Kidney

Angiotensin II Type 2–Receptor Agonist C21 Reduces Proteinuria and Oxidative Stress in Kidney of High-Salt–Fed Obese Zucker Rats

Sanket N. Patel, Quaisar Ali, Tahir Hussain

Abstract—Oxidative and nitrosative stress have been implicated in high-sodium diet (HSD)–related hypertensive renal injury. In this study, we investigated angiotensin II type 2–receptor–mediated renoprotection in obese Zucker rats fed HSD. Obese Zucker rats were fed normal sodium diet or HSD 4%, for 14 days, with/without angiotensin II type 2–receptor agonist C21, delivered subcutaneously via osmotic pump, 1 mg/kg per day. Compared with normal sodium diet controls, HSD rats exhibited increase in cortical nicotinamide adenine dinucleotide phosphate oxidase activity, urinary H₂O₂, and 8-isoprostanestes, which were associated with severe glomerulosclerosis, interstitial fibrosis, decline in estimated glomerular filtration rate, and an increase in urinary leak and activity of N-acetyl-β-D-glucosaminidase, a lysosomal enzyme and a marker of tubular damage. These changes were improved by C21 treatment. Cortical expression of endothelial nitric oxide synthase, phospo-endothelial nitric oxide synthase (Ser1177), and plasma nitrites were reduced after HSD intake, whereas nitrosative stress (3-nitrotyrosine) and enzymatic defense (superoxide dismutase-to-catalase activity) remained unaltered. However, C21 preserved plasma nitrites in HSD-fed obese Zucker rat. C21 treatment reduced protein-to-creatinine, albumin-to-creatinine, as well as fractional excretion of protein and albumin in HSD-fed obese Zucker rat, which is independent of changes in protein recycling receptors, megalin, and cubilin. HSD intake also altered renal excretory and reabsorptive capacity as evident by elevated plasma urea nitrogen-to-creatinine and fractional excretion of urea nitrogen, and reduced urine-to-plasma creatinine, which were modestly, but insignificantly, improved by C21 treatment. Together results demonstrate that angiotensin II type 2–receptor activation protects against HSD-induced kidney damage in obesity plausibly by reducing nicotinamide adenine dinucleotide phosphate oxidase activity and rescuing nitrites. (Hypertension. 2016;67:906-915. DOI: 10.1161/HYPERTENSIONAHA.115.06881.)

Online Data Supplement

Key Words: AT₂ receptor ■ NADPH oxidase ■ oxidative stress ■ rats, Zucker

Hyperactive local angiotensin (Ang) II type 1 receptor (AT₁R) signaling in kidney can operate independently of a systemic renin angiotensin system and is a key player in pathogenesis of progressive hypertensive chronic kidney diseases (CKD).¹–¹¹ Recent analysis revealed that much of continuing increase in number of patients with CKD is seen in obese hypertensive patients.⁴ Various animal models have been used to gain more insight into the pathogenesis of CKD associated with obesity hypertension. One of the validated models is the obese Zucker rat (OZR). These rats may or may not be hypertensive as such, but exhibit gradual increase in blood pressure⁵ and classical morphological characteristics of nonimmune-spontaneous focal segmental glomerulosclerosis just in 14 days when fed high-sodium diet (HSD; 4%).⁶ HSD intake also increased renal content of Ang-II in obese rats.⁵⁷ Lavaud et al⁸ and others⁹,¹⁰ held nonhemodynamic factors responsible for this event, because glomerular hemodynamic function remained unchanged in obese and lean Zucker rat. Experimental and clinical reports point at essential events associated with HSD-related pathologies of hypertension and CKD: oxidative¹¹–¹³ and nitrosative stress and renal immune cell infiltration.

