Chronic candesartan alters expression and activity of NKCC2, NCC, and ENaC in the obese Zucker rat

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Halogappa VK, Tiwari S, Riazi S, Hu X, Ecelbarger CM. Chronic candesartan alters expression and activity of NKCC2, NCC, and ENaC in the obese Zucker rat. Am J Physiol Renal Physiol 294: F1222–F1231, 2008. First published February 27, 2008; doi:10.1152/ajprenal.00604.2007.—The obese Zucker rat reportedly has increased activity of the intrarenal renin-angiotensin-aldosterone system, which conceptually could contribute to elevated salt sensitivity and blood pressure (BP). Our aim was to determine whether there was increased angiotensin II type 1 receptor (AT1R)-mediated upregulation of expression or activity of the bumetanide-sensitive Na-K-2Cl cotransporter (NCC), and/or the epithelial sodium channel (ENaC) in obese vs. lean Zucker rats. Male obese and lean Zucker rats (10-wk old) were fed either 1) control chow (1% NaCl) or 2) chow with candesartan (CAN), an AT1R antagonist (25 mg/kg · diet) for 14 wk (n = 8/treatment/body type). BP measured by radiotelemetry, was markedly reduced by CAN (~20–25 mmHg) in both lean and obese rats with no body-type differences. Obese rats had significantly greater net natriuretic response to single injections of hydrochlorothiazide and benzamil, suggesting increased activity of NCC and ENaC, respectively; however, only the response to benzamil was reduced by CAN. CAN led to a significant reduction in whole kidney levels of NCC and γ-ENaC (70-kDa band) in both lean and obese rats. However, it significantly increased α-ENaC and Na-K-2Cl cotransporter levels, and these increases were greater in obese rats. These studies suggest that relatively increased ENaC, but not NCC activity, in obese rats is due to enhanced AT1R activity. CAN attenuated the reduction of several renal transporters in the obese rat kidney. Finally, differences in intrarenal AT1R activity do not seem directly responsible for BP differences between lean and obese rats.

**THE LEPTIN RECEPTOR-DEFICIENT obese Zucker rat (faffa) is grossly obese, markedly insulin resistant, and exhibits modest hypertension.** Several studies (1, 3, 13) have reported increased angiotensin II (ANG II) activity in these rats, relative to their lean littermates, as determined by decreased blood pressure (BP). The obese Zucker rat reportedly has increased activity of the intrarenal renin-angiotensin-aldosterone system, which conceptually could contribute to elevated salt sensitivity and blood pressure (BP). Our aim was to determine whether there was increased angiotensin II type 1 receptor (AT1R)-mediated upregulation of expression or activity of the bumetanide-sensitive Na-K-2Cl cotransporter (NCC), and/or the epithelial sodium channel (ENaC) in obese vs. lean Zucker rats. Male obese and lean Zucker rats (10-wk old) were fed either 1) control chow (1% NaCl) or 2) chow with candesartan (CAN), an AT1R antagonist (25 mg/kg · diet) for 14 wk (n = 8/treatment/body type). BP measured by radiotelemetry, was markedly reduced by CAN (~20–25 mmHg) in both lean and obese rats. However, it significantly increased α-ENaC and Na-K-2Cl cotransporter levels, and these increases were greater in obese rats. These studies suggest that relatively increased ENaC, but not NCC activity, in obese rats is due to enhanced AT1R activity. CAN attenuated the reduction of several renal transporters in the obese rat kidney. Finally, differences in intrarenal AT1R activity do not seem directly responsible for BP differences between lean and obese rats.

**The leptin receptor-deficient obese Zucker rat (faffa) is grossly obese, markedly insulin resistant, and exhibits modest hypertension.** Several studies have reported increased angiotensin II (ANG II) activity in these rats, relative to their lean littermates, as determined by decreased blood pressure (BP) in response to ANG II receptor antagonism (1), increased BP response to infused ANG II (2), and increased ANG II type 1 receptor (AT1R) binding sites, at least in proximal tubule (3). Our laboratory previously reported increased renal abundance and urine excretion of the thiazide-sensitive Na-K cotransporter (NCC) in obese Zucker rats, relative to lean age-mates (4, 6, 16) and thus hypothesized that increased activity of the renin-angiotensin system (RAS) in these rats might contribute to these changes. However, whether or not NCC, localized in the distal convoluted tubule (DCT), is a direct target of ANG II is not entirely clear.

