Angiotensin AT2 receptor agonist prevents salt-sensitive hypertension in obese Zucker rats

Quaisar Ali, Sanket Patel, and Tahir Hussain

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

Submitted 6 January 2015; accepted in final form 7 April 2015

Ali Q. Patel S, Hussain T. Angiotensin AT2 receptor agonist prevents salt-sensitive hypertension in obese Zucker rats. Am J Physiol Renal Physiol 308: F1379–F1385, 2015. First published April 8, 2015; doi:10.1152/ajprenal.00002.2015.—High-sodium intake is a risk factor for the pathogenesis of hypertension, especially in obesity. The present study is designed to investigate whether angiotensin type 2 receptor (AT2R) activation with selective agonist C21 prevents high-sodium diet (HSD)-induced hypertension in obese animals. Male obese rats were treated with AT2R agonist C21 (1 mg·kg−1·day−1, oral) while maintained on either normal-sodium diet (NSD; 0.4%) or HSD (4%) for 2 wk. Radiotelemetric recording showed a time-dependent increase in systolic blood pressure in HSD-fed obese rats, being maximal increase (~27 mmHg) at day 12 of the HSD regimen. C21 treatment completely prevented the increase in blood pressure of HSD-fed rats. Compared with NSD controls, HSD-fed obese rats had greater natriuresis/diuresis and urinary levels of nitrates, and these parameters were further increased by C21 treatment. Also, C21 treatment improved glomerular filtration rate in HSD-fed rats. HSD-fed rats expressed higher level of cortical ANG II, which was reduced to 50% by C21 treatment. HSD feeding and/or C21 treatment had no effects on cortical renin activity and the expression of angiotensin-converting enzyme (ACE) and chymase, which are ANG II-producing enzymes. However, ANG(1–7) concentration and ACE2 activity in the renal cortex were reduced by HSD feeding, and C21 treatment rescued both the parameters. Also, C21 treatment reduced the cortical expression of AT2R in HSD-fed rats, but had no effect on AT3R expression. We conclude that chronic treatment with the AT2R agonist C21 prevents salt-sensitive hypertension in obese rats, and a reduction in the renal ANG II/AT1R and enhanced ACE2/ANG(1–7) levels may play a potential role in this phenomenon.

Address for reprint requests and other correspondence: T. Hussain, Univ. of Houston, Houston, TX 77204-5037 (e-mail: thussain@central.uh.edu).

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peritoneal cavity of the rats under anesthesia with isoflurane. On days 13 and 14, blood (80 μl) samples from tail vein were collected. The FITC-inulin fluorescent counts in plasma samples and in urine collected on the same days (described above) were determined using spectrophotofluorometer (Cytofluor Series 4000, Applied Biosystem). The glomerular filtration rate (GFR) was calculated using the formula GFR = (urine fluorescence counts/24 h)/(plasma fluorescence counts/μl) and reported as μl/min (5, 39).

Blood Pressure and Heart Rate by Telemetry

Obese rats were fasted overnight and treated presurgically with anesthetic Marcaine. After midline incision, the telemetry probe (model PA C-40, Data Sciences International, St. Paul, MN) was implanted in the abdominal aorta under isoflurane anesthesia (5% induction, 3–4% maintenance at 1.5 l/min). After implantation, the muscular abdominal wall was closed with 4-0 polyester suture, which also anchored the telemetry probe to the anterior abdominal wall. Skin was closed with wound clips and carprofen (5 mg/kg body wt ip) was injected every 24 h for 3 days post-surgery. The animals were allowed to recover from implantation surgery for 7 days before the treatment with AT2R agonist C21 was initiated. Animals were housed singly with each cage placed over a receiver used to monitor the blood pressure and heart rate telemetry signal. Data acquisition software (Data Quest ART, Data Sciences) was configured to sample a 15-s blood pressure reading every 15 min over the course of the study. Twenty-four-hour readings were averaged to provide the daily blood pressure reading. At the end of the blood pressure measurements and metabolic cage study, the animals were euthanized and the kidneys were removed, pat-dried, weighed, and stored frozen at −80°C for biochemical assays.

