Angiotensin II Type 2 Receptor Agonist Directly Inhibits Proximal Tubule Sodium Pump Activity in Obese But Not in Lean Zucker Rats

Amer C. Hakam, Tahir Hussain

Abstract—We have reported recently that the renal angiotensin II type 2 (AT$_2$) receptors are upregulated and involved in promoting natriuresis/diuresis in obese but not in lean Zucker rats. In the present study, we tested the hypothesis that there is an enhanced AT$_2$ receptor signaling via NO/cGMP pathway leading to greater inhibition of the Na$^+$, K$^+$-ATPase (NKA) activity in the proximal tubules (PT) of obese rather than lean Zucker rats. The AT$_2$ agonist CGP42112 (0.1 to 100 nmol/L) inhibited (33% at 100 nmol/L) the NKA activity in the PTs of obese but not in lean Zucker rats. The AT$_2$ antagonist PD123319 (1 μmol/L), not the angiotensin II type 1 antagonist losartan (1 μmol/L), significantly diminished the CGP42112-induced inhibition of the NKA activity in obese rats. The AT$_2$ agonist (10 nmol/L)–induced NKA inhibition was abolished by the soluble guanylate cyclase inhibitor 1H-[1,2,4] oxadiazolo-[4,3-a] quinoxalin-1-one (10 μmol/L), the NO synthase inhibitor N$^6$-nitro-l-arginine methyl ester (100 μmol/L), and the protein kinase G inhibitor K1388 (2 μmol/L). CGP42112 (10 nmol/L) caused an increase in serine phosphorylation of NKA α1-subunit in PT of obese rats. Measurement of cGMP and NO revealed that CGP42112 (0.1 to 100 nmol/L) increased cGMP and NO accumulation in the PTs of obese but not lean rats. The CGP42112-induced stimulation of NO and cGMP was blocked by PD123319 (1 μmol/L), N$^6$-nitro-l-arginine methyl ester (100 μmol/L), and 1H-[1,2,4] oxadiazolo-[4,3-a] quinoxalin-1-one (10 μmol/L) but not by losartan (1 μmol/L). The data suggest that the AT$_2$ receptor activation via stimulation of the NO/cGMP/protein kinase G pathway directly inhibits the tubular NKA activity that provides as a mechanism responsible for the AT$_2$ receptor–mediated natriuresis in obese but not in lean Zucker rats. (Hypertension. 2006;47:1117-1124.)

Key Words: diabetes mellitus ■ hypertension, sodium-dependent ■ kidney ■ sodium ■ epithelium

The renin–angiotensin system (RAS) is a major hormonal regulator of sodium homeostasis. The RAS mediates most of its effects via the octapeptide angiotensin II (Ang II). Ang II signals are mediated via 2 major receptor subtypes, Ang II type 1 (AT$_1$) and Ang II type 2 (AT$_2$), belonging to the G protein–coupled receptor superfamily. The AT$_1$ receptors are ubiquitously expressed and are involved in mediating the Ang II–associated vasoconstriction, cell growth, and sodium retention. The AT$_2$ receptors are expressed in various tissues and are associated with vasodilatation, apoptosis, antiproliferation, and natriuresis and, therefore, can be considered as functional antagonists to the AT$_1$ receptors.

We have shown recently that the AT$_2$ receptors are upregulated in the proximal tubular membranes of obese Zucker rats. Selective activation of the AT$_2$ receptors by CGP42112 promotes natriuresis/diuresis to a greater degree in obese compared with lean Zucker rats. The obese Zucker rat is a genetic model of insulin resistance that develops mild hypertension. It has been shown that the RAS is overactive in obesity and that obesity-associated hypertension is linked to increased sodium reabsorption. The proximal tubules (PT) are involved in reabsorbing $\approx$75% of the filtered sodium. Ang II, via the activation of the AT$_1$ receptors expressed on the PT membranes, activates the Na$^+$, K$^+$-ATPase (NKA), Na$^+$, H$^+$ exchanger, and Na$^+$, HCO$_3^-$ cotransporter and thereby contributes to sodium retention.