Progression of CKD is predicted by (1) glomerular filtration rate (GFR), (2) fractional excretion of protein (FEₚro) and albumin (FEₜₐl), and (3) an absolute comparison of urinary albumin-to-protein ratio. Under normal conditions, the glomerulus filters significant protein, most of which is retrieved by tubular receptors (megalin and cubilin), processed through lysosomal pathway in amino acids which are delivered back into circulation. Thus, under normal circumstances, protein remains undetected in urine. FEₚro, FEₜₐl, and urinary albumin-to-protein ratio have reliable predictive values in characterizing renal damage, glomerular (albuminuria>proteinuria), or tubular (proteinuria>albuminuria), because it represents glomerular...
filtration function with increased glomerular permeability. In addition to these indices, determination of urinary activity of N-acetyl-β-d-glucosaminidase (NAG), one of the best characterized tubular lysosomal enzyme helps distinguish renal tubular injury. Their comprehensive assessment in OZR model of salt-induced kidney damage has not been reported. Renal AT₉R, while sparsely expressed in healthy tissues, is upregulated in obesity, diabetes mellitus, and tissue damage and considered endogenous defense by virtue of its own actions and by antagonizing AT₁R-mediated deleterious actions. Recently, AT₉R activation by preferential agonists C21 and CGP42122A has showed natriuretic and blood pressure reducing effects, as well as GFR improvement in HSD-fed obese rats. AT₉R also have anti-inflammatory, antiproliferative, and antifibrotic effects, which are important in preventing disease progression. Also, there is indication that AT₉Rs may be involved in antioxidative stress activities. Pharmacological inhibition of AT₉R by PD123319 has shown to increase oxidative stress in vitro and in vivo. AT₉R stimulation by CGP42112 caused a decrease in (rotenone+Ang-II)–induced nitric oxide adenine dinucleotide phosphate oxidase (NOX) activity and mRNA expression of NOX units in in vitro CATH animal model of Parkinson disease. Recently, C21, which is a nonpeptide orally active AT₉R agonist, has been shown to reduce superoxide formation in cryo-sections of aorta of stroke-prone spontaneously hypertensive rats and frozen sections of ischemic cerebral hemisphere. However, in study of Rehman et al, C21 did not reduce NOX activity. It is unknown whether direct AT₉R stimulation by a preferential agonist reduces oxidative stress and protects against salt-induced kidney damage in obesity. Therefore, this study investigated the role of AT₉R selective activation by C21 in oxidative stress and renoprotection in terms of kidney structure and function in high-salt–fed OZRs.

### Methods

#### Experimental Protocol

Male OZRs (12–14 weeks) were purchased from Harlan, Indianapolis, IN. After arrival, the animals were housed in the University of Houston animal care facility. Animal experimental protocols used in this study were approved by the IACUC at the University of Houston and adhere to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Animals were treated with AT₁R agonist C21 (1 mg/kg per day) subcutaneously via Alzet osmotic pump (Model 2ML2; Durect Corporation, Cupertino, CA) while maintained on either normal (0.4%) or HSD (4%) for 14 days. In this study, the subcutaneous delivery rate of 0.42 μg/min (or 605 μg/d) was chosen to maintain the daily dose of C21 (1 mg/kg per day) as we reported earlier. This dose of C21 is considered AT₉R selective as another study from our laboratory showed that C21 (1 and 5 μg/kg per minute) IV–induced natriuresis was blocked by AT₁R antagonist PD123319. Control obese rats (CT) were implanted with osmotic pumps filled with normal sterile saline while maintained on either NSD or HSD for 14 days. Each of the groups had n=8 animals. Rats were placed in metabolic cages during terminal 4 days for urine collection for the last 3 days. Twenty-four–hour food and water intake was recorded. After treatment, blood was collected via cardiac puncture, processed for plasma preparation, and stored at –80°C. Kidney cortices were snap-frozen and stored at –80°C. The kidney cortices were homogenized in the buffer containing (in mmol/L) Tris 50, EDTA 10, PMSF 1, and protease inhibitors. Details on various assay methods are available in the online-only Data Supplement.

#### Statistical Analysis

Data are presented as mean±SEM. The data were analyzed using GraphPad Prism 5 and subjected to 1-way ANOVA with Bonferroni multiple comparison post hoc test, P<0.05, n=8 per group.