Urinary Volume and Sodium

The urine collected every 24-h period over 3 consecutive days was averaged for each animal and presented as 24-h urine volume, ml/day. Sodium in each urine sample was determined by Perkin Elmer Analyst 400 atomic absorption spectrometer. Similar to the urine volume, values of urinary Na volume (UNaV) in each urine sample collected over 3 days for each animal were averaged and presented as μmol/day.

Urinary Nitrates

Nitrates in urine were measured by Griess reagent. Briefly, 2-ml fraction of 24-h urine was filtered using Amicon Ultracel-10K centrifugal filters for 1 h and filtrate was diluted 10 times. For nitrate determination, 50 μl of filtered urine were allowed to react with 10 μl nitrate reductase enzyme preparation and 10 μl cofactor preparation with gentle shaking for 15 min. After those samples, nitrate standards (0–100 μM) and blanks (distilled water) were allowed to react with 50 μl 2% sulfanilamide in 5% phosphoric acid for 20 min in the dark with gentle shaking, and then with 50 μl 0.2% NEDD for 20 min in the dark with gentle shaking. Azo dye formed was measured at 540 nm and urinary nitrates were reported as μmol/day.

RAS Components

Western blotting. The expression of AT1R, AT2R, ACE, and chymase in the kidney cortex was determined by Western blotting. For this purpose, the kidney cortices were homogenized in the buffer containing (in mM) 50 Tris, 10 EDTA, 1 PMSF, and protease inhibitors. Equal amounts of protein (30 μg for AT1R, 60 μg for AT2R, 40 μg for ACE, 40 μg for chymase) from various rat groups were subjected to SDS-PAGE and Western blotting using relevant primary antibodies for the AT1R, AT2R, ACE, and chymase. Following the incubation with the primary antibodies, the blots were incubated with relevant horseradish peroxidase-conjugated IgG. The signal was detected by ECL system, recorded, and analyzed for the bands densitometry by Fluorchem 8800 (Alpha Innotech Imaging System, San Leandro, CA). The blots were stripped and reprobed with β-actin mouse monoclonal antibody.

Renin and ACE2 activity. Renin and angiotensin-converting enzyme type 2 (ACE2) activity in kidney cortex were measured by SensoLyte 520 and SensoLyte 390 activity kit (Anaspec, Fremont, CA), respectively. These kits provide a Mca/Dnp fluorescence resonance energy transfer (FRET) peptide as a matrix metalloproteinases (MMPs) substrate. In the intact FRET peptide, the fluorescence of Mca is quenched by Dnp. Upon cleavage into two separate fragments by MMPs, the fluorescence of Mca is recovered, and can be monitored at excitation/emission wavelengths (490 nm/520 nm for renin and 330 nm/390 nm for ACE2).

Angiotensin peptides. Levels of ANG II and ANG(1–7) peptides in the kidney cortex were quantified using LC/MS, as we described recently (6). The tissue was homogenized in lysis buffer (10 mM Tris, pH 7.4), centrifuged for 15 min at 1,600 g, a solution containing supernatant was loaded onto an C18-E (5 μm, 70A) cartridge, and equilibrated with 60% acetonitrile, 1% trifluoroacetic acid, and 39% distilled water. The column was eluted with the equilibrium buffer and collected in a 15-ml tube. The eluent was dried and reconstituted in 80% acetonitrile and 0.1% formic acid before LC/MS analysis of the peptides.

Chemicals

C21 [butyl][3-(4-((1H-imidazol-1-yl)methyl)phenyl)-5-isobutylthiophen-2-yl] sulphonylcarbamate] was custom synthesized (SPS AlfaChem). Antibodies for ACE (sc-12188) and β-actin (sc-47778) were purchased from Santa Cruz. Chymase antibody (MA5-11717) was purchased from Thermo Scientific (Rockford, IL), AT2R antibody (EZBiolab, Westfield, IL) and AT1R antibody (Biomolecular Integrations, Little Rock, AR) were custom raised in rabbit. All other chemicals used were of standard grade and purchased from Sigma (St. Louis, MO). Nitrate reductase enzyme (780010) and cofactor preparation (780012) were purchased from Cayman Chemical (Ann Arbor, MI).