The NKA is a major active transporter involved in pumping sodium from the cytoplasm to the interstitium against its density gradient. We have reported recently that AT$_2$ receptor activation causes NKA inhibition via the NO/cGMP pathway in the PTs of Sprague Dawley rats. Because AT$_2$ receptors are upregulated in PTs, and the selective activation of the AT$_2$ receptors causes greater natriuresis in obese Zucker rats, we hypothesized on an enhanced AT$_2$ receptor–mediated NKA inhibition because of enhanced NO/cGMP signaling in PTs of obese Zucker rats. We found that selective activation of the AT$_2$ receptors mediates an inhibitory effect on the NKA activity associated with enhanced serine phosphorylation of the NKA in the PT isolated from obese Zucker rats but not in lean rats. We also found that this inhibition was reversed by the soluble guanylate cyclase inhibitor 1H-[1,2,4] oxadiazolo-[4,3-a] quinoxalin-1-one (10 μmol/L), the NO synthase inhibitor N$^6$-nitro-l-arginine methyl ester (100 μmol/L), and 1H-[1,2,4] oxadiazolo-[4,3-a] quinoxalin-1-one (10 μmol/L) but not by losartan (1 μmol/L). The data suggest that the AT$_2$ receptor activation via stimulation of the NO/cGMP/protein kinase G pathway directly inhibits the tubular NKA activity that provides as a mechanism responsible for the AT$_2$ receptor–mediated natriuresis in obese but not in lean Zucker rats.
mediated via an NO/cGMP/protein kinase G (PKG) pathway, and the AT$_2$ receptor activation stimulates NO/cGMP production in the PTs of obese but not lean rats.

**Methods**

**Animals**

Male obese and lean Zucker rats (10 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA). The animals were housed in the University of Houston animal care facility and had free access to standard rat chow and tap water. The Institutional Animal Use and Care Committee approved animal experimental protocols.

**Preparation of Renal Proximal Tubular Suspensions**

Renal proximal tubular suspensions were prepared as described previously. Rats were anesthetized with sodium pentobarbital (50 mg/kg, IP). After a midline incision, selective perfusion of the kidneys was performed with modified Krebs–Henseleit buffer containing collagenase type IV (230 U/mL) and hyaluronidase type III (250 U/mL). Kidneys were excised, and the outer cortex was removed, minced into fine pieces, and digested with collagenase–hyaluronidase solution under 95% O$_2$:5% CO$_2$ atmosphere until uniformly dispersed. Enrichment of PTs was carried out using 20% Ficoll density gradient. Trypan blue exclusion test was used to determine tubule cell viability. More than 95% of the tubules resulted in a single layer. Enriched PTs were centrifuged at 600 g for 8 minutes and the pellet was suspended in reaction mixture A (mmol/L: NaCl 70, KCl 5, MgCl$_2$ 5, CaCl$_2$ 1, Tris-HCl 150; pH 7.4). The average optical density was calculated. The values are represented as a percentage of nitrate/nitrite per milligram of protein.

**Immunoprecipitation of NKA**

Proximal tubular suspensions isolated from lean and obese Zucker rats were incubated in the absence and presence of CGP42112 (10 nmol/L) for 30 minutes as described above. After incubation, the samples were frozen on acetone and dry ice and stored at $-70\,^\circ$C until use. The immunoprecipitation was carried out as described previously.

**Western Blotting**

The Western Blotting was performed on PVDF membranes (blot). The blots were incubated with mouse monoclonal phosphoserine antibody or mouse monoclonal $\alpha_1$-subunit NKA antibody for 1 hour. Anti-mouse IgG-horseradish peroxidase conjugate and chemiluminescent substrate were used to detect the signal that was recorded on x-ray film. The values are presented as the ratio of the phosphoband to the total NKA band in respective lanes. The specificity of the phosphoserine and NKA antibodies has been confirmed by us earlier.
**Materials**

CGP42112, PD123319, L-NAME, ODQ, K3761, K1388, and all of the other chemicals were purchased from Sigma Aldrich. Losartan was a generous gift from Merck Sharp & Dohme. Antibody for NKA serine antibody was purchased from Calbiochem.

**Statistical Analysis**

Data are presented as mean±SEM. One-way ANOVA with post-hoc tests (Neumann–Keul) was used to analyze variation within the group. Student t test was used to compare variation between groups. We used a total of 9 lean and 11 obese rats. All of the statistical analyses were done using Graph Pad Prism, version 3.02 (GraphPad Software). A value of P<0.05 was considered statistically significant.

