Renal Angiotensin II Type-2 Receptors Are Upregulated and Mediate the Candesartan-Induced Natriuresis/Diuresis in Obese Zucker Rats

Amer C. Hakam, Tahir Hussain

Abstract—Recently, there has been a growing interest in studying the role of angiotensin II type-2 (AT2) receptor in renal/cardiovascular function in pathological conditions. The present study was designed to determine the functional role of the AT2 receptors on natriuresis/diuresis and compare the level of the tubular AT2 receptor expression in obese and lean Zucker rats (12 weeks old). Under anesthesia, candesartan (angiotensin II type 1 [AT1]–specific antagonist; 100 μg/kg bolus) produced natriuresis/diuresis to a greater degree in obese than in lean rats. The specific AT2 antagonist PD123319 (50 μg/kg per minute) after candesartan administration abolished the natriuretic/diuretic effects of candesartan in obese rats but not in lean rats. Infusion of AT2 receptor agonist, CGP-42112A (1 μg/kg per minute), produced greater increase in sodium and urine excretion over basal in obese than in lean rats. The presence of the AT2 receptor expression in the brush-border and basolateral membranes was confirmed by Western blotting using specific antibody and antigen-blocking peptide. Densitometric analysis of the bands revealed 1.5- to 2.0-fold increase in the AT2 receptor proteins in both membranes of obese compared with lean rats. Our results suggest upregulation of the AT2 receptors, which play a role in mediating the natriuretic/diuretic effects of AT1 receptor blockers in obese Zucker rats. We speculate that AT2 receptors, by promoting sodium excretion, may protect obese Zucker rats against blood pressure increase associated with sodium and water retention. (Hypertension. 2005;45:270-275.)

Key Words: angiotensin II receptors ■ obesity ■ angiotensin II ■ kidney ■ angiotensin I ■ rats, Zucker

Angiotensin II (Ang II), a major effector hormone of the renin-angiotensin system (RAS), signals via 2 major receptor subtypes: Ang II type-1 (AT1) and Ang II type-2 (AT2).1 The AT1 receptor is predominant in adult tissues, and most of its mediated effects, such as vasoconstriction, hypertrophy, and sodium retention,2 are well documented. Recently, there has been a growing interest in studying the role of the AT2 receptor in renal/cardiovascular function in pathological conditions. Contrary to the initial notion, the presence of AT2 receptors in various tissues of adult animal models has been reported. Recent evidence implicated AT2 receptors in the regulation of renal and cardiovascular function, including vasodilation and natriuresis.2 Also, the AT2 receptors have antiproliferative and antigrowth effects, and they promote apoptosis.2–3 All of these characteristics are very important in preventing tissue remodeling and, therefore, disease progression. It has been shown that AT2 receptor knockout mice have higher blood pressure and exaggerated response to Ang II infusion on blood pressure.4 Acute and chronic blockade of AT1 receptors prevented the hypotensive effects of AT1 receptor antagonist in normal rats.5 Cardiac overexpression of AT2 receptors diminished the AT1 receptor–mediated pressor response.6 AT2 receptors are present in adult rat kidneys7 and are involved in interstitial fluid cGMP modulation under low-salt condition.8 Zucker rats, a genetic model of obesity and hyperinsulinemia, exhibit hyperactive RAS9–11 that is manifested in a mildly elevated blood pressure.10 Obese Zucker rats have greater reduction in blood pressure when treated with AT1 receptor antagonist compared with lean rats.10 The natriuretic/diuretic response to AT1 receptor antagonist is exaggerated in obese Zucker rats when compared with lean rats.12 The expression and functional role of AT2 receptors, especially on AT1 receptor antagonist–mediated renal effects in obese Zucker rats, are not known. Therefore, the present study was designed to determine the expression of AT2 receptors in proximal tubular membrane and their functional role on natriuresis/diuresis in obese Zucker rats. We observed that AT2 receptors were upregulated and mediated the candesartan-induced sodium excretion in obese Zucker rats.

Methods

Animals
Male obese and lean Zucker rats (10 weeks of age) were purchased from Charles River Laboratories. Animals were housed in the University of Houston animal care facility and had free access to

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Intravenous infusion of PD123319 (50 μmol/L) was used to displace with varying concentration of unlabeled Sar1–Ang II (500 pmol/L to 10 nmol/L). The nonspecific binding was determined using 125I-Sar1–Ang II Binding buffer at 25°C for 15 minutes. The radioligand was displaced with varying concentration of unlabeled Sar1–Ang II (500 pmol/L to 10 nmol/L). The nonspecific binding was determined using 125I-Sar1–Ang II. The radioligand was then removed by washing the membrane three times with ice-cold binding buffer. The membrane was then dried and counted for radioactivity. The Bmax (binding capacity) and Kd (dissociation constant) were calculated using computer software by Biosoft. All experiments were performed in triplicate and the data were analyzed using Scatchard analysis for Bmax and Kd calculation.