Statistical Analysis

Data are presented as means ± SE. The data were analyzed using GraphPad Prism 4 (GraphPad, San Diego, CA) and subjected to one-way ANOVA with Newman-Keuls post hoc test with n = 5 in each group as detailed in the figure legends. A P value of <0.05 was considered statistically significant.

RESULTS

General Parameters

The amount of food intake was similar in all the rat groups (Table 1). Water consumption was significantly increased in HSD-fed rats compared with NSD-fed controls (HSD 44±2 vs. NSD 32±2 ml/day). C21 treatment did not affect water or food intake in rats fed with either NSD or HSD (Table 1).

The rats fed with HSD and/or treated with C21 had similar body weight (Table 1); although a significant higher body weight gain was observed in HSD-fed rats (HSD 74±5 vs. NSD 45±8 g over respective basal weight) over 2-wk period of treatment. C21 treatment prevented increase in body weight gain of HSD-fed rats. Kidney weight was unchanged among study groups (Table 1).

GFR

The GFR was decreased significantly by HSD feeding compared with NSD-fed controls (NSD 654±82 vs. HSD 202±81 μl/min). C21 treatment rescued GFR in the HSD-fed animals.
rate and diastolic blood pressure remained similar in all the NSD-fed rats was not affected by the C21 treatment. The heart induced increase in the blood pressure. The blood pressure in maximum. C21 treatment completely prevented the HSD appeared to plateaued off, being 27-mmHg increase as the increase in blood pressure was gradual over the course of HSD sure over 2-wk period of the dietary regimen. Although the intake caused a significant increase in the systolic blood pres-

**Table 1. General and renal parameters of control and C21-treated obese rats fed with either NSD or HSD**

<table>
<thead>
<tr>
<th>Parameters/Rats Groups</th>
<th>CT</th>
<th>C21</th>
<th>HSD</th>
<th>HSD+C21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food, g/day</td>
<td>31 ± 1.7</td>
<td>29 ± 2</td>
<td>34 ± 1</td>
<td>30 ± 6</td>
</tr>
<tr>
<td>Water, ml/day</td>
<td>32 ± 2</td>
<td>30 ± 3</td>
<td>44 ± 2‡</td>
<td>51 ± 4</td>
</tr>
<tr>
<td>Body wt (before study), g</td>
<td>527 ± 7</td>
<td>536 ± 4</td>
<td>515 ± 8</td>
<td>520 ± 4</td>
</tr>
<tr>
<td>Body wt (after study), g</td>
<td>569 ± 9</td>
<td>583 ± 8</td>
<td>587 ± 8</td>
<td>564 ± 5</td>
</tr>
<tr>
<td>Gain in body wt, g</td>
<td>45 ± 8</td>
<td>48 ± 6</td>
<td>74 ± 5‡</td>
<td>43 ± 6‡</td>
</tr>
<tr>
<td>Kidney wt/body wt, mg/g</td>
<td>3.24 ± 0.13</td>
<td>3.16 ± 0.06</td>
<td>3.14 ± 0.10</td>
<td>2.97 ± 0.07</td>
</tr>
<tr>
<td>Glomerular filtration rate, µl/min</td>
<td>654 ± 82</td>
<td>847 ± 107</td>
<td>202.7 ± 81‡</td>
<td>482 ± 47‡</td>
</tr>
<tr>
<td>Urinary volume, ml/day</td>
<td>9 ± 1</td>
<td>13 ± 1†</td>
<td>19 ± 0.8†</td>
<td>25 ± 0.7‡</td>
</tr>
<tr>
<td>Urinary Na excretion, µmol/day</td>
<td>0.69 ± 0.07</td>
<td>0.72 ± 0.07</td>
<td>1.45 ± 0.22‡</td>
<td>2.06 ± 0.23‡</td>
</tr>
<tr>
<td>Heart rate, beats/min*</td>
<td>353 ± 12</td>
<td>340 ± 18</td>
<td>352 ± 14</td>
<td>342 ± 15</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg*</td>
<td>94 ± 8</td>
<td>92 ± 5</td>
<td>95 ± 4</td>
<td>99 ± 10</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg*</td>
<td>130 ± 3</td>
<td>127 ± 5</td>
<td>154 ± 8‡</td>
<td>131 ± 5‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; 1-way ANOVA followed by Newman-Keuls test, *P < 0.05; n = 5 in each group. NSD, normal-sodium diet; CT, obese control; C21, obese treated with C21; HSD, obese treated with high-sodium diet; HSD+C21, obese treated with C21 and HSD. *Values are average of 24 h on day 14. †Significantly different from control obese. ‡Significantly different from HSD-fed rats.