**Results**

**Effect of AT2 Receptor Agonist on NKA Activity**

The AT2 receptor agonist CGP42112, in a dose-dependent manner (0.1 to 100 nmol/L), inhibited the NKA activity (Figure 1) in PTs isolated from obese but not lean Zucker rats. The minimal inhibitory effect of ~6% was observed at 100 pmol/L, and the maximal inhibitory effect of ~33% was observed at 100 nmol/L of the agonist. The ouabain-insensitive (Mg^-ATPase) activity was not affected by the CGP42112 treatment (data not shown). The presence of the AT2 receptor agonist (PD123319, 1 μmol/L) attenuated the CGP42112-mediated inhibition at all of the concentrations in obese rats while not affecting the NKA activity in lean rats. This suggested that the inhibition of NKA in PTs isolated from obese rats was AT2 receptor mediated (Figure 1). PD123319 by itself did not affect the basal NKA activity in obese (basal, 158±5 versus PD123319, 163±6 nmol Pi/mg protein per minute) or in lean (basal, 167±15 versus PD123319, 170±13 nmol Pi/mg protein per minute) rats. We tested various inhibitors to investigate the potential pathway involved in mediating the CGP42112 (100 nmol/L)-induced inhibition of the NKA activity in obese rats. The AT2 receptor agonist losartan (1 μmol/L) did not alter the inhibitory effect of CGP42112 (10 nmol/L) on the NKA activity in obese or lean rats. PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase.

**Figure 1.** Effect of CGP42112 (0.1 to 100 nmol/L) in the absence and presence of the AT2 antagonist PD123319 (1 μmol/L) on the NKA activity in the proximal tubular suspension isolated from lean and obese Zucker rats. Values are represented as mean±SEM (4 lean rats and 6 obese rats); *P<0.05 vs basal (ANOVA followed by Newman–Keuls multiple comparison test), #P<0.05 vs CGP42112+PD123319 (Student t test) in obese rats, $P<0.05 vs lean CGP42112 (Student t test).

**Figure 2.** (A) Effect of CGP42112 (10 nmol/L) on the NKA activity in the proximal tubular suspension isolated from lean and obese Zucker rats, in the absence and presence of PD123319 (1 μmol/L), losartan (1 μmol/L), L-NAME (100 μmol/L), and ODQ (10 μmol/L). Values are represented as mean±SEM (4 lean rats and 6 obese rats); *P<0.05 vs basal within the same group (ANOVA followed by Newman–Keuls multiple comparison test), #P<0.05 vs CGP42112 (10 nmol/L) within the same group (ANOVA followed by Newman–Keuls multiple comparison test), PD123319, L-NAME, and ODQ did not affect the basal activity in obese rats (basal, 158±5 vs L-NAME, 154±6 and ODQ, 155±4 nmol Pi/mg protein per minute). (B) Effect of CGP42112 (10 nmol/L) on the NKA activity in the proximal tubular suspension isolated from lean and obese Zucker rats, in the absence and presence of the PKA inhibitor K3761 (500 nmol/L) and the PKG inhibitor K1388 (2 μmol/L). Values are represented as mean±SEM (5 lean rats and 5 obese rats); *P<0.05 vs basal within the same group (ANOVA followed by Newman–Keuls multiple comparison test), #P<0.05 vs CGP42112 (10 nmol/L) within the same group (ANOVA followed by Newman–Keuls multiple comparison test). K3761 and K1388 did not affect the basal NKA activity in obese or lean rats. PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase.
The AT2 receptor agonist (CGP42112) in a dose-dependent manner (0.1 to 100 nmol/L) abolished cAMP accumulation to a similar extent in the PTs (Figure 5A) isolated from lean and obese rats. Preincubating the PTs with the AT2 receptor antagonist PD123319 (1 μmol/L) abolished the CGP42112 (10 nmol/L)-induced cAMP accumulation (Figure 5B), whereas the AT1 antagonist losartan (1 μmol/L) did not affect the inhibition in obese and lean rats, suggesting the involvement of the AT2 receptors. Neither the NO synthase inhibitor l-NAME (100 μmol/L) nor the NO-dependent soluble guanylate cyclase inhibitor ODQ (10 μmol/L) affected basal cAMP accumulation in the PTs isolated from lean and obese rats (Figure 5A and 5B).
fected the CGP42112-induced cAMP inhibition (Figure 5B) in lean and obese Zucker rats, suggesting that the cAMP inhibition observed is not NO synthase dependent and soluble guanylate cyclase mediated as observed with the NKA inhibition. Both of the inhibitors alone did not significantly alter the basal cAMP levels in obese rats (basal, 0.045 ± 0.005 versus ODQ, 0.04 ± 0.005 and L-NAME, 0.046 ± 0.007 pmol/mg protein).