Western Blot Analysis

The Western blot analysis was performed as described previously.12,13 Briefly, tissues from lean and obese Zucker rats were used for Western blotting using AT2 receptor polyclonal antibody. The antibodies were purchased from Santa Cruz Biotechnology.125I-Sar1–Ang II was purchased from American Radiolabeled Chemicals. Anti-rabbit IgG was purchased from Bio-Rad. antibody for AT2 receptor and its antigen-blocking peptide were purchased from Santa Cruz Biotechnology. Binding data were subjected to Scatchard analysis for Bmax and Kd calculation. All Western blotting data were analyzed using GraphPad Prism, version 6.0 software. A value of P<0.05 was considered statistically significant.

Chemicals

Antibody for AT1 receptor and its antigen-blocking peptide were purchased from Alpha Diagnostics. Anti-rabbit IgG was purchased from Santa Cruz Biotechnology. Antibodies for AT2 receptor and its antigen-blocking peptide were purchased from Santa Cruz Biotechnology. Binding data were subjected to Scatchard analysis for Bmax and Kd calculation. All Western blotting data were analyzed using GraphPad Prism, version 6.0 software. A value of P<0.05 was considered statistically significant.

Statistical Analysis

Data are presented as mean±SEM. One-way ANOVA with post hoc tests (Newman–Keuls) was used to analyze variation within the group. Student t test was used to compare variation between groups. Binding data were analyzed using computer software by Biosoft. All other statistical analyses were done using GraphPad Prism, version 3.02 (GraphPad Software). A value of P<0.05 was considered statistically significant.

Results

General Parameters

As shown in Table 1, obese rats had significantly higher body weight and significantly lower kidney-to-body weight ratio.
Similarly, bolus dose of candesartan modestly increased UF and GFR in obese rats (Table 2), suggesting a tubular effect of candesartan infusion. MAP and GFR were not altered in lean or obese rats. The GFR and MAP were measured for 2 hours after bolus was statistically significant in obese rats but not in Zucker rats that was sustained for 2 hours. The increase in UF, UNaV, and FENa over basal was not changed by CGP-42112A in lean (92 ± 5.8 versus 91 ± 4.3). The MAP in obese rats was modestly but insignificantly decreased by CGP-42112A (113 ± 9 versus 103 ± 9). The basal UNaV and FENa were lower in this experiment compared with the previous one; however, the magnitude of difference between lean and obese rats was similar.

**TABLE 2. Effect of Candesartan (100 μg/kg bolus) Alone and PD123319 (50 μg/kg per minute infusion) Alone on Kidney Function in Zucker Rats**

<table>
<thead>
<tr>
<th>Strain/Parameter</th>
<th>Pre-Cand</th>
<th>Cand1</th>
<th>Cand2</th>
<th>Cand3</th>
<th>Cand4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean UF, μL/min</td>
<td>1.45±0.13</td>
<td>2.10±0.22</td>
<td>2.27±0.27</td>
<td>2.20±0.28</td>
<td>2.40±0.37</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>65.00±3.40</td>
<td>62.25±6.25</td>
<td>55.25±3.90</td>
<td>55.30±4.50</td>
<td>53.50±2.60</td>
</tr>
<tr>
<td>GFR, mL/min</td>
<td>0.70±0.089</td>
<td>0.86±0.120</td>
<td>0.92±0.130</td>
<td>0.93±0.110</td>
<td>0.82±0.170</td>
</tr>
<tr>
<td>Obese UF, μL/min</td>
<td>2.10±0.32</td>
<td>3.85±0.60*</td>
<td>4.48±0.68*</td>
<td>4.70±0.76*</td>
<td>4.70±0.80*</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>92.00±2.9</td>
<td>89.50±2.1</td>
<td>85.75±5.5</td>
<td>82.75±4.5</td>
<td>81.00±4.7</td>
</tr>
<tr>
<td>GFR, mL/min</td>
<td>0.80±0.12</td>
<td>1.00±0.15</td>
<td>1.10±0.09</td>
<td>1.13±0.14</td>
<td>1.08±0.10</td>
</tr>
</tbody>
</table>

Top, each No. represents the UF (μL/min), MAP (mm Hg), or GFR (mL/min) before (pre-cand) and after (cand) candesartan bolus injection. Data are presented as mean±SEM of 4 to 6 experiments. *P<0.001 compared with basal (ANOVA followed by Newman–Keuls comparison test).

Bottom, each No. represents value before (Pre-PD) and during (PD1 and PD2) PD123319 continuous infusion. Urine was collected in 30-minute periods. Data are presented as mean±SEM of 4 experiments.