(HSD+C21 482±47 µl/min), but had no effect in NSD-fed rats (Table 1).

**Urinary Volume and Sodium Excretion**

Compared with NSD controls, HSD-fed rats excreted greater amount of urine volume (HSD 19±2 vs. NSD 9±1 ml/day) and urinary sodium (HSD 8±0.6 vs. NSD 3±0.4 µmol/day). C21 treatment caused significant increases in both the urine volume and sodium excretion in rats fed with NSD or HSD (Table 1 and Fig. 1).

**Urinary Nitrate Excretion**

Urinary excretion of nitrates (HSD 1.45±0.22 vs. CT 0.69±0.07 µmol/day) was found significantly increased in HSD-fed animals compared with that of control animals. C21 treatment caused further increase in the urinary nitrates in HSD-fed rats (Table 1).

**Systolic Blood Pressure**

As shown in Fig. 2, telemetry monitoring revealed that HSD intake caused a significant increase in the systolic blood pressure over 2-wk period of the dietary regimen. Although the increase in blood pressure was gradual over the course of HSD feeding, the increase in the second week of HSD feeding appeared to be plateauled off, being 27-mmHg increase as the maximum. C21 treatment completely prevented the HSD-induced increase in the blood pressure. The blood pressure in NSD-fed rats was not affected by the C21 treatment. The heart rate and diastolic blood pressure remained similar in all the groups during treatment period (Table 1, only day 14 data are presented).

**RAS Components**

**AT1R and AT2R.** The HSD feeding or C21 treatment of NSD-fed rats had no effect on the cortical AT1R (43-kDa band) expression. However, AT1R expression in HSD-fed rats compared with any of the groups was reduced to half by C21 treatment (Fig. 3A). Western blot shows presence of AT1R as three bands (44/45 and 39 kDa) in the renal cortex. These three bands of AT2R revealed that the cortical expression of AT2R was not affected either by HSD feeding and/or C21 treatment (Fig. 3B).

**Renin activity and chymase expression.** Cortical renin activity in all treatment groups remained unaffected (Fig. 4A). Western blot of kidney cortex revealed 35-kDa band for chymase that also remained unaffected by HSD and/or C21 treatments (Fig. 4B).

**ACE expression and ACE2 activity.** Western blot of the kidney cortex detected a 170-kDa band for ACE (Fig. 4C). Densitometry of these bands suggested that ACE expression was not affected by HSD feeding and/or C21 treatment. On the
Contrary, ACE2 activity in the kidney cortex was greatly reduced after HSD feeding, and this reduction was prevented by C21 treatment (Fig. 4D).

Angiotensin peptides. ANG II levels in the kidney cortex of HSD-fed rats were almost fourfold higher than NSD controls. C21 treatment attenuated HSD-induced ANG II increase in obese rats (Fig. 4E). Contrary to the ANG II changes, ANG(1–7) level was reduced by HSD feeding and this reduction was rescued to normal level by C21 treatment (Fig. 4F).

DISCUSSION

Enhanced renal Na reabsorption is a major contributor to the pathogenesis of obesity hypertension. However, the exact mechanism is unclear but exaggerated renal Na reabsorption associated with volume expansion leading to increased peripheral resistance is considered an overall mechanism (22). In the present study, the AT2R agonist C21 induced diuresis/natriuresis and possibly reduction in intravascular volume, as indicated by reduced weight gain may constitute a global mechanism responsible for lowering of blood pressure in HSD-fed obese rats. We also observed that HSD-fed obese rats exhibited a reduced GFR, an indication of chronic kidney disease (50), which could be a consequence of sustained hypertension. Obese rats start exhibiting glomerulosclerosis at earlier age (12); it is likely that in addition to higher blood pressure, high-sodium intake per se might have accelerated glomerular injury in HSD-fed rats. AT2R activation seems to be protective, as we suggested earlier (12). Since a reduced GFR is associated with enhanced renal sodium retention, it is likely that increase in GFR in HSD-fed rats in response to C21 treatment led to restoration of urinary sodium excretion and thus protection against blood pressure increase in these animals.

