Renal angiotensin II AT₂ receptors promote natriuresis in streptozotocin-induced diabetic rats

Amer C. Hakam, Athar H. Siddiqui, and Tahir Hussain

Department of Pharmacological and Pharmaceutical Sciences, University of Houston, Houston, Texas

Submitted 7 May 2005; accepted in final form 19 September 2005

Hakam, Amer C., Athar H. Siddiqui, and Tahir Hussain. Renal angiotensin II AT₂ receptors promote natriuresis in streptozotocin-induced diabetic rats. Am J Physiol Renal Physiol 290: F503–F508, 2006. —Angiotensin II AT₂ receptors have been implicated to play a role in the regulation of renal/cardiovascular functions under pathological conditions. The present study was designed to investigate the function of the AT₂ receptors on renal sodium excretion and AT₂ receptor expression in the cortical membranes of streptozotocin (STZ)-induced diabetic rats. The STZ treatment led to a significant weight loss, hyperglycemia, and decrease in plasma insulin levels compared with control rats. STZ-induced diabetic rats had significantly elevated basal urine flow, urinary sodium excretion rate (UNaV), urinary fractional sodium excretion, and urinary cGMP compared with control rats. Infusion of PD-123319, an AT₂ receptor antagonist, caused a significant decrease in UNaV (µmol/min) in STZ-induced diabetic rats (1 ± 0.09 vs. 0.45 ± 0.1) but not in control rats (0.35 ± 0.08 vs. 0.4 ± 0.07). The decrease in UNaV was associated with a significant decrease in urinary cGMP levels (pmol/min) in STZ-induced diabetic rats (21 ± 2 vs. 10 ± 0.8) but not in control rats (11.75 ± 3 vs. 12.6 ± 2). The infusion of PD-123319 did not alter glomerular filtration rate (STZ: 0.3 ± 0.02 vs. 0.25 ± 0.03; control: 1.4 ± 0.05 vs. 1.5 ± 0.09 ml/min) or mean arterial pressure (STZ: 82 ± 3 vs. 79 ± 3.5; control: 90 ± 4 vs. 89 ± 4 mmHg), suggesting a tubular effect of the drug. Western blot analysis using an AT₂ receptor antibody revealed a significantly enhanced expression of the AT₂ receptor protein (~45 kDa) in brush-border (~50-fold) and basolateral membranes (~80-fold) of STZ-induced diabetic compared with control rats. In conclusion, our data suggest that the tubular AT₂ receptors in diabetic rats are profoundly enhanced and possibly via a cGMP pathway promote sodium excretion in this model of diabetes.

OF THE ANGIOTENSIN II receptors, AT₁ receptors are predominately expressed in adult tissues and perform most of the known ANG II-elicted functions such as vasoconstriction, hypertrophy, and sodium/fluiddretention (26). AT₂ receptor expression in adult tissues is relatively low; however, AT₂ receptors are implicated in the cellular and physiological functions that are opposite to the functions mediated by the AT₁ receptors (10, 18, 19). The activation of AT₂ receptors has been shown to promote cell differentiation and apoptosis (18, 26). The AT₂ receptors are also implicated in blood pressure regulation via vasodilatation and possibly affecting fluid/sodium homeostasis (6, 12, 15).

There is evidence that the expression and function of the AT₂ receptors become relevant under pathophysiological conditions (16, 18), such as diabetes. Diabetic patients and animal models exhibit altered sodium/fluidd metabolism associated with changes in the renin-angiotensin system (RAS) (1, 27). Because evidence suggests that some of the diabetic animal models may not have altered production of ANG II (8, 12, 21), the altered expression and function of the ANG II receptors may be the site of regulation that affects sodium metabolism in diabetes. Recently, we showed that the tubular AT₂ receptor in obese Zucker rats, a model of type 2 diabetes, is upregulated and mediates the natriuretic effects of an AT₁ receptor antagonist (16).

The streptozotocin (STZ)-treated rat is used as a model of type 1 diabetes, which is associated with low plasma insulin and severe hyperglycemia (7, 14, 24, 34). Several studies have shown a decrease in the expression of the AT₁ receptors in the kidneys of type 1 diabetic animal models (5, 8, 14, 21). Other studies have shown an upregulation of AT₁ receptor protein in kidney (8, 34) and other tissues (19). There are no reports of the functional role of the renal AT₂ receptor on sodium metabolism in STZ-induced diabetic rats. Therefore, this study was designed to determine the expression of AT₂ receptor protein in proximal tubular membrane and to assess the functional role of AT₂ receptors on natriuresis-diuresis in STZ-induced diabetic rats. We found a profound increased expression of the tubular AT₂ receptors that contribute to the enhanced urinary sodium excretion in STZ-treated rats.