#### Results

### General Parameters

In comparison with obese control rats, HSD-fed rats drank more water (HSD: 32.96±3.69 versus CT: 23.54±2.1 mL/d) and exhibited marked diuresis (HSD: 16.33±1.78 versus CT: 8.96±0.88 mL/d).

### Methods

#### Experimental Protocol

Male OZRs (12–14 weeks) were purchased from Harlan, Indianapolis, IN. After arrival, the animals were housed in the University of Houston animal care facility. Animal experimental protocols used in this study were approved by the IACUC at the University of Houston and adhere to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Animals were treated with AT₁R agonist C21 (1 mg/kg per day) subcutaneously via Alzet osmotic pump (Model 2ML2; Durect Corporation, Cupertino, CA) while maintained on either normal (0.4%) or HSD (4%) for 14 days. In this study, the subcutaneous delivery rate of 0.42 μg/min (or 605 μg/d) was chosen to maintain the daily dose of C21 (1 mg/kg per day) as we reported earlier. This dose of C21 is considered AT₉R selective as another study from our laboratory showed that C21 (1 and 5 μg/kg per minute) IV–induced natriuresis was blocked by AT₁R antagonist PD123319. Control obese rats (CT) were implanted with osmotic pumps filled with normal sterile saline while maintained on either NSD or HSD for 14 days. Each of the groups had n=8 animals. Rats were placed in metabolic cages during terminal 4 days for urine collection for the last 3 days. Twenty-four–hour food and water intake was recorded. After treatment, blood was collected via cardiac puncture, processed for plasma preparation, and stored at –80°C. Kidney cortices were snap-frozen and stored at –80°C. The kidney cortices were homogenized in the buffer containing (in mmol/L) Tris 50, EDTA 10, PMSF 1, and protease inhibitors. Details on various assay methods are available in the online-only Data Supplement.

#### Statistical Analysis

Data are presented as mean±SEM. The data were analyzed using GraphPad Prism 5 and subjected to 1-way ANOVA with Bonferroni multiple comparison post hoc test, P<0.05, n=8 per group.

#### Results

### General Parameters

In comparison with obese control rats, HSD-fed rats drank more water (HSD: 32.96±3.69 versus CT: 23.54±2.1 mL/d) and exhibited marked diuresis (HSD: 16.33±1.78 versus CT: 8.96±0.88 mL/d).
8.96±0.88 mL/d). C21 treatment did not affect water consumption and diuresis in rats fed with either NSD or HSD. Food consumption, body weight, and changes in total kidney weight of all animal groups remained unchanged during 2-week study period (Table 1). No animals died during the study.

Indices of Oxidative Stress In Vivo

HSD feeding significantly increased the activity of NOX, a major superoxide radical anion (O$_2^-$)--producing enzyme (HSD: 0.37±0.07 versus CT: 0.13±0.04 ΔRLU/μg protein per minute), which was reduced by C21 in HSD-fed rats (HSD+C21: 0.11±0.04 ΔRLU/μg protein per minute), but not in NSD-fed control rats (Figure 1A). Renal activities of superoxide dismutase, superoxide dismutating enzyme (Figure 1B), and catalase, H$_2$O$_2$ metabolizing enzyme (Figure 1C), remained unchanged. Mean plasma content of H$_2$O$_2$ (HSD: 323.80±25.85 versus CT: 173.20±38.58 μmol/L per milliliter; Figure 1D) and urinary excretion of H$_2$O$_2$ (HSD: 41.70±4.72 versus CT: 23.01±2.13 μmol/L per milligram Cr (creatinine); Figure 1E) were increased in HSD-fed rats. Cotreatment with C21 reduced urinary (HSD+C21: 30.51±3.00 μmol/L per milligram Cr, but not plasma content of H$_2$O$_2$ (HSD+C21: 328.80±22.31 μmol/L per milliliter) in HSD-fed rats. Urinary excretion of 8-isoprostane was increased as well in HSD-fed rats (HSD: 7.90±1.65 pg/μg Cr), which was reduced by C21 treatment (HSD+C21: 4.12±1.01 pg/μg Cr; Figure 1F). Renal carbonyl content was also reduced by C21 treatment in HSD-fed rats (HSD+C21: 5.47±1.42 versus HSD: 10.51±0.92 μmol/L per milligram protein; Figure 1G). C21 did not affect carbonyl content in NSD fed obese rats.