ANG II receptors have been localized by 125I-labeled binding studies in microdissected renal tubules with the greatest density in proximal tubules (25). However, all major sodium-reabsorptive epithelial cell types, including the thick ascending limb (TAL) and collecting duct, were shown to express binding sites. ANG II receptors fall into two pharmacologically distinct classes: AT1 and AT2 (35). AT1R are further subdivided into AT1A and AT1B. The AT1R activation (in particular the AT1A subtype in kidney) has been associated with increased BP and sodium reabsorption. These receptors have been localized by PCR to the proximal tubule, TAL, and collecting duct (7). ANG II also has been shown to directly regulate ion transport in collecting duct and connecting tubule using micropuncture and patch-clamp analysis (2, 21, 37). Recently, Sandberg and colleagues (29) demonstrated that acute ANG II promotes apical redistribution of NCC in the DCT in rat kidney, which would support direct regulation of NCC in the DCT by ANG II.

ANG II or ANG II antagonism has been demonstrated to regulate proteins, both by increasing the total pool size, i.e., whole cell abundances, and by affecting the proportion of transporters in the apical membrane, i.e., trafficking (4, 8, 15, 20, 36, 39, 40). In chronic studies, both forms of regulation might be expected to occur. Brooks and colleagues (8) showed that mice with knockout of the AT1AR had reduced renal abundance of NCC and the α-subunit of epithelial sodium channel (ENaC), with no measurable change in plasma aldosterone levels, suggesting the abundances of these proteins, found in the distal tubule, were upregulated by ANG II via the AT1R. Yang and colleagues (40) found that chronic enalapril (angiotensin-converting enzyme inhibitor) treatment decreased apical membrane associated sodium-phosphate cotransporter (NaPi-2), sodium/hydrogen exchanger (NHE3), NCC, and Na-K-2Cl cotransporter (NKCC2) in the spontaneously hypertensive rat (SHR), but reduced total cell abundance of only NaPi-2.

With regard to the TAL, Klein et al. (19) reported a decrease in the protein abundance of the primary apical sodium transporter, NKCC2, in rats treated 14 days with ANG II infusion at a rate of 0.75 mg · kg⁻¹ · day⁻¹. On the other hand, Kwon et al. (20) reported an increase in the levels of this protein, with ANG II infusion ranging from 50 to 200 ng/min for 7 days. Finally, Beutler et al. (4) found no effect of candesartan (CAN) treatment on NKCC2 abundance in low-NaCl-fed rats after 2
days of treatment. Thus regulation of TAL NKCC2 levels by the AT\(_{1}\)R is not well understood.

Therefore, this study was designed to determine whether the increased NCC expression and excretion observed in obese Zucker rats was due to relatively increased AT\(_{1}\)R activity. If so, chronic CAN treatment should eliminate these differences. Furthermore, using radiotelemetry, we determined whether BP response was indeed more sensitive to CAN in obese relative to lean Zucker rats. If so, this would support a causative role for elevated RAS in the obese Zucker rat as a determinant of higher BP. We, furthermore, examined the regulation of renal abundance and activity of NKCC2 and ENaC subunits using Western blotting, as well as the natriuretic response to select additional vital sodium reabsorptive proteins, expressed in the post-proximal regions of the renal tubule. Finally, we evaluated the regulation of protein abundance of the major proximal tubule apical sodium transporters, NHE3 and NaPi-2, since the proximal tubule is also a major site for ANG II action in the kidney (7, 14, 25).

METHODS

Animals, study design, and BP monitoring. Thirty-two male Zucker rats (16 lean and 16 obese), were obtained from Charles River Laboratories (Wilmington, MA). Twenty-four were implanted with radiotelemetry transmitters, as previously described (32), to continuously measure BP (Data Sciences International, St. Paul, MN). Rats were housed singly in microfilter top, plastic cages with a normal 12:12-h light-dark cycle, according to protocols approved by the Georgetown Animal Care and Use Committee. After 1 wk of baseline recording (at ~9 wk of age), 8 rats from each body type were randomly assigned to either ground control diet (Purina 5001 Rodent Chow, Purina Mills, St. Louis, MO) solidified in agar with 70% water, or the same base diet with 23.5 mg ground CAN cilexetil (Atacand, AstraZeneca Pharmaceuticals, Wilmington, DE) incorporated per kilogram diet (wet weight) to provide a dose of CAN in the range of 3–5 mg·kg body wt\(^{-1}\)·day\(^{-1}\), similar to oral doses used by other investigators (26, 34, 36). Rats were weighed weekly and fed diets and received water ad libitum for 14 wk. The study length was chosen based on previous studies in which we found this was the time period in which obese Zucker rats progress from insulin resistant to Type 2 diabetic (5, 6, 16, 28). Food intake records were made in metabolic cages for measurement of sodium and urinary proteins (see below).