METHODS

Animal model. Age-matched male Sprague-Dawley rats, weighing 200–250 g and purchased from Harlan (Indianapolis, IN), were used in this study. 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. Type 1 diabetes was induced with a single intraperitoneal injection of STZ (55 mg/kg) dissolved in citrate buffer. Control rats were injected with vehicle only. Forty-eight hours postinjection, blood glucose was measured and rats with plasma glucose above 300 mg/dl were included in the study. All experiments were performed 2 wk after diabetes induction.

Urinary, plasma, and hemodynamic parameters. Fasting blood glucose was measured using a glucometer (The BioScanner 2000, Polymer Technology Systems, Indianapolis, IN). An RIA kit (Linco Research, St. Charles, MO) was utilized to determine plasma insulin levels. Urinary and plasma creatinine levels were determined using a creatinine analyzer (model 2, Beckman, CA). Plasma and urine levels of Na were measured using a flame photometer (Ciba Corning Diagnostics, Norwood, MA).

Urinary cGMP measurement. Urinary cGMP was measured using ELISA kit (R&D Systems, Minneapolis, MN). Urine that was collected from the functional study was diluted 100-fold according to the manufacturer recommendation and assayed in duplicate. A set of

Address for reprints and other correspondence: T. Hussain, Dept. of Pharmacological and Pharmaceutical Sciences, Science and Research Bldg. 2, Univ. of Houston, 4800 Calhoun, Houston, TX 77204-5037 (e-mail: thussain2@uh.edu).

http://www.ajprenal.org 0363-6127/06 $8.00 Copyright © 2006 the American Physiological Society
standards (0.4–500 pmol/ml) was assayed in duplicate at the same time. Nonspecific binding and the background were subtracted from each reading, and the average optic density was calculated. The data were processed using GraphPad Prism, and the concentration was extrapolated from the standard curve and then the 100-fold dilution was accounted for. The final concentration was multiplied by the urine flow rate (UF) to calculate the concentration per unit of time.

Experiment protocol for renal function. Rat surgery and kidney function were performed as described earlier (16, 25, 30). Briefly, rats were anesthetized using Inactin (100–160 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. Normal saline was continuously infused at a fixed rate of 1% body wt to maintain a constant hydration. After a stabilization period of 1 h, we collected urine at 30-min intervals. The first two periods were used to compute the basal parameters. The following is the schematic representation of the protocol:

<table>
<thead>
<tr>
<th>Stabilization</th>
<th>Basal</th>
<th>Basal</th>
<th>PD-123319</th>
<th>PD-123319</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>50 µg·kg⁻¹·min⁻¹</td>
<td></td>
</tr>
</tbody>
</table>

At the end of each urine collection period, the urine volume was measured and UF was calculated (µl/min). The urinary sodium excretion rate (U₅Na; µmol/min) was computed as UF × urinary sodium concentration (µmol/l). The glomerular filtration rate (GFR; ml/min) was calculated based on creatinine clearance. The U₅Na was divided by the plasma sodium concentration (mg/dl) and GFR to compute the fraction of sodium excreted in the urine (FESTNa, %).

Membrane preparation. Animals were anesthetized using pentobarbital sodium (50 mg/kg ip). After a midline incision, the kidneys were excised and cut sagittally. The outer cortices were used for the preparation of the basolateral (BLM) and brush-border (BBM) membranes (16, 28). Briefly, the cortices were homogenized in Tris-barbital sodium (50 mg/kg ip). The homogenate was centrifuged at 24,000 g for 20 min. The fluffy layer of the pellets was removed and suspended in buffer A, which Percoll was added. The suspension was thoroughly mixed and centrifuged at 30,000 g for 35 min. This resulted in two layers, upper light cloudy (BLM) and lower dense layer (BBM), which were separately collected. The two layers were washed three times with buffer containing 100 mM KCl, 100 mM mannitol, and 5 mM HEPES, pH 7.2 by centrifugation at 34,000 g. Finally, pellets were collected and suspended in buffer A. We determined earlier that the BLM fraction showed a strong presence of the α₁-subunit of Na-K-ATPase (NKA) and lacked Na-H exchanger 3 (NHE3), whereas the BBM fraction showed a strong band for NHE3 and lacked NKA (data not shown). Protein estimation of these samples was done using a BCA protein assay kit (Pierce).

