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, hypertension, 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 AT1 receptors diminished the AT1 receptor–mediated pressor response.6 AT1 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 μg/L) was used to determine plasma insulin levels. Urinary and plasma creatinine levels were determined using a creatinine analyzer (model 2; Beckman). Plasma and urine levels of Na were measured using a flame photometer (Ciba Corning Diagnostics).

Kidney Function Experiment Protocol

Rat surgery and kidney function were performed as described previously. Briefly, rats were anesthetized using Inactin (100 to 160 mg/kg IP). The left jugular vein and carotid artery were cannulated for saline/drug infusion and blood pressure measurement, respectively. The ureter is cannulated for urine collection. After a stabilization period of 1 hour, we collected urine in 30-minute intervals. The first 2 periods were used to compute the basal parameters. After the second period, we delivered a bolus dose of candesartan (100 μg/kg), an AT1 receptor antagonist, followed by a 30-minute period of urine collection. After this period, we started an intravenous infusion of PD123319 (50 μg/kg per minute). An AT2 receptor–specific antagonist, and collected urine for 2 periods. Urine samples were placed on ice. Blood pressure was monitored throughout the experiment. At the end of the protocol, kidneys were excised, patted dry, and weighed. A semi-automatized representation of the different protocols was used is shown in Figure 1. At the end of each urine collection period, urine volume was measured, and urine flow rate (UF) was calculated (μL/min). The urinary sodium excretion rate (urinary sodium volume [U\text{Na}\text{V}]; μmol/min) was computed as UF×urinary sodium concentration (μmol/μL). The glomerular filtration rate (GFR; mL/min) was calculated based on creatinine clearance. The U\text{Na}\text{V} (μmol/min) was divided by the plasma sodium concentration (mg/dL) to compute the fraction of sodium excreted in urine (F\text{E}\text{Na}; percentage).

Proximal Tubule Membrane Preparation

Animals were anesthetized using sodium pentobarbital (50 mg/kg IP). After a midline incision, kidneys were excised and cut sagittally. Outer cortices were used for preparation of basolateral membrane (BLM) and brush-border membrane (BBM) as described previously. The BLM and BBM were characterized with the presence and absence of Na-K-ATPase, respectively. Protein estimation of these samples was done using bicinchoninic acid protein assay kit (Pierce).

Table 1. General and Hemodynamic Parameters in Zucker Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LZR</th>
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<tbody>
<tr>
<td>BW, g</td>
<td>292.5±5.3</td>
<td>447.3±11.7*</td>
</tr>
<tr>
<td>KW/BW</td>
<td>0.91±0.010</td>
<td>0.78±0.019*</td>
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<td>FBG, mg/dL</td>
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<td>Plasma insulin, ng/mL</td>
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BW, indicates body weight; KW, kidney weight; FBG, fasting blood glucose; HR, heart rate; Cr, creatinine; LZR, lean Zucker rats; OZR, obese Zucker rats. Values are presented as mean±SEM of 12 to 14 experiments.

Western Blot Analysis

Equal amounts (40 μg protein) of BLM and BBM proteins from lean and obese Zucker rats were used for Western blotting using AT2 receptor antibody. Anti-rabbit IgG–horseradish peroxidase conjugate and chemiluminescent substrate were used to detect the signal that was recorded on x-ray film. The band was densiometrically quantified. Antigen peptide was used to determine the AT2-specific band.

Enzymatic Deglycosylation of the AT2 Receptor Protein

The deglycosylation experiment was performed as recommended by the kit manufacturer (ProZyme). Briefly, samples from BBM were incubated with denaturing solution and heated at 100°C for 5 minutes. Once cooled to room temperature, the samples were incubated with N-glycanase at 37°C for 3 hours. After incubation, samples were used for Western blot analysis as described above.

125I-Sar1–Ang II Binding

Binding of 125I-Sar1–Ang II to BLM and BLM was performed according to the method described previously. Briefly, 50 μg of protein was incubated with the ligand (25 fmol/L) in a 200 μL (final volume) of 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 10 μmol/L Sar1–Ang II. Losartan (1 μmol/L) was used to determine the AT1-specific binding. Binding data were subjected to Scatchard analysis for Bmax and Kd calculation.