Natriuretic-response tests. As a measure of NKCC2, ENaC, and NCC activity, sodium excreted in response to single injections (intraperitoneal [ip]) of FUR (12 mg/kg body wt), BNZ (0.7 mg/kg body wt), and hydrochlorothiazide (HCTZ, 3.75 mg/kg body wt) was measured at 10, 11, and 12 wk, respectively. Urine was collected in metabolic cages for 0–3 h and from 3–24 h following administration of each natriuretic agent. Before these tests, a similar natriuretic response test to vehicle (water) ip administered was performed on each rat. Urine was measured for volume and sodium. Net sodium excretion in response to FUR, HCTZ, and BNZ above that particular rat’s response to vehicle was determined and considered an index of the relative in vivo “activity” of NKCC2, NCC, and ENaC.

Kidneys and blood collection. Rats were euthanized by decapitation after being kept on diet for 14 wk. Trunk blood was collected into both K\(_2\)-EDTA- and Na\(^{+}\)-heparin-containing vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ). The right and left kidneys were rapidly removed. The left kidney was processed as a whole kidney homogenate (WKH) for immunoblotting. The right kidney was dissected by a razor blade and sharp-curved scissors into inner medulla and processed as a whole cell homogenate (IMH; white inner portion with papilla), inner stripe of the outer medulla (OMH; dark red ring outside inner medulla), and cortex (CTXH; tan, majority of kidney). The WKH and CTXH were homogenized in 10-mL isolation buffer, and the OMH and IMH in 1 mL. Protein concentrations of each sample were determined by bichinoninic acid determination (Pierce).

Plasma and urine analyses. Aldosterone and renin activity were analyzed in blood collected at euthanasia by RIA kits, as previously described (5, 31). Urine (at 7 wk) was collected for measures of urine NCC and NKCC2. Twenty-four-hour urine was collected into 15 mL of ice-cold 1 M Tris·Cl (pH 6.8) containing 1 mg/ml leupeptin, 1 mM sodium azide, and 0.1 mg/ml phenylmethylsulfonfluoride, as previously described (24). Exactly one-sixth of the day’s collected volume from each rat was brought up to 10 mL with distilled water and centrifuged at 1,000 g for 5 min to spin down whole cells, which were discarded. The supernatants were then spun at 200,000 g for 120 min to obtain a protein pellet. Pellets were resuspended in 150–\(\mu\)L isolation solution and heated to 60°C for 15 min after the addition of 50 \(\mu\)L of 4× Laemmli sample buffer. After solubilization, 30 \(\mu\)L (1/30th of the day’s total volume) were loaded in each lane and probed with our primary antibodies against NCC and NKCC2 (see below).

Immunoblotting of kidney. Kidney samples of whole left kidney (WKH), cortex (CTXH), outer medulla (OMH), and/or inner medulla (IMH) from the right kidney were immunoblotted for NHE3, NaPi-2, NCC, NKCC2, and the three subunits of ENaC, according to previously defined protocols (10), using our own rabbit polyclonal antibodies against sequences previously described (17, 18, 23). Equal amounts of total protein from each kidney or region were loaded in each lane from each rat.

Correction for renal hypertrophy. Western blotting semiquantitative determination of renal protein levels in rats can be confounded by renal hypertrophy [see our laboratory’s previous findings (5)]. Hypertrophy may include tubule and nontubule proteins; the relative increase in each is not clear. These additional proteins could potentially be dilutional, where total transporter levels per epithelial cell may not be affected. Therefore, WKH blots were statistically evaluated in two ways: 1) normalized band densities were compared relative to total protein loaded (conventional approach); and 2) absolute band densities were multiplied by a correction factor, which considered increased (or decreased) total protein in the kidney. The correction factor was determined by first quantifying total protein in the left kidney from each rat by multiplying protein concentration times the dilution volume (10 mL of isolation buffer). The average total amount of protein was calculated for the lean control rats and set to 100%. Total protein determined for each rat for their left kidneys was converted to a percentage of that found in the average of the lean control, e.g., 317 mg of total protein (obese control rat 4) = 117%. Thus band densities for obese control rat 4 were multiplied by 1.17 to determine the “absolute” values. Our laboratory has utilized this method previously (5).

Statistics. Most data were analyzed by two-way (body type × treatment) ANOVA (Sigma Stat, Chicago, IL). One-way ANOVA was also performed and followed by Tukey’s or Dunn’s multiple-comparison test when the ANOVA was significant to determine differences between individual pairs of means. BP data were analyzed by three-way repeated-measures ANOVA (body type × treatment × time; NCSS, Kaysville, UT). \(P < 0.05\) was considered significant for all comparisons.