Isolation of proximal tubules and glomeruli. The proximal tubules and glomeruli were isolated using the Percoll gradient centrifugation method (33). Briefly, the cortices were minced and digested with collagenase type IV in Krebs-Hanseleit solution (KHS), pH 7.4, with constant oxygenation until a uniform suspension is formed. The suspension was filtered through a nylon 250-µm sieve and centrifuged at 100 g for 1 min. The pellets were suspended and washed two times in KHS. The pellet suspension in KHS was mixed thoroughly with 40% Percoll and centrifuged at 26,000 g for 30 min. Four distinct bands (F1-F4) were separated. The F1 band was collected and enriched for glomeruli by passing sequentially through 105- and 80-mm nylon sieve (3, 4). The sample retained at the 80-mm sieve was a pure glomerular fraction, as determined under a light microscope. The F4 band, highly enriched proximal tubule fraction (33), was carefully collected, suspended, and washed in KHS. The cells in both the glomeruli and the proximal tubule preparations were intact, as determined by their ability to exclude Trypan blue.

Western blot analysis. Equal amounts (40 µg protein for AT₂ and 10 µg protein for AT₁) of BBM and BLM proteins, as previously described (16), from control and STZ-treated rats were used for Western blotting using polyclonal AT₂ and AT₁ receptor antibodies. For Western blotting of the AT₂ receptors in the proximal tubule preparation, we used 80 µg protein from control and STZ-treated rats. Anti-rabbit IgG-horseradish peroxidase (HRP) conjugate and chemiluminescent substrate were used to detect the signal that was recorded on X-ray film. The bands were densitometrically quantified and compared between control and STZ-treated rats.

Chemicals. Antibody for AT₂ receptor (cat. no. AT21-A) was purchased from Alpha Diagnostics (San Antonio, TX). According to the manufacturer’s information, an 18aa peptide sequence (KRE SMS CRK SSS LRE MET) near the COOH terminus of the human AT₂ receptor was used to generate the anti-AT₂ antibody. This peptide sequence is 88% identical between human and rat and has no sequence homology to other G protein-coupled receptors. The antibody for the AT₁ receptor was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). According to the manufacturer’s information, the AT₁ antibody is raised against a peptide mapping near the NH² terminus of the human AT₁ receptor; this region is identical to the corresponding rat sequence. This antibody is tested by the manufacturer and does not cross react with the AT₂ receptor. HRP-linked anti-rabbit IgG was purchased from Sigma (St. Louis, MO).

Statistical analysis. Data are presented as means ± SE. One-way ANOVA with post hoc tests (Newman-Keuls) was used to analyze variation within the group. Student’s t-test was used to compare variation between groups. All statistical analyses were done using GraphPad Prism, version 3.02 (GraphPad Software, San Diego, CA). A value of P < 0.05 was considered statistically significant.

RESULTS

Effect of STZ on general and hemodynamic parameters. Two weeks after the induction of diabetes, STZ-treated rats had significantly lower body weight, plasma insulin, heart rate, and mean arterial pressure (MAP) compared with control rats (Table 1). Fasting blood glucose was significantly elevated in STZ-treated rats compared with control rats (Table 1). Higher plasma glucose and very low plasma insulin are the characteristics of type 1 diabetes. Plasma creatinine and kidney weight were significantly higher in STZ-treated rats compared with control rats suggesting the presence of kidney damage and hypertrophy (Table 1).

Effect of PD-123319 on natriuresis-diuresis in STZ-treated rats. STZ-treated rats had significantly higher basal UF, U₅Na, and FESTNa compared with control rats; however, the GFR was...
significantly lower in STZ-treated rats (Fig. 1). Infusion of PD-123319 (50 μg·kg⁻¹·min⁻¹) did not significantly affect UF in control or STZ-treated rats (Fig. 1A); however, it significantly decreased UNaV and FENa in STZ-treated but not in control rats (Fig. 1B and C). The infusion of PD-123319 did not produce any significant change in GFR (Fig. 1D) or MAP in control (pre-PD-123319: 90 ± 3.9 mmHg; post-PD-123319: 89 ± 4 mmHg) or in STZ-treated (pre-PD-123319: 82 ± 3 mmHg; post-PD-123319: 79 ± 3.5 mmHg) rats, suggesting tubular effects of the drug.

Effect of PD-123319 on urinary cGMP levels. Basal levels of urinary cGMP were significantly higher in STZ-induced diabetic rats compared with control rats (Fig. 2). The intravenous infusion of the AT2 antagonist PD-123319 significantly decreased urinary cGMP levels in STZ-treated rats but had no effect on urinary cGMP levels in control rats.