The blood pressure of NSD-fed obese rats was not affected by C21 treatment, which is contrary to our previous report (5). The discrepancy, however, could be based on 1) agonist: in an earlier study, CGP42112A, a peptide agonist, was delivered via osmotic pump implanted subcutaneously; in the present study, C21, a novel orally active nonpeptide agonist, was given orally to the animals, and 2) methods used to measure blood pressure: in the earlier studies, blood pressure was measured only under stress (anesthesia or tail cuff) (5, 42) causing the blood pressure to increase (28), which is prevented in CGP42112A-treated animals (5). The present study utilized radiotelemetry approach to measure blood pressure continuously over 2-wk period in freely moving animals. Some studies (33), including our unpublished data (mean arterial pressure: obese 107 ± 1, lean 103 ± 1 mmHg), suggest that obese rats in this age group may not be hypertensive, therefore C21 is not expected to lower blood pressure in control obese rats.

The role of AT2R in lowering blood pressure has been debated whether its activation alone is sufficient to regulate blood pressure. Some studies have reported the antihypertensive activity of AT2R activation only in the presence of AT1R blockers or ACE inhibitors (8,10). Obese Zucker rats, a model of obesity with insulin resistance and modest hyperglycemia, express higher AT2R in the kidney proximal tubules (19); hyperglycemia appears to be driving the increase in AT2R expression (4). In vitro and in vivo studies suggest that AT2R activation directly stimulates nitric oxide (NO)/cGMP production, inhibits Na pump activity in the proximal tubules, and causes natriuresis, primarily via proximal tubule mechanisms in obese Zucker rats (3, 18). In SHR and Dahl salt-sensitive rats, kidney proximal tubules AT2R expression is similar compared with their respective controls, and the AT2R that are present in the kidney of these hypertensive animals are functionally defective (26). The antihypertensive activity of AT2R...
agonist in the presence of AT1R blockade in SHR could be based on the vascular AT2R activation and not the renal AT2R (8). Overall, we speculated that hyperglycemia is one of the metabolic conditions driving AT2R's natriuretic function regulating salt-sensitive hypertension in obesity. Enhanced AT2R expression has been reported in the diabetic human kidney (35) and in streptozotocin-induced diabetic rats (20). The recent study, however, demonstrated that C21 treatment, without manipulating AT1R or ACE activity, reduced blood pressure in ANG II-induced hypertension in Sprague-Dawley rats (30). Parallel to this study (35), HSD-fed Zucker rats express higher level of renal ANG II, which could be a factor contributing to the increased blood pressure that was protected by C21 treatment.

There are several cellular and whole animal studies demonstrating the direct role of renal AT2R in inhibiting tubular sodium transport and promoting natriuresis/diuresis in animals, including in obese and diabetic rats (18, 20). It is reasonable to suggest that AT2R activation directly affects natriuresis and thereby prevents blood pressure increase in HSD-fed obese rats. However, this is interesting to observe an enhanced natriuresis even at 2 wk of treatment with the agonist; sodium balance should have occurred within few days of C21 treatment. In our earlier study (5), daily sodium excretion in obese rats treated with the AT2R agonist CGP42112A was tracked over 2-wk period while the animals were maintained on NSD. We found urinary sodium excretion increased within 2 days of the treatment and remained elevated throughout the treatment period. In a recent study (30), C21-treated group maintained higher urinary sodium excretion at day 7 of the drug treatment period. Since sodium delivery to the animals was not controlled and other sodium excretory pathways, except urine, were not assessed, it would be difficult to come on definite conclusion as to why enhanced urinary sodium excretion continued at a later stage of the treatment period. To study the role of kidney in sodium balance in response to AT2R agonist may require a controlled sodium delivery.