RESULTS

CAN reduced renal hypertrophy and increased plasma renin activity. As expected, obese rats ate more food and were significantly heavier at the end of the study (Table 1). Fourteen
drug administration (left)
tion was measured. When considering the first 3 h following
approximated 3.0 –3.4 mg
records, was not significantly different between body types and
lean or obese Zucker rats. CAN dose, estimated by food intake
weeks of CAN treatment did not affect body weights in either
between treatments or body types.
activity was significantly increased by CAN and not affected
was markedly attenuated with CAN treatment. Plasma renin
significantly larger in the obese control rats, and this increase
in both lean and obese body types (Fig. 1). Nonetheless,
stabilized 20 –35 mmHg lower than control by the second week
three-way repeated measures indicated obese rats did have
in both lean and obese body types (Fig. 1). Nonetheless,
Factors
Body type <0.001 0.21 (by unpaired t-test) <0.001 <0.001 0.40 0.61
Treatment 0.39 0.28 0.032 <0.001 0.44
Interaction 0.59 0.46 0.061 0.52 0.67

Values are means ± SE; n = 7 or 8/group. The 24-h dry food intake and calculated candesartan (CAN) dose were measured three times at 9, 10, and 11 wk. Plasma renin activity is generated angiotensin I. **Results of Tukey’s multiple-comparison test following a significant one-way ANOVA (** is highest mean, followed by †). Means with symbols in common are not different from each other. P values in boldface are <0.05 by two-way ANOVA (significant).

weeks of CAN treatment did not affect body weights in either
lean or obese Zucker rats. CAN dose, estimated by food intake
records, was not significantly different between body types and
approximated 3.0 –3.4 mg·kg·body wt⁻¹·day⁻¹. Kidneys were
significantly larger in the obese control rats, and this increase
was markedly attenuated with CAN treatment. Plasma renin
activity was significantly increased by CAN and not affected
by body type, whereas plasma aldosterone was not different
between treatments or body types.

CAN markedly reduced BP in lean and obese rats. BP fell by
15–20 mmHg within the first week of CAN treatment and
stabilized 20–35 mmHg lower than control by the second week
in both lean and obese body types (Fig. 1). Nonetheless,
three-way repeated measures indicated obese rats did have
significantly higher mean arterial pressure over the course of
the study, regardless of treatment. BP change from baseline
(Fig. 1B) was greater in CAN-treated rats and not affected by
body type. Thus obese rats did not appear to have a more
sensitive BP response to CAN than did lean rats.

CAN reduces 24-h natriuretic response to BNZ, ENaC
antagonist. As an index of relative in vivo activity of NKCC2,
NCC, and ENaC, rats were treated with single doses of FUR
(Fig. 2A), HCTZ (Fig. 2B), and BNZ (Fig. 2C), respectively, at
different times, as described in METHODS. Urine sodium excretion was measured. When considering the first 3 h following drug administration (left bars), it was clear that FUR was the strongest natriuretic agent, as evident by the greatest degree of natriuresis, and that obese rats excreted significantly more sodium (above their own baselines) than did lean rats in response to all agents. Furthermore, CAN significantly blunted early natriuretic response to FUR and HCTZ and showed a strong trend (P = 0.075) for BNZ.

However, because we were concerned that the fall in BP with CAN might independently blunt or delay natriuretic response to these agents, we also examined net sodium excretion over the entire 24 h following the administration of the drugs (Fig. 2, right bars). In this case, a different pattern emerged. Natriuretic response to FUR was significantly increased by CAN (two-way ANOVA), especially in obese rats. Natriuretic response to HCTZ was decreased by CAN in lean rats, but increased in obese rats, giving a significant interaction by two-way ANOVA. The response to BNZ was qualitatively unchanged; however, a much greater difference between lean
and obese rats emerged, with obese rats excreting over 10×
more sodium (above their own baselines) in response to BNZ,
relative to lean. Chronic CAN cut in half sodium excretion in
response to BNZ in obese rats, but it was still 5× higher than
that in lean rats.
Hypertrophy reflects increased total renal protein in obese rats. Total renal protein per kidney was increased in obese rats. CAN reduced this increase substantially, but did not affect total protein in lean rats. Protein levels were as follows (%lean control): 1.00 ± 0.059 (lean control); 1.00 ± 0.080 (lean CAN); 1.37 ± 0.057 (obese control), and 1.18 ± 0.084 (obese CAN). The ratio of protein to kidney weight was also calculated and was found to be decreased in obese rats and increased some with CAN treatment. This ratio was as follows (mg protein/g kidney wet wt): 212 ± 16 (lean control), 217 ± 13 (lean CAN), 167 ± 13 (obese control), and 176 ± 6 (obese CAN); P = 0.004 for body type.

NCC is decreased by CAN. Renal NCC protein levels were variable, especially in the CAN-treated obese rats (Fig. 3). However, two-way ANOVA indicated a significant effect of CAN to reduce the abundance of this protein in WKH, regardless of whether data were expressed in the normalized format or as absolute numbers (see METHODS). The same was true for cortex homogenates derived from the right kidney of the same animals (Table 2). Body type did not affect sensitivity to CAN. No significant differences between individual groups were found by one-way ANOVA.