Superoxide Formation In Vitro

To selectively isolate the effect of AT$_2$R activation on NOX-mediated superoxide formation in the absence of in vivo hemodynamic or renal changes, we have stimulated human kidney-2 cells with high salt (0.25 M NaCl, final) to generate superoxide formation (control treatment had 113 mmol/L NaCl). Salt induced significant superoxide formation (≈12-fold compared with control) which was reduced by AT$_2$R agonist C21 (1 μmol/L; ≈3-fold compared with salt). Pretreatment with AT$_2$R antagonist PD123319 (1 μmol/L) abolished C21-inhibition of salt-induced superoxide formation. In fact under normal salt condition, treatment with PD123319 increased superoxide formation (≈9-fold; Figure 1H).

Expression of Endothelial Nitric Oxide Synthase, Phosphoendothelial Nitric Oxide Synthase (Ser$^{1177}$) and 3-Nitrotyrosine

In comparison with obese control rats, HSD fed rats show a decreased expression of endothelial nitric oxide synthase (eNOS; ≈130 kDa) by ≈50%, which was not affected by C21 treatment (Figure 2A). Phospho-eNOS remained statistically unchanged among treatment groups (Figure 2B). Changes in renal expression of 3-nitrotyrosine were remained nonsignificant. Although, there was a trend of decrease in the expression of 3-nitrotyrosine because of HSD feeding (by ≈20%), which was further decreased by C21 in HSD-fed rats (by ≈20%; Figure 2C).

Plasma Nitrites

HSD feeding of obese rats significantly reduced plasma nitrites (HSD: 15.35±0.49 versus CT: 23.67±1.75 μmol/L). This decrease in plasma nitrites was prevented by concurrent treatment with C21 (HSD+C21: 22.08±2.16 μmol/L; Table 1).

Morphological Assessment of Kidney

Periodic acid-Schiff staining was performed to study renal structural changes (Figure 3; Table 2). In NSD-fed OZR, glomeruli were characterized by mild to moderate focal segmental hypercellularity, mesangial expansion, matrix deposition, and thickening of basement membrane and narrowing of Bowman’s space; tubules were characterized by mild increase in cellularity, moderate vacuolization, lipid deposition, narrowing of lumen, and sloughing of brush border and basement membranes; interstitium showed mild infiltration of immune cells. HSD feeding of obese rats accelerated such structural changes; in glomeruli, it extended to crescent formation,
visceral epithelial cell hypertrophy in urinary space, focal segmental collapse of tuft, breaking of capillaries, and loss of nephron elements; in tubules, it extended to dilatation, cast formation and atrophy; in interstitium, it extended to significant infiltration and fibrosis. Our findings are in agreement with other reports. C21 treatment significantly prevented these structural changes, albeit reduction in glomerular indices was higher than that of tubular (Table 2). Other improvements in tubular structural changes by C21 such as cast formation, lipid deposition, and narrowing of tubular lumen, vacuolization, sloughing of brush border and basement membranes, and visceral epithelial cell hypertrophy may have been insufficient to be detected by our semiquantitative assessment.