**Effect of AT2 Receptor Agonist on NKA Phosphorylation**

The monoclonal antibody against the NKA α1-subunit detected a single band (≈100 kDa) in the immunoprecipitated samples (Figure 6A, bottom). The phosphoserine antibody also detected a major band at ≈100 kDa (Figure 6A, top). The effect of the AT2 agonist CGP42112 on NKA phosphorylation was presented as the ratio of the density of phosphorylated NKA to the total NKA protein (Figure 6B). CGP42112 (10 nmol/L) enhanced the level by 50% of phosphorylated NKA in PT isolated from obese but not lean rats (Figure 6B).

The enhanced level of phosphorylation of the NKA suggests a direct effect of the AT2 receptor activation on the NKA activity.

**Discussion**

Obesity is a major risk factor for the development of hypertension. Although the mechanism responsible for obesity-related hypertension is not understood, numerous reports suggest that a balance between antinatriuretic and natriuretic hormone function on renal sodium metabolism is impaired, which contributes to the development of hypertension in obesity. The obese Zucker rat is a genetic model of insulin resistance and exhibits mildly elevated blood pressure. This animal model has close resemblance to the human metabolic syndrome. We have shown that, on one hand, the renal dopamine D1 receptor, a natriuretic hormone receptor, function is defective, and on the other hand, Ang II function with an antinatriuretic effect is hyperactive in obese Zucker rats. However, we reported recently that AT2 receptors are upregulated in the cortical membranes of obese Zucker rats, and on selective activation, the AT2 receptors...
mediate greater natriuresis in these animals. Obese Zucker rats respond with greater natriuresis/diuresis when infused with candesartan, an AT2 receptor antagonist, which was initially believed to be because of upregulation of the AT1 receptor function. However, the AT2 receptor antagonist PD 123319 infusion abolished candesartan-induced natriuresis in obese rats. Also, selective stimulation of the AT2 receptors by CGP42112 produced, without affecting glomerular filtration rate or blood pressure, greater diuresis/natriuresis in obese rats compared with lean rats.

In an attempt to investigate the signaling mechanism of AT2 receptor function, we have reported that AT2 receptor activation via the NO/cGMP pathway inhibits NKA activity in the PTs of Sprague Dawley rats, where we have also shown that AT2 receptors mediate natriuresis. In the present study, we tested the hypothesis that AT2 receptor activation produces greater inhibition in the NKA activity in the isolated PTs of obese compared with lean Zucker rats. Interestingly, we found that AT2 receptor agonist CGP42112 produced concentration-dependent inhibition of the NKA activity in the PT suspension prepared only from obese but not lean Zucker rats. Because L-NAME and ODQ abolished the CGP42112-induced NKA inhibition in obese Zucker rats, it suggested the role of NO/cGMP pathway in the AT2 receptor-mediated NKA inhibition. Earlier, NO was suggested as the primary second messenger leading to the physiological effects mediated by the AT1 receptor activation. The AT2 receptor activation increases NO and cGMP production in the kidney, including in the PTs. The cGMP is known to mediate a vast array of signaling pathways, including stimulation of cGMP-dependent protein kinase (PKG) and modulation of phosphodiesterase activity. The PKG is a potential enzyme that may affect NKA activity directly, and phosphodiesterase causes changes in the cAMP metabolism, hence affecting the activity of PKA, an enzyme shown to regulate NKA activity. Here we found that PKG inhibitor, not PKA inhibitor, completely abolished the CGP42112-induced NKA inhibition in obese Zucker rats, suggesting the role of PKG in AT2 receptor-mediated NKA inhibition.

The NKA inhibition by CGP42112 may explain the exaggerated sodium excretion reported previously in obese Zucker rats in response to AT2 receptor agonist. However, it is interesting to note that, whereas AT2 receptors also are expressed in cortical membrane of lean rats, the AT2 receptor agonist CGP42122 completely failed to affect NKA activity, as well as to increase NO/cGMP levels in the PTs of lean Zucker rats. Similarly, we have shown that the AT2 receptor agonist also failed to promote natriuresis in lean Zucker rats. This may not be attributed to the expression of the nonfunctional AT2 receptors in lean rats, because the AT2 receptor agonist CGP42112, independent of the NO/cGMP pathway, reduces cAMP accumulation in the PTs of both lean and obese Zucker rats equally. This also suggests that, whereas the AT2 receptor activation inhibits cAMP generation, it does not seem to participate in the AT2 receptor–mediated NKA inhibition. The AT2 receptors are also expressed in distal nephron segments; therefore, it is likely that the activation of the AT2 receptor will also lead to the NKA inhibition distal tubules in obese rats. Also, it is expected that, similar to the PTs, distal tubule NKA in lean Zucker rats may not be sensitive to the AT2 receptor activation.