NKCC2 is increased by CAN. In agreement with increased 24-h natriuretic response to FUR, CAN led to a marked increase in WKH protein levels of NKCC2 (Fig. 4) in both lean and obese rats. This effect was present in the cortex (CTXH) and inner stripe of the outer medulla (OMH), as well (Tables 2 and 3). For NKCC2, there was a strong, significant interactive term with two-way ANOVA, indicating the obese rats responded to the CAN with a greater relative increase in NKCC2 than did lean rats.

α-ENaC is increased by CAN. Similarly, two-way ANOVA indicated that the abundance of α-ENaC in whole kidney was
considered. However, examination of regional expression especially when hypertrophy (absolute levels, rats.

In WKH, -ENaC, but only in obese rats.

Table 2. Cortex homogenate densitometry summary (% of lean control)

<table>
<thead>
<tr>
<th>Factors</th>
<th>NCC</th>
<th>NKCC2</th>
<th>α-ENaC</th>
<th>β-ENaC</th>
<th>γ-ENaC (85-kDa Band)</th>
<th>γ-ENaC (70-kDa Band)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Lean control</td>
<td>100±11*</td>
<td>100±20†‡</td>
<td>100±19*</td>
<td>100±7*</td>
<td>100±6*</td>
<td>100±6</td>
</tr>
<tr>
<td>Lean CAN</td>
<td>48±2‡</td>
<td>187±32†‡</td>
<td>83±9†‡</td>
<td>70±3†‡</td>
<td>93±5†‡</td>
<td>78±4</td>
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<tr>
<td>Obese control</td>
<td>48±4*†‡</td>
<td>82±12†‡</td>
<td>31±4†‡</td>
<td>55±8†‡</td>
<td>71±6*†‡</td>
<td>69±5</td>
</tr>
<tr>
<td>Obese CAN</td>
<td>52±13**‡</td>
<td>232±41*</td>
<td>116±24*</td>
<td>49±6†‡</td>
<td>62±13†‡</td>
<td>69±7</td>
</tr>
</tbody>
</table>

Results of two-way ANOVA for above parameters (P values)

Values are means ± SE in %lean control; n = 6 for lean control, lean CAN, and obese control, and n = 5 for obese CAN. NCC, Na-Cl cotransporter; NKCC2, Na-K-2Cl cotransporter; ENaC, epithelial sodium channel. *†‡Results of Tukey’s multiple-comparison test following a significant one-way ANOVA (* is highest mean, followed by † and ‡). Means with symbols in common are not different from each other. P values in boldface are <0.05 by two-way ANOVA (significant).

increased by CAN (Fig. 5); furthermore, the increase was significantly greater in obese rats (significant interactive term). When CTXH, OMH, and IMH from the right kidney were evaluated separately (Tables 2–4), CAN led to increased α-ENaC, but only in obese rats.

β-ENaC is differentially regulated by CAN in lean and obese rats. In WKH, β-ENaC abundance was significantly higher in obese rats, relative to lean (Fig. 6) by two-way ANOVA, especially when hypertrophy (absolute levels, right bars) was considered. However, examination of regional expression

Fig. 4. NKCC2 protein. A: representative immunoblots of WKH and outer medullary homogenate (OMH) with a different rat’s sample in each lane (n = 6 per group, except for obese CAN, where n = 5). Coomassie-stained loading gels were used to evaluate accuracy of protein loading. B: densitometry summary of WKH expressed as normalized by total protein (left) or absolute protein levels (right). A,BMeans with letters in common are not significantly different from each other by Tukey’s multiple-comparisons test following a significant one-way ANOVA (“A” is assigned to the highest mean and significantly different from “B”, but not from “AB”). Two-way ANOVA P values are displayed below bar graph; boldface highlights a significant (<0.05) P value. CAN significantly increased NKCC2 in lean and obese rats, an effect that was significantly greater in obese rats.

Table 3. Outer medullary homogenate densitometry summary (% of lean control)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NCC</th>
<th>NKCC2</th>
<th>α-ENaC</th>
<th>β-ENaC</th>
<th>γ-ENaC (85-kDa Band)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean control</td>
<td>100±16†‡</td>
<td>100±12†‡</td>
<td>100±14†‡</td>
<td>100±13</td>
<td></td>
</tr>
<tr>
<td>Lean CAN</td>
<td>191±11*</td>
<td>99±9*†</td>
<td>80±19†‡</td>
<td>77±9</td>
<td></td>
</tr>
<tr>
<td>Obese control</td>
<td>26±10†</td>
<td>36±13†</td>
<td>74±16†‡</td>
<td>94±15</td>
<td></td>
</tr>
<tr>
<td>Obese CAN</td>
<td>119±26†</td>
<td>158±10*</td>
<td>194±28*</td>
<td>116±14</td>
<td></td>
</tr>
</tbody>
</table>

Results of two-way ANOVA for above parameters (P values)

Values are means ± SE in %lean control; n = 6 for lean control, lean CAN, and obese control, and n = 5 for obese CAN. *†‡Results of Tukey’s multiple-comparison test following a significant one-way ANOVA (* is highest mean, followed by † and ‡). Means with symbols in common are not different from each other. P values in boldface are <0.05 by two-way ANOVA (significant).
obese rats (a significant interactive term), so that body type differences no longer existed.