Indices of Renal Function
HSD feeding caused a significant decrease in urinary excretion of Cr (HSD: 7.83±0.34 versus CT: 20.47±2.41 mg/dL) and urea nitrogen (UN; HSD: 27.63±0.21 versus CT: 32.58±0.66; Table 1), which were not affected by C21 cotreatment. These HSD-related urinary changes were associated with elevated plasma levels of Cr (HSD: 5.98±0.44 versus CT: 3.69±0.26 mg/dL) and UN (HSD: 36.03±2.14 versus CT: 26.51±3.08 mg/dL) in HSD-fed rats (Table 1), which were also not reduced with C21 treatment. Based on plasma and urinary values of Cr and UN, GFR was estimated (Figure 4A). HSD feeding significantly reduced GFR (HSD: 15.15±2.3 versus CT: 43.50±9.9 μL/min), which was improved with C21 treatment (HSD+C21: 27.43±4.91 μL/min), albeit at nonsignificant extent compared either with HSD or with CT. HSD feeding significantly decreased urine-to-plasma Cr (U/Pcr; HSD: 1.37±0.14 versus CT: 5.80±0.89; Figure 4B) and increased FE_{cr} values (HSD: 60.45±5.83 versus CT: 26.08±3.68; Figure 4C). C21-treatment increased U/Pcr (HSD+C21: 2.32±0.40) and decreased FE_{cr} (HSD+C21: 46.05±9.30), albeit at nonsignificant extent. Surprisingly, HSD intake did not alter plasma UN-to-Cr in obese rats (HSD: 6.17±0.40 versus CT: 7.31±0.92). C21 treatment increased plasma UN-to-Cr values in obese rats fed with either NSD (C21: 11.16±2.19) or HSD (HSD+C21: 8.66±1.74; Figure 4D). FE_{cr} was increased by HSD intake (HSD: 0.18±0.02 versus CT: 0.12±0.02 μg/min; Figure 5A), whereas FE_{un} remained unchanged (Figure 5B). C21 was able to reduce significantly, both, FE_{cr} (HSD+C21: 0.11±0.02 μg/min) and FE_{un} (HSD+C21: 0.08±0.01 μg/min) in HSD-fed rats. HSD feeding of obese rats showed a significant increase in uPcr (HSD: 36±1.63 versus CT: 17±1.14; Figure 5C), but did not affect uAccr (Figure 5D). C21 treatment of HSD-fed rats decreased both, uPcr (HSD+C21: 29±1.56) and uAccr (HSD+C21: 22±2.47). Similarly, urinary albumin-to-protein ratio was also decreased in urine of HSD-fed rats (HSD: 89±15 versus CT: 268±55%), which was not affected by C21 (Figure 5E). Intake of HSD resulted in tubular damage and leakage of NAG in urine. NAG activity was significantly increased in urine of HSD-fed rats compared with NSD controls (HSD: 3.32±0.31 versus CT: 2.22±0.24 RFU/30 min per microgram Cr). Treatment of HSD-fed rats with AT_{2}R agonist C21 reduced tubular damage and urinary NAG activity (HSD+C21: 2.46±0.13 RFU/30 min per microgram Cr; Figure 5F). Dot blot analysis of kidney homogenate of HSD-fed rats showed significantly reduced expression of protein recycling receptors, cubilin (HSD: 0.77±0.16 versus CT: 1.27±0.14; Figure 5G) and megalin (HSD: 0.25±0.02 versus CT: 0.47±0.07; Figure 5H); C21 had no further effects on the expression of these proteins. Qualitative assessment of all biochemical indices is summarized in Table 3.

Discussion
A novel and significant contribution of our study is to demonstrate that AT_{2}R activation exerts antioxidative effects and preserve kidney structure and function under HSD feeding in obese rats. This finding is supported by reduced renal NOX activity, urinary excretion of H_{2}O_{2}, 8-isoprostanes, and renal protein carbonyls in AT_{2}R selective agonist C21-treated HSD-fed rats. Interestingly, C21 was unable to lower plasma content of H_{2}O_{2} which indicates renal specific effects of AT_{2}R agonist...
treatment. Unlike our previous report, we did not observe C21-mediated diuretic and reduced body weight gain effect in HSD-fed OZR. This may have been because of different route of C21 administration, that is, oral bolus earlier versus osmotic pump continuous delivery in this study.