ANG II AT2 and AT1 receptors expression. We determined the expression of the AT2 receptor proteins by Western blot analysis of the BBM and BLM of control and STZ-treated rats. The AT2 receptor antibody detected an approximately 45-kDa band of molecular weight that was displaced by the antigen peptide (Fig. 3A). Densitometric analysis of the bands showed a significant (50- to 80-fold) increase in the expression of AT2 receptor protein in both BBM and BLM in STZ-treated rats compared with control rats (Fig. 3A). An increase (~25-fold) in the AT2 receptor expression was also observed in the proximal tubule preparations of STZ-treated compared with control rats (Fig. 3A). We also used AT2 receptor antibody, obtained from Santa Cruz Biotechnology, to label AT2 receptors in the proximal tubule preparations. Similar to the Alpha Diagnostic AT2 receptor antibody, the Santa Cruz AT2 antibody also detected a band with similar increased intensity in STZ-treated compared with control rats (data not shown). Unlike in the cortical membrane and proximal tubule preparations, AT2 receptor expression was not detected in glomeruli of either control or STZ-treated rats (data not shown).

AT1 receptor protein expression was also determined using Western blot analysis of the BBM and BLM of control and

Fig. 1. Effect of PD-123319 (50 μg·kg⁻¹·min⁻¹ infusion) on urine flow (UF; A), urinary sodium volume (UNaV; B), fraction of sodium excreted in urine (FENa; C), and glomerular filtration rate (GFR; D) in control and streptozotocin (STZ)-treated rats. Values are means ± SE. *P < 0.05 vs. basal within the same group (Student’s t-test, n = 4 – 6). #P < 0.05 vs. basal control (Student’s t-test).

Fig. 2. Effect of PD-123319 (50 μg·kg⁻¹·min⁻¹ infusion) on urinary cGMP levels in control and STZ-treated rats. Values are means ± SE. *P < 0.05 vs. basal within the same group (Student’s t-test). #P < 0.05 vs. basal control (Student’s t-test, n = 4 – 6).
STZ-treated rats. The AT1 receptor antibody detected two bands ~40 and 50 kDa in size, which could be due to different degree of glycosylation, as reported earlier (32). Both bands were displaced by the antigen peptide (Fig. 3B). Densiometric analysis of both bands revealed a 50% increase in the AT1 receptor protein expression in the BBM and no change in the BLM of STZ-treated compared with control rats (Fig. 3B).

**DISCUSSION**

In the present study, we demonstrate that the greater urinary sodium excretion in STZ-induced diabetic rats is mediated by tubular ANG II type 2 receptors. The sodium excretory function of the AT2 receptor was associated with a profound increase in AT2 receptor expression in cortical membrane and proximal tubule preparations and an increase in urinary cGMP excretion in STZ-treated compared with control rats.

STZ treatment of rats is known to cause, as we also observed in the present study, a reduction in plasma insulin and severe hyperglycemia. Body weight loss, development of renal hypertrophy, and later diabetic nephropathy are some of the consequences of diabetes, which are similar to the human type 1 diabetes. In our study, we observed a possible renal injury in STZ-treated rats, as indicated by an increase in kidney weight and plasma creatinine. The STZ-treated rats excrete higher urine volume and urinary sodium compared with control rats. This may be owing to the severe hyperglycemia in these animals, which is reported in many studies (7, 14, 24, 32). However, the involvement of any receptor system in the enhanced diuresis and natriuresis in this model of diabetes is not known. In the present study, we intravenously infused PD-123319, an AT2 receptor antagonist that produced antinatriuresis in the STZ-treated, and not in control rats, suggesting a role of the AT2 receptors in renal sodium metabolism in diabetes. Because infusion of the antagonist did not affect blood pressure or GFR, a tubular effect of the AT2 receptor on sodium excretion is suggested.

In the recent past, the AT2 receptors, because of their anti-AT1 receptors function, have generated special interest. However, of the functions associated with the AT2 receptors, sodium metabolism is the least known phenomenon (11). Recently, we provided evidence suggesting the role of the AT2 receptors in the renal sodium excretion in obese Zucker rats (16). In that study (16), we found that while an AT2 receptor antagonist does not affect the basal urine or sodium excretion, it does abolish the natriuretic-diuretic effects induced by the AT1 receptor antagonist in obese Zucker rats. In the present study, it is especially interesting to note that the increased sodium excretion in the STZ-treated rats was almost entirely blocked by the AT2 receptor antagonist. This decrease in UNaV was accompanied by a 60% decrease in the fraction of sodium excreted. The lack of complete blockade of the enhanced fraction of sodium excreted suggests that other intrarenal factors are also involved in the sodium homeostasis in STZ-induced diabetes. One of the factors responsible for such a drastic effect of the AT2 receptor antagonist on sodium metabolism may be the greater expression of AT2 receptors on the proximal tubules of STZ-treated compared with control rats. This notion is supported by some of the studies suggesting that the plasma renin and renal contents of the renin, angiotensinogen, and angiotensin-converting enzyme, indexes of ANG II production, in STZ-induced diabetic rats are similar or lower than in control rats (13, 14, 34).