Comparison of protein changes to diuretic responses. Qualitative changes in the whole kidney protein levels due to obesity and CAN are compared with diuretic responses in Fig. 9. Untreated obese rats had relatively lower levels of sodium transporters expressed in the proximal tubule and TAL. The greater acute response to FUR in obese rats suggested maintenance of active TAL NKCC2 in these rats, despite differences no longer existed.

Trykey’s multiple-comparisons test following a significant one-way ANOVA ("A" is assigned to the highest mean and significantly different from "B", but not from "AB"). Two-way ANOVA P values are displayed below bar graph; boldface highlights a significant (<0.05) P value. CAN increased α-ENaC, an effect that was significantly greater in obese rats.

proximal tubule diuretics. In the distal tubule, increases or no changes were observed for most proteins with obesity. This, in general, matched with increased responses to both HCTZ and BNZ acutely and BNZ chronically.

The effects of CAN on protein levels and diuretic responses are separately evaluated in Fig. 10. CAN reduced acute response to all three diuretics in both lean and obese rats. This matched with decreased NCC and β- and γ-ENaC (both bands) in lean rats and with decreased NCC and the 70-kDa band of γ-ENaC in obese rats. However, NKCC2 abundance was increased by CAN in both lean and obese rats. This matched only with the chronic response to FUR in obese rats. Thus overall NKCC2 and α-ENaC abundances matched least with expected activity, whereas the 70-kDa band for γ-ENaC, β-ENaC, and NCC matched fairly closely.

Reduction in urine excretion of NKCC2 and NCC in lean and CAN-treated rats. Urine excretion of NKCC2 and NCC was measured to determine whether they would correlate with activity or renal expression of the proteins. Obese rats excreted relatively much higher amounts of NCC and NKCC2 in their urine (Fig. 10). Band densities of the upper molecular mass (320 kDa) bands associated with both proteins were measured. This excretion was substantially attenuated by treatment with CAN. Therefore, urine excretion of NCC and NKCC2 in obese rats did not positively correlate with renal levels or estimated activity, but was higher in the rats with greater renal hypertro-

Table 4. Inner medullary homogenate densitometry summary (% of lean control)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>α-ENaC</th>
<th>β-ENaC</th>
<th>γ-ENaC (85-kDa Band)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean control</td>
<td>100±18</td>
<td>100±16</td>
<td>100±22†</td>
</tr>
<tr>
<td>Lean CAN</td>
<td>86±10</td>
<td>62±14</td>
<td>115±22†</td>
</tr>
<tr>
<td>Obese control</td>
<td>116±12</td>
<td>108±11†</td>
<td>167±12†</td>
</tr>
<tr>
<td>Obese CAN</td>
<td>140±20</td>
<td>147±17*</td>
<td>190±28*</td>
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</table>

Results of two-way ANOVA for above parameters (P values)

<table>
<thead>
<tr>
<th>Factors</th>
<th>P value</th>
<th>P value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body type</td>
<td>0.036†</td>
<td>0.005†</td>
<td>0.016</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.74</td>
<td>0.97</td>
<td>0.037†</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.23</td>
<td>0.017</td>
<td>0.074</td>
</tr>
</tbody>
</table>

Values are mean ± SE in %lean control; n = 6 for lean control, lean CAN, and obese control; and n = 5 for obese CAN. †Results of Tukey’s multiple-comparisons test following a significant one-way ANOVA (* is the highest mean, followed by †). Means with symbols in common are not significantly different from each other. P values in boldface are <0.05 by two-way ANOVA (significant).
DISCUSSION

Elevated intrarenal activity of the RAS may be an important determinant of sodium retention and BP in obesity, even in the absence of measurable increases in systemic RAS activity, e.g., plasma renin activity (1, 11, 22, 30). In fact, our laboratory and others have reported unchanged or even decreased plasma renin activity in obese vs. lean Zucker rats (1, 16). Nonetheless, numerous reports suggest increased sensitivity of a variety of physiological parameters to infused ANG II, AT1R antagonists, and angiotensin-converting enzyme inhibitors in the obese vs. lean Zucker rat (1, 3, 9, 12, 38).