Effects of PD123319 on Candesartan-Induced Natriuresis/Diuresis in Obese Rats

In the initial set of experiments, we determined the effect of candesartan and PD123319 alone on UF and hemodynamic parameters. As shown in Table 2, a bolus dose (100 μg/kg IV; protocol 1) of candesartan increased UF in lean and obese Zucker rats that was sustained for 2 hours. The increase in UF over basal was statistically significant in obese rats but not in lean rats. The GFR and MAP were measured for 2 hours after candesartan infusion. MAP and GFR were not altered in lean or in obese rats (Table 2), suggesting a tubular effect of candesartan. In a separate set of experiments, PD123319 infusion (50 μg/kg per minute IV; protocol 2) did not alter UF significantly in lean or obese rats and also caused no changes in GFR and MAP (Table 2). PD infusion did increase the UNaV in lean rats, but the change did not reach statistical significance.

As indicated in experiment protocol 3, we assessed the effect of PD123319 on candesartan-induced changes in UF and UNaV. Candesartan (100 μg/kg IV bolus) produced a significant increase in UF, UNaV, and FENa over basal values in obese rats. Similarly, bolus dose of candesartan modestly increased UF and FENa over basal in lean rats (Figure 2A through 2C). However, the natriuretic/diuretic response to candesartan was more exaggerated in obese compared with lean Zucker rats. The infusion of PD123319 (50 μg/kg/min IV) almost abolished the natriuresis/diuresis produced by candesartan in obese rats, whereas in lean rats, PD123319 did not alter the candesartan-induced natriuresis/diuresis. Basal UF, UNaV, and FENa were higher in obese than in lean Zucker rats (Figure 2A through 2C).

**Effect of CGP-42112A on Natriuresis and Diuresis in Obese Zucker Rats**

In this experiment, we determined the effect of the specific AT2 agonist10 CGP-42112A on natriuresis and diuresis in lean and obese Zucker rats. The UF and UNaV in response to CGP-42112A infusion (1 μg/kg per minute; protocol 4) were more increased in obese rats than in lean rats (Figure 3A and 3B). Similarly, FENa in response to CGP-42112A infusion was greater in obese than in lean rats (Figure 3C). This dose of CGP-42112A did not alter GFR significantly (lean 0.56±0.06 versus 0.57±0.03; obese 0.71±0.04 versus 0.69±0.08). The MAP was not changed by CGP-42112A in lean (92±5.8 versus 91±4.3). The MAP in obese rats was modestly but insignificantly decreased by CGP-42112A (113±9 versus 103±9). The basal UNaV and FENa were lower in this experiment compared with the previous one; however, the magnitude of difference between lean and obese rats was similar.

**AT2 Receptor Expression**

We determined the expression of the AT2 receptor protein by Western blotting analysis of the BBM and BLM of lean and obese Zucker rats. The AT2 receptor antibody detected 3 bands at 50, 45, and 30 kDa (Figure 4A, lane 1). All 3 bands were displaced by antigen peptide of the AT2 receptor (Figure 4A, lane 2). The densitometric analysis of all 3 bands revealed a significant increase in the density of the AT2 receptor protein in BBM (50%) and BLM (85%) of obese Zucker rats compared with lean rats (Figure 4B).

It has been reported that the AT2 receptor has 5 different potential sites for N-glycosylation.17 We investigated whether
a different level of glycosylation is responsible for the different bands, as detected in Figure 3. Treatment with N-glycanase, specific N-linked deglycosylation enzyme, shifted all 3 bands to 1 band (28 kDa), as detected with AT2 antibody (Figure 3A, lane 3). This band was displaced by the AT2-blocking peptide (Figure 3A, lane 4), suggesting the band is AT2 receptor specific.

125I-Sar1–Ang II Binding

Scatchard plot analysis of the binding data revealed no change in total receptor number (Figure 5A) in the BBM and BLM of obese compared with lean (Bmax BBM 257±8.6 in lean versus 203±38 in obese rats; Bmax BLM 360±11 in lean versus 373±32 in obese rats). Kd values were similar in lean and obese in BBM and BLM (Figure 5B). Bmax and Kd values were calculated using Sar1–Ang II, a nonspecific antagonist. We also determined whether there is a difference in the AT1 binding between lean and obese. We used losartan (1 μmol/L) to determine the non-AT1 binding. We found similar AT1 binding in lean and obese in BBM and BLM (Figure 5C).