One of the mechanisms by which chronic salt intake in obesity causes formation of superoxide is activation of the renal renin angiotensin system component, Ang-II-AT1R leading to NOX activation and oxidative stress. Our study shows that the HSD-induced increase in superoxide and oxidative stress is because of increased NOX activity, a major superoxide-generating enzyme and not because of superoxide catabolizing enzymes, superoxide dismutase and catalase, which were not altered by HSD intake in obese rat kidneys. These findings are consistent with earlier studies. Superoxide can directly influence tubular reabsorption of sodium in setting of hypertension and renal injury in obese rats as seen in this study and in Dahl S (salt-sensitive) rats. The AT1R agonist C21 treatment has shown to reduce renal Ang-II expression of p47phox—NOX subunit, superoxide formation, and salt-sensitive rise in blood pressure. Because Ang-II via AT1R and high blood pressure are potent stimuli to cause oxidative stress, it is likely that a reduction in renal Ang-II levels and blood pressure in response to C21 contributed to the antioxidative properties of the AT1R in these animals. On the other hand, our in vitro study performed in human kidney-2 cells and another in vivo study provide evidence suggesting that AT2R activation may have a direct role in reducing oxidative stress. However, present in vivo study does not allow us to conclude whether C21 actually reduced oxidative stress independent of the changes in blood pressure in obese animals. A separate study is warranted to delineate AT1R-linked...
antioxidative stress activity from hypertension and Ang-II in salt-induced renal injury in obese rats.

Our study does not support the role of nitrosative stress in HSD-related renal pathologies in obesity. OZRs have reduced expression and activity of NOS and bioavailability of nitric oxide. HSD intake further reduced eNOS expression and induced superoxide formation. If increase in expression/activity of NOS is involved per se, we would expect a change in renal 3-nitrotyrosine during HSD intake because HSD intake increases superoxide formation. However, 3-nitrotyrosine remained unchanged; therefore in such conditions, we do not expect peroxynitrite and nitrosative stress to rise. This notion is supported by our results of reduced expression of renal eNOS and plasma nitrites and unchanged renal 3-nitrotyrosine in HSD-fed obese rats. C21 did not prevent reductions of eNOS or p-eNOS, but preserved plasma nitrites in obese rats fed HSD, and this effect may be owed to reduction of NOX activity, superoxide formation, and oxidative stress indices.

OZRs exhibit modest monocyte-macrophage infiltration, glomerular hypertrophy, mesangial matrix expansion, and basement membrane thickening as early as 7 to 8 weeks of age, which is resolvable by AT₄R agonist C21. At this age, animals do not show membranoproliferative focal segmental glomerulosclerosis, tubular fibrosis, or proteinuria. Older OZRs (12–14 weeks of age) exhibit mild focal segmental glomerulosclerosis and fibrosis and decline in GFR at 30 weeks of age. Our study at 12 to 14 weeks of age showed morphological changes which remained statistically unchanged by C21 treatment. This discrepancy observed with C21 treatment in our earlier and present study may be because of age of OZR (7–8 versus 12–14 weeks). The data of this study suggest that HSD intake caused an interstitial infil- tration and hastened tubulointerstitial fibrosis secondary to focal segmental glomerulosclerosis and reduced GFR. Similar changes also have been observed in HSD-fed Dahl salt-sensitive and obese spontaneously hypertensive rats. Thus, immune cell infiltration and fibrosis may represent a nonspecific response to HSD intake, which can plausibly contribute to kidney damage, for example, by augmenting oxidative stress. Our recent studies, both in vivo and in vitro, indicated that AT₄R activation with C21 exert anti-inflammatory response via altering cytokine profiles of renal epithelial cells and macrophages. Consistent with those findings, it is indicated that AT₄R activation by C21 significantly reduced interstitial infiltration of immune cells in HSD-fed OZR and in turn may have been a reason for reduced tubulointerstitial fibrosis. Anti-inflammatory properties of AT₄R activation are dependent on activation of phosphatases or decrease in tissue inhibitors of