In this study, we evaluated the effects of chronic CAN treatment on BP, natriuretic responses to select sodium transporter/channel antagonists, sodium transporter and channel subunit protein expression, and excretion in obese vs. lean Zucker rats. Previously, our laboratory showed increased renal levels of the thiazide-sensitive NCC in young (2–4 mo old), obese Zucker rats relative to lean age-mates (6, 16). This difference was attenuated as rats aged and renal hypertrophy developed (5). Here we show that CAN was able to significantly reduce renal NCC and natriuretic response to HCTZ, but it did so in both lean and obese rats, and the reduction was no greater in the obese. Moreover, chronic CAN treatment could not reduce the relatively elevated 24-h natriuretic response to HCTZ in the obese rats. Taken together, these findings suggest that NCC protein levels may indeed be directly upregulated by AT1R activity; however, they also suggest that the relatively increased expression and/or activity in the obese vs. the lean Zucker rat cannot be explained by increased AT1R activity. On the other hand, ENaC activity, as determined by natriuretic response to BNZ, was markedly increased in the obese rats (over 10-fold) and decreased by CAN. These studies are the first to demonstrate an increase in ENaC activity in the obese vs. lean Zucker rat, by any measure. Previously, our laboratory demonstrated increased α2ENaC abundance in young Zucker rats (23, 24). Here we show that chronic CAN treatment could not reduce the relatively elevated α2ENaC abundance in the obese Zucker rats.

Fig. 7. γ-ENaC protein. A: representative immunoblots of WKH, OMH, and IMH with a different rat’s sample in each lane (n = 6 per group, except for obese CAN, where n = 5). Coomassie-stained loading gels were used to evaluate accuracy of protein loading. Densitometry summary of 85-kDa band (B) and 70-kDa band (C) in WKH expressed as normalized by total protein (left) or absolute protein levels (right). A,B,CMeans with letters in common are not significantly different from each other by Tukey’s multiple-comparisons test following a significant one-way ANOVA (“A” is assigned to the highest mean and significantly different from “B”, but not from “AB”). Two-way ANOVA P values are displayed below bar graph; boldface highlights a significant (<0.05) P value. CAN significantly decreased the 70-kDa band in lean and obese rats.

Fig. 8. Sodium-phosphate cotransporter (NaPi-2) and sodium/hydrogen exchanger (NHE3) proteins. A: representative immunoblots of cortex homogenate (CTXH) NaPi-2 (top) and NHE3 (bottom) with a different rat’s sample in each lane (n = 6 per group, except for obese CAN, where n = 5). Coomassie-stained loading gels were used to evaluate accuracy of protein loading. B: densitometry summary of NaPi-2 and NHE3 expressed as normalized by total protein. A,B,CMeans with letters in common are not significantly different from each other by Tukey’s multiple-comparisons test following a significant one-way ANOVA (“A” is assigned to the highest mean and significantly different from “B”, but not from “AB”). Two-way ANOVA P values are displayed below bar graph; boldface highlights a significant (<0.05) P value. Obese rats had significantly less NaPi-2. CAN reduced NHE3 in lean, but increased it in obese rats.
young obese rats (6) and an increase in all three subunits when aldosterone levels were clamped (27). Here we showed, with obesity, an increase in \(\alpha\)-ENaC (both bands), but no increase in \(\gamma\)-ENaC. Chronic CAN reduced renal levels of \(\beta\)- and \(\gamma\)-ENaC, but mainly in lean rats. In fact, \(\alpha\)-ENaC was substantially increased by CAN in obese rats. These findings were somewhat different than those reported by Beutler and associates (4) with regard to the regulation of ENaC subunits by ANG II. In their studies, \(\gamma\)-ENaC abundance was decreased by CAN, and \(\alpha\)- and \(\beta\)-ENaC were relatively increased. Significant differences between their studies and ours include that they were examining a model with high plasma renin activity, i.e., normal rats fed a low-NaCl diet, and our study was considerably longer, i.e., 14 wk vs. 2 days. Yang et al. (40) treated SHR rats for 4 wk with enalapril and found a significant decrease in \(\alpha\)-ENaC, in agreement with our findings for lean rats, but no changes in \(\beta\)- or \(\gamma\)-ENaC. However, the SHR has much higher BP to start with, compared with the obese Zucker rat, and enalapril will reduce ANG II levels and thus activity at both AT1R and AT2R, both which may have contributed to differences between these studies. Finally, we suspect that the differential responses in lean vs. obese rats to CAN therapy, with regard to protein levels of the ENaC subunits, likely had to do with the renal restorative aspects of the CAN therapy in obese rats, which are discussed in a subsequent paragraph.