The enhanced natriuresis in HSD-fed C21-treated rats is associated with parallel changes in the urinary nitrates, an index of NO production. High-sodium intake has been reported to cause an increase in NO production and NO synthase 16 expression in the kidney (34). Such an increase in NO production could be a compensatory mechanism leading to enhanced natriuresis during high-sodium intake. It is likely that the activation of AT2R, which is linked to endothelial NO synthase and NO production (45, 49), causes further increase in NO production leading to natriuresis. However, it is also likely that reduced levels of the anti-natriuretic ANG II/AT1R in the kidney cortex of C21-treated HSD-fed rats might have contributed to the enhanced natriuresis and the reduction in blood pressure in these animals. ANG II peptide, which is almost fourfold higher in HSD-fed rats compared with NSD rats, is reduced to half and the AT1R expression is also reduced to 50% in C21-treated HSD-fed rats. The reduction in AT1R by AT2R agonist treatment is supported by in vitro studies (5, 51). It has been suggested that the reduction in AT1R receptor...
expression and function involves AT$_3$R-mediated stimulation of NO/cGMP/sp1 pathway (51). There are several studies suggesting an enhanced renal AT$_1$R function in obese rats as a cause of obesity hypertension (7, 9). From the present study, it is clear that HSD feeding in obese rats increased renal ANG II levels, which were reduced by C21 treatment.

It is interesting to note that the changes in ANG II levels in response to HSD feeding alone or in combination with C21 treatment were not associated with parallel changes in the renal renin activity and ACE expression, which are the primary enzymes regulating ANG II levels. These findings suggest that the cause for the enhanced ANG II levels in response to HSD could be non-ACE pathway and/or altered degradation of the peptide. Chymase, which is known to produce ANG II directly from ANG I bypassing ACE pathway, especially during diabetes, has not changed either by HSD or HSD+C21 treatment. Thus, involvement of chymase as a cause of higher ANG II is ruled out. However, ACE-independent chymase-mediated ANG II formation was found in the ischemic kidney (43) and severe hyperglycemia (27), and inhibitors of ACE/chymase did not block formation of ANG II. It was concluded that chymase is not a significant contributor to increased renal ANG II levels (47).

Changes in ANG(1–7) levels, in part, explain the changes in ANG II levels in HSD and/or HSD+C21-treated groups. Conversion of ANG II by ACE2 to ANG(1–7) is one of the pathways that can attenuate the levels and activity of ANG II (17, 24). The present findings suggest that lowering the ACE2 activity and thus reduced ANG(1–7) production might have led to the accumulation of renal ANG II in HSD-fed rats. Since the changes (fmol/mg tissue) in ANG II are larger than the changes in ANG(1–7), ACE2 pathway alone may not be responsible for the changes in ANG II levels in HSD or HSD+C21-treated groups. Other pathways via aminopeptidases that produce or degrade ANG II (13, 14, 47) may also be involved and altered by HSD and/or AT$_2$R agonist treatment. Since this is not the focus of the study, a separate systematic study is warranted to understand ANG II metabolism in obesity during high-salt intake. In addition to the suppression of ANG II, increase in the renal levels of ANG(1–7) may also have contributed to the anti-hypertensive effect of C21 treatment. There are numerous studies suggesting the pronatriuretic and anti-hypertensive role of ANG(1–7) in animal models of hypertension (11). Collectively, C21 treatment during HSD intake reverses the imbalance of ANG II and ANG(1–7) levels with a potential of proportional changes in their physiological significance. We reported earlier that AT$_3$R agonist treatment upregulates ACE2 expression in obese rat kidney and human kidney-2 cells (5). Although the molecular mechanism responsible for enhanced ACE2 activity by AT$_3$R agonist has not been explored, a reduced ANG II/AT$_1$R expression in response to AT$_3$R agonist might have contributed indirectly to the enhanced ACE2 activity/ANG(1–7). Activation of AT$_3$R has been suggested to suppress ACE2 expression/activity in myocytes and fibroblasts (15).

In summary, the present study demonstrates that AT$_3$R activation with a novel agonist C21 prevents salt-induced increase in blood pressure in obese Zucker rats. Further C21 treatment causes a decrease in the expression of the antinatriuretic ANG II/AT$_1$R levels and pronatriuretic ANG(1–7) levels, suggesting their potential involvement in the anti-hypertensive effects of AT$_3$R activation in obesity with high-sodium intake.

**REFERENCES**


ROLE OF AT2R IN Na-SENSITIVE HYPERTENSION