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BW, indicates body weight; KW, kidney weight; FBG, fasting blood glucose; HR, heart rate; Cr, creatinine; LZR, lean Zucker rats; OZR, obese Zucker rats. Values are presented as mean±SEM of 12 to 14 experiments.

*P<0.05 vs LZR (unpaired Student t test).

Statistical Analysis

Data are presented as mean±SE. One-way ANOVA with post hoc tests (Newmann–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 Graph Pad 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...
when compared with lean rats. Fasting blood glucose and plasma insulin levels were significantly elevated in obese rats compared with lean rats. Plasma creatinine level was greater in obese rats when compared with lean rats, suggesting possible kidney damage. Obese rats had significantly higher blood pressure (mean arterial pressure [MAP]) and similar heart rate when compared with lean rats.

**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; 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 agonist\(^5\) 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

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**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 (cand1 through cand4) 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.
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 lean rats 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 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 obese rats. AT2 receptor expression in the cortical membranes BBM and BLM of obese rats was approximately 1.5- to 2-fold greater than in lean rats. Although alone, PD123319 infusion had no significant effect on urine and sodium excretion in lean and obese rats, the blockade of AT1 receptors leaves AT2 receptors to function unopposed. It is likely that the level of the AT2 receptor expression in lean rats is not enough to produce natriuresis in lean rats as in obese rats, in which the AT2 receptor expression is greater. This notion is further supported by the results of the AT2 receptor agonist–induced natriuresis/diuresis in obese and not in lean rats. The use of losartan, an AT1 receptor antagonist, showed that there was no difference in the AT1 receptor–binding sites on BLM or BBM between lean and obese rats.

It was surprising that whereas PD 123319 did not change the basal tone of urine/sodium excretion significantly in obese rats, the AT2 agonist does show an increase in the urine/sodium output in obese rats. The response to the AT2 agonist in lean rats was minimal but insignificant compared with the basal levels. The reason for the inability of PD123319 to affect urine/sodium output is not clear. However, it may be extrapolated from other hormonal responses such as dopamine. Dopamine, which activates D1 receptor present on the proximal tubules, is an autocrine/paracrine natriuretic hormone.25 It is reported that D1 receptor agonists, and not the antagonist, promotes natriuresis/diuresis in rats. The effect of D1 antagonist is reported only under acute volume expansion.25 Based on these reports, it may be speculated that under conditions such as sodium load, the effect of AT2 antagonist becomes obvious; however, that is yet to be investigated.

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.18 In rabbit proximal tubule, it has been shown that AT2 receptors on the BBM mediate activation of phospholipase A2 and increase the release of

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**Figure 4.** A is a representative Western blot of the AT2 protein before and after deglycosylation and in the presence and absence of blocking peptide. B, Expression of the AT2 receptor protein in the BBM and BLM of obese Zucker rats as percentage of lean rats (12 weeks old). Bars in B represent the mean intensity of all 3 bands. Values are represented as mean±SEM. *P<0.05 compared with lean of the same group (Student t test; n=6).

**Figure 5.** 125I-Sar1–Ang II binding to the BBM and BLM obtained from lean and obese Zucker rats. Binding data were subjected to Scatchard analysis using LIGAND computer program. The maximum binding sites (B max), Kd, and dissociation constant (Kd; B) were calculated. C represents total (AT1 and AT2) and AT1-specific binding using Sar1–Ang II (10 μmol/L) and losartan (1 μmol/L) for nonspecific binding, respectively. Values are represented as mean±SEM of 5 experiments. *P<0.009 compared with lean and obese of the same group (Student t test). #P<0.05 compared with lean and obese BBM (Student t test).
Role of AT2 Receptors in Kidney Function

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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 AT1 receptors have been blocked by candesartan or the AT2 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 AT2 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. AT2 receptor knockout mice have elevated basal blood pressure and exaggerated pressor response to exogenous Ang II infusion when compared with wild-type mice.4 In spontaneously hypertensive rats, the AT2 receptor agonist produced hypertension. AT2 receptor knockout mice have elevated basal blood pressure of obese rats treated with losartan. 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.

Acknowledgments

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