The selective activation of the AT2 receptor signaling via NO/cGMP in obese Zucker rats seems to be proximal to the NO/cGMP and is not because of higher activity of the NO/cGMP pathway, per se, in the PTs of obese rats. This notion is supported by the reports that cortical NO synthase activity was lower and sodium nitroprusside, an NO donor that directly activates soluble guanylate cyclase, produced a similar degree of NKA inhibition in obese and lean Zucker rats. To test whether AT2 receptor-mediated NKA inhibition was a direct effect or whether changes in the sodium entry from apical side were responsible for this inhibition, we performed 2 sets of experiments. In 1 set, gramicidin, which disrupts the sodium gradient between the intracellular and extracellular spaces, did not affect the AT2-mediated inhibition of the NKA activity in obese rats. In the second experiment, we found that CGP42112 induced a significant increase in serine phosphorylation of the NKA α1-subunit in the PTs of obese and not in lean rats. The gramicidin and phosphorylation data suggest that AT2 receptor–mediated inhibition of the NKA activity is direct and not dependent on apical sodium entry. Other studies have shown that NO, which is a mediator of NKA inhibition in the present study, inhibits the NKA activity independent of Na,H-exchange inhibition or apical sodium entry into the cell. Serine phosphorylation of the NKA α1-subunit has been suggested as a mechanism of inhibition induced by dopamine and parathyroid hormone. Because the signaling (NO/cGMP/PKG) involved in AT2 receptor–mediated NKA inhibition is different than dopamine D1 and parathyroid hormone receptors (cAMP and protein kinase C), the intermediary mechanisms of NKA phosphorylation...
and inhibition by AT₂ receptor may be different. The NKA protein expression, per se, may not contribute to the enhanced NKA inhibition by AT₁ receptor, because the basal NKA activity observed in the present study and reported earlier, as well as the NKA protein expression, are similar in the PTs of lean and obese Zucker rats.

It is believed that hyperactivity of antinatriuretic hormones and defective natriuretic hormones contribute to the increased renal sodium absorption and the development of hypertension in obese Zucker rats. In numerous studies, we have shown that dopamine D₁ receptor, which is known to increase tubular sodium excretion, is defective and unable to inhibit NKA activity in the PTs and promote natriuresis in response to D₁ receptor agonist in obese rats. Although D₁ receptor agonist was reported recently to promote AT₁ receptor translocation to the membrane and thereby enhance the AT₁ receptor function to promote natriuresis, it is unlikely that defective D₁ receptor contributed to the enhanced AT₁ receptor function as a compensatory mechanism in obese Zucker rats. Similarly, an upregulation of the renal AT₁ receptor function on tubular sodium metabolism was found in streptozotocin-induced diabetic rats, where dopamine D₁ receptor also are shown to be defective. In addition to the defective dopamine D₁ receptor, we and others have reported enhanced Ang II function on tubular sodium transport and hyperantinatriuretic activity in obese compared with lean Zucker rats. Compared with the AT₂ receptors, in general, the AT₁ receptors are the predominant receptor subtype; therefore, the net effect of Ang II is believed to be mediated via the activation of the AT₁ receptors. It is likely that the enhanced AT₁ receptor function, along with defective dopamine D₁ receptor function, lead to increased renal sodium retention in obese rats. It is important to note that because of likely dominance of the AT₁ receptors, the effect of AT₂ receptors is evident only by selectively activating with AT₂ agonist or by blocking the AT₁ receptor with AT₂ antagonist. The AT₂ receptor antagonist PD123319 alone did not affect the basal sodium excretion in obese Zucker rats. Although selective activation of the AT₂ receptor inhibits sodium transport and increases its urinary excretion, whether AT₂ receptors participate and provide a compensatory mechanism is yet to be determined. Long-term treatment of obese Zucker rats with selective AT₂ receptor antagonist may shed light as to whether AT₂ receptors are protective against enhanced renal sodium absorption and the development of severe hypertension. It should be noted that obese Zucker rats develop only mild hypertension.

Perspectives
Recent focus on the understanding of the AT₂ receptor function in renal sodium metabolism provides novel aspects of the RAS in the regulation of renal function under normal and pathophysiological conditions. Whereas the AT₁ receptor is implicated in the stimulation of the tubular sodium transport, the AT₂ receptor not only antagonizes the AT₁ receptor function, but it also seems to produce direct effect on sodium excretion by inhibiting tubular NKA activity. These findings support the notion that selective activation of the AT₂ receptors, in addition to selective inhibition of the AT₁ receptors, may provide a better therapeutic approach to treat salt-sensitive hypertension, although it remains to be a subject of further investigation.

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References


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