). The AT2R 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).

AT2Rs were expressed in resistance arteries from normotensive and hypertensive subjects at a very low density, as assessed by confocal microscopy (Figure 3a and 3b). AT2R expression increased significantly in hypertensive type 2 diabetic patient arteries only after 1-year treatment with valsartan (Figure 3a and 3b). Expression of AT2R was observed in the entire vascular wall, albeit mainly in the media (Figure 4a). Specificity of labeling of AT2R by the antibody was demonstrated by preventing antibody binding with AT2R 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 AT1R antagonist exhibit enhanced AT2R expression and associated AT2R-mediated vasodilation in response to Ang II. In adult animals, AT2R expression is variable in different tissues in normal and pathological conditions. In the vasculature of normotensive and hypertensive rats, low levels of AT2R mRNA and protein have been detected. AT2Rs are upregulated and participate in vasodilation after in vivo chronic AT1R blockade only in the vasculature of hypertensive animals. Moreover, in normotensive and hypertensive rats, evidence suggests that pharmacological stimulation of AT2R contributes to BP reduction in the presence of AT1R blockade. In endothelial cells transfected with the AT2R promoter, Ang II–induced expression of AT2R mRNA was abolished when AT1R was active and was restored after AT1R blockade. Thus, in vitro and in vivo experiments indicate that AT2R expression is downregulated by AT1R stimulation and is restored after AT1R inhibition, mostly in pathophysiological conditions. Our results in humans are consistent with these findings, because AT2R 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 AT2R 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) or AT2R blockade with PD123319. Ang II-induced dilatation 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 ≈65% of the patients were being treated with an angiotensin-converting enzyme inhibitor and ≈50% with a calcium channels blocker,18 both of which may already have improved endothelial function.17,35

AT₂R 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 AT₂R on vascular smooth muscle cells. Indeed, we demonstrated recently that Ang II, through its binding with AT₂R, negatively regulates the Rho/Rho kinase pathway (which is involved in vascular contraction and cell growth) in vascular smooth muscle cells and in the vascularity of hypertensive rats chronically treated with AT₁R blockers.7 This effect was associated with Ang II–induced vasodilation.

We reported recently that tight BP control with the AT₁R 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 AT₂R regulation. Equally effective BP control, ARBs may exert beneficial actions on vascular remodeling acting as a vasodilator by blocking AT₁R. Furthermore, the functional expression of AT₂R on resistance arteries of hypertensive diabetic patients treated with an ARB may also participate in these beneficial effects. In presence of AT₁R antagonism, Ang II may stimulate unblocked AT₂R, 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 AT₂R-mediated vasodilation to antihypertensive effects of selective AT₁R blockade with ARBs in high cardiovascular risk patients. Another potentially important consequence of the role of AT₂R 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 AT₂R blockers compared with other antihypertensive drugs, including angiotensin-converting enzyme inhibitors (which would not provide the benefit of AT₂ stimulation), in hypertensive diabetic subjects.

Sources of Funding
This study was supported by grant 13570 (to E.L.S.) and a group grant to the Multidisciplinary Research Group on Hypertension, both from the Canadian Institutes of Health Research, as well as a research grant from Novartis Pharma Canada (to E.L.S.). C.S. was supported in part by a fellowship from the Italian Society of Hypertension and from Chair of Cardiology 2nd Faculty of Medicine University of Rome “La Sapienza.” R.M.T. is recipent of a Canada Research Chair in Hypertension Research at the University of Ottawa, and E.L.S. is recipent of a Canada Research Chair in Hypertension and Vascular Research at McGill University.

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


Angiotensin Type 2 Receptor in Resistance Arteries of Type 2 Diabetic Hypertensive Patients
Carmine Savoia, Rhian M. Touyz, Massimo Volpe and Ernesto L. Schiffrin

Hypertension. 2007;49:341-346; originally published online December 11, 2006; doi: 10.1161/01.HYP.0000253968.95136.b8
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/49/2/341

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/