Our BP data indicate that AT1R activity is important in the maintenance of normal BP in lean and obese rats, as BP fell dramatically in both body types. This was in agreement with Alonso-Galicia et al. (1), who used losartan rather than CAN. However, using radiotelemetry, we did not find evidence of a significantly greater fall in BP in the obese rats. In fact, CAN did not completely abolish the BP difference between lean and obese (Fig. 1A), suggesting that another factor may be responsible for this difference. One possible candidate of several is insulin. We have shown that treatment with an insulin-sensitizing agent, rosiglitazone, a peroxisome proliferator activated receptor (subtype \(\gamma\); PPAR-\(\gamma\)), reduces circulating insulin and nearly normalizes BP in obese Zucker rats. However, PPAR-\(\gamma\) agonists are associated with a plethora of effects, and additional studies will be needed to examine this more thoroughly.

We found a marked increase in NKCC2 in the cortex and medulla of rats treated with CAN. This included the lean control rats on our standard 1% NaCl diet, which would not have been expected to have significantly elevated activity of ANG II. This agreed with, and was the reciprocal of, the findings of Klein et al. (19) of reduced NKCC2 in ANG II-treated rats. This group found that raising BP by another means, i.e., norepinephrine administration, also resulted in downregulation of NKCC2, suggesting that this was not a direct effect of ANG II, but of elevated BP. Thus we suspect...
that NKCC2 upregulation in our rats may be a compensatory means to try to increase BP and maintain sodium balance in these rats. We believe that our rats were in sodium balance due to the chronic nature of our experiment and the fact that body weight was not affected by CAN treatment.

It is likely that proximal tubule and post-macula densa sodium reabsorption were decreased; thus the TAL may be the only site able to compensate. Indeed, CAN significantly reduced the proximal tubule sodium transporters NHE3 and NaPi-2 in lean and obese rats, respectively. Decreased NaPi-2 with CAN agreed with the findings of Yang et al. (40) in the SHR treated with enalapril. Despite TAL compensation, BP was severely compromised. Another possible reason for NKCC2 downregulation could be that ANG II binds to AT2R was severely compromised. Another possible reason for NKCC2 downregulation could be that ANG II binds to AT2R in TAL, due to blockade of AT1R, and that this may decrease NKCC2 expression. Further studies will be needed to better elucidate regulation of NKCC2 by ANG II.

It is possible that our results might have been different had we used younger rats without the confounding influence of hypertrophy. Our laboratory has found previously that hypertrophy of the kidneys results in a general downregulation of many transporter and channel proteins, with some proteins clearly more sensitive than others (5, 28). CAN therapy did a superb job in preventing hypertrophy and protein downregulation, and, in some cases, increased affected proteins to levels higher than in the lean, e.g., α-ENaC and medullary β-ENaC. We are fairly certain that CAN prevents the development of hypertrophy, rather than reverses existing hypertrophy, due to our observation of kidney size in younger 4-mo-old Zucker rats (6). In those studies, kidney weights were not significantly different between lean and obese rats. Proteins particularly sensitive to downregulation in the obese rats were NKCC2, α-ENaC, and NHE3. Thus we believe that there is likely elevated intrarenal activity of the RAS in the obese rats, and that it plays a strong contributory role in the downregulation of these proteins. This agrees with what Jensen and colleagues (15) have reported in another pathological model, ureteral obstruction, and also in agreement with what our laboratory has shown previously (5, 28). On the other hand, NHE3 was significantly downregulated in lean rats treated with CAN, in agreement with anti-natriuretic action of ANG II through the AT1R in the proximal tubule. Therefore, the change in NHE3 may be primarily dependent on different factors in lean and obese rats. In the obese rats, AT1R blockade may be more important in preventing renal disease, which leads to downregulation of NHE3. In lean rats, there is no renal disease, so the direct effect of AT1R blockade on NHE3 can be observed. By what means CAN is protective is not entirely clear. One possibility is by reducing inflammatory pathways. Recently, Topcu et al. (36) demonstrated increased renal NF-κB expression in rats with ureteral obstruction that was preventable by CAN. This represents one possible mechanism. Our laboratory (28) has also reported similar “protection” of these proteins in obese rats treated with rosiglitazone, a PPAR-γ agonist. A microarray analysis of kidney genes showed that tumor necrosis factor-α, which is directly upstream of NF-κB, was a major locus of significantly altered mRNA expression pathways in obese rats in response to rosiglitazone (33). Regardless of the mechanism, preservation of the expression level of these proteins may be vital in maintenance of normal salt and water homeostasis.

Overall, these studies show a clear protective effect of CAN, independent of body weight, in the obese Zucker rat to preserve the expression of several renal transporters and reduce renal hypertrophy. This confirms other reports of altered RAS activity in these animals. However, these data do not support a role for increased AT1R in determining the BP differences between the lean and obese rats, since BP was substantially, but not differentially, reduced in both body types by CAN. Nonetheless, overactivity of NCC and ENaC in obese rats may still have a role in BP differences, but likely due to mechanisms other than increased AT1R activity.

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REFERENCES

AT1R blockade in the Zucker rat