We found that the AT1 receptor protein expression is enhanced (50%) in the BBM while the expression was not altered in the BLM of STZ-induced diabetic rats compared with control rats. Our observation is in agreement with other reports of enhanced AT1 receptor expression in STZ-induced diabetes (8, 34). However, some reports suggested that STZ treatment caused a decrease in renal AT1 receptor expression (5, 8, 21). It appears that while AT1 receptor protein expression in the kidney of STZ-induced diabetic rats may vary depending on the kidney region or the methodology, the tubular AT1 receptors lack functionality, as demonstrated by the absence of diuresis-natriuresis in response to candesartan or losartan (AT1 receptor antagonists) infusions in STZ-treated rats (2, 30). Under this scenario, when ANG II production may be similar and the AT1 receptors lack functionality, the enhanced expression of the AT2 receptor may be responsible, in part, for the excessive fluid and sodium excretion in STZ-treated rats. The minimal effect of PD-123319 on UF could be partially due to...
the increased urine osmolality secondary to the presence of glucose and proteins. In the presence of such a high osmolality, it may be difficult to alter the UF by pharmacologically manipulating the sodium reabsorption.

In our study, while we found an upregulation of AT2 receptors in the proximal tubules, a decrease in AT2 receptors was reported in the glomeruli and the tubular epithelial cells of STZ-treated compared with control rats (7, 34). We did not detect AT2 receptor expression in rat glomeruli of either control or STZ-treated rats, suggesting a lack of AT2 receptor expression. Similarly, Tejera et al. (32) reported that while AT2 receptors are expressed in the proximal and distal tubules, no appreciable expression of the AT2 receptor was detected in the glomeruli. From these studies, it is not known whether the discrepancy in detecting the changes in AT2 expression in proximal tubules or the absence and presence per se of the AT2 receptors in glomeruli is due to the source of antibody. However, we support the presence and upregulation of the tubular AT2 receptors in STZ-treated rats. The renal AT2 receptors can mediate the production of bradykinin and nitric oxide (NO) and, therefore, increase the levels of cGMP (10, 29). NO is known to act as a natriuretic factor that has direct tubular action to inhibit sodium reabsorption in the proximal tubules and collecting ducts by inhibiting Na+/H+ exchanger and Na+-K+-ATPase activity (23). NO can also stimulate soluble guanylyl cyclase and increase the levels of cGMP (22), which is also a natriuretic factor (20) and has an inhibitory effect on Na+-K+-ATPase activity in the proximal tubular cells (15). It is likely that the upregulated AT2 receptors via NO/cGMP pathways mediate tubular sodium transport inhibition in STZ-treated rats, leading to enhance urinary sodium excretion.

To our knowledge, this is the first report that shows a functional role for the AT2 receptor in obesity homeostasis in STZ-induced diabetic rats. We previously (16) showed a similar role for the AT2 pathway in obese Zucker rats. Both our reports and reports from other groups (10, 12, 29) indicate an important role for the AT2 receptors in modulating renal and cardiovascular functions in pathological conditions. The pronatriuretic effect of the renal AT2 receptors that we observed in these studies is of great physiological/therapeutic relevance as the kidney is a major cardiovascular organ with great influence on sodium and water balance and, therefore, blood pressure maintenance. We also found that STZ treatment decreased MAP significantly as measured under anesthesia, and this finding is in agreement with a previous report indicating a decrease in systolic blood pressure in spontaneously hypertensive rats after STZ treatment (7). We speculate that the upregulated AT2 receptors in the kidney may play a role in the lower blood pressure in STZ-treated rats secondary to their natriuretic effect. We need to acknowledge that in the present study, the blood pressure was measured under anesthesia and, although Inactin, a drug known to have minimal effect on renal and cardiovascular systems (9), was used, we do not know the effect of the anesthesia on blood pressure.

In summary, we found that tubular AT2 receptors were upregulated and play a role in renal sodium excretion in STZ-induced diabetic rats. We speculate that such a role of the AT2 receptor may be physiologically relevant to compensate for the enhanced fluid intake observed in STZ-induced diabetic rats. However, it is yet to be determined whether chronic blockade of the AT2 receptor will have an effect on pressure-natriuresis, leading to blood pressure elevation.

GRANTS

This work is supported by National Institutes of Health Grant R01-DK-61578.

REFERENCES