Discussion

Ang II is a potent antinatriuretic and antidiuretic hormone and, thereby, plays an important role in the maintenance of sodium and fluid homeostasis. Increased function of Ang II, either attributable to increased production of the hormone or increased activity of the AT1 receptor, contributes to development of hypertension. The use of selective AT1 receptor antagonists leads to reduction in blood pressure in human as well as in experimental animal models, including obese Zucker rats. The greater reduction in blood pressure and greater natriuresis in the absence of enhanced renin activity in the plasma or the kidney of obese Zucker rats may be attributed to increased activity of the AT1 receptors. However, there has been a growing interest to understand the role of the AT2 receptors, which are known to counteract the effects of the AT1 receptors, in the renal cardiovascular regulation. In the present study, we found that intravenous infusion of PD123319, an AT2 receptor antagonist, abolished the candesartan-induced natriuresis/diuresis in obese Zucker rats. On the other hand, PD123319 had no effect on candesartan-induced natriuresis/diuresis in lean rats. We also found that direct stimulation of the AT2 receptors with CGP-42112A produced a significant natriuresis/diuresis in obese but not in lean rats.

Candesartan, PD123319 alone or in combination, and CGP-42112A did not alter the GFR significantly in lean or obese rats. These drugs also did not alter MAP in lean rats and obese rats, except for a modest but insignificant effect of CGP-42112A on MAP in obese rats. The decrease in MAP could be attributed to the effect of CGP-42112A on AT2 receptors in the blood vessels...
where AT₂ receptor upregulation has been shown previously.²³ Despite a decrease in MAP, CGP-42112A continued to promote natriuresis in obese rats; therefore, the natriuretic effects may be attributed to the activation of the AT₂ receptors in the tubular membranes. In these experiments, inactin was used as anesthetic agent. Although it has minimal effects on the cardiovascular system,²⁴ we should still be cautious when interpreting the results.

The complete reversal by AT₂ receptor antagonist of candesartan-induced natriuresis and diuresis in obese and not in lean rats indicates a role for AT₂ receptors in obese but not in lean rats. The obvious role of AT₂ receptors in obese and not in lean rats could be attributed to the level of AT₂ receptor expression in the tubular membranes. In these experiments, inactin was used as anesthetic agent. Although it has minimal effects on the cardiovascular system,²⁴ we should still be cautious when interpreting the results.

The mechanism by which AT₂ promotes natriuresis/diuresis in obese Zucker rats is not known. However, it has been shown that renal AT₂ receptors in Sprague-Dawley rats can mediate the production of bradykinin and NO and therefore increase the levels of cGMP.¹⁻⁸ In rabbit proximal tubule, it has been shown that AT₂ receptors on the BBM mediate activation of phospholipase A2 and increase the release of
arachidonic acid. In vitro studies have shown that AT2 receptors on proximal tubules mediate inhibitory effects on sodium and bicarbonate absorption. It is likely that the increased expression of AT2 receptors on the proximal tubular membranes of obese Zucker rats stimulate the above-mentioned mechanisms affecting the tubular sodium transport when the AT2 receptors have been blocked by candesartan or the AT1 receptors were selectively activated by an agonist. However, the functional status of the AT2 receptors in terms of the second messenger and cellular mechanisms is yet to be determined in the kidney of obese Zucker rats.

The increased renal function of AT2 receptors on sodium metabolism may have important physiological consequences in obesity-related hypertension. Obese Zucker rat is a model of insulin resistance and development of mild hypertension. It has been reported that treatment with losartan, an AT1 receptor antagonist, lowers blood pressure to greater extent in obese than in lean Zucker rats. On the basis of our results, it can be speculated that overexpression of the tubular AT2 receptors might have contributed to the greater reduction in blood pressure of obese rats treated with losartan. However, the direct role of AT1 receptors in blood pressure regulation in this model of obesity with insulin resistance is yet to be determined. Previous studies in animal models have shown that the AT2 receptor plays a role in altering blood pressure. AR2 receptor knockout mice have elevated basal blood pressure and exaggerated pressor response to exogenous Ang II infusion when compared with wild-type mice. In spontaneous hypertensive rats, the AT2 receptor agonist produced vasodilatation in the presence of AT1 receptor antagonist, a response that was abolished by the AT2 receptor antagonist PD123319. Carey et al reported that the AT2 receptor stimulation using specific AT2 receptor agonist decreases arterial pressure in normal rats, an effect that was abolished in the presence of the specific AT1 receptor antagonist.

Perspectives

Present studies demonstrate a functional role of the renal AT2 receptors in obese Zucker rats. The enhanced expression of the tubular AT2 receptors mediates the natriuresis/diuresis induced by the AT1 receptor blocker (ARB) or by the selective activation of the AT2 receptors. ARBs and angiotensin-converting enzyme inhibitors (ACEIs) are used to improve renal function in diabetes and to treat hypertension. Recent scientific data have initiated a debate of preferential use of ARBs over ACEIs. The basis of such preference lies in that ARBs selectively block AT1 receptors and to treat hypertension. Recent scientific data have initiated a debate of preferential use of ARBs over ACEIs. The basis of such preference lies in that ARBs selectively block AT1 receptors.

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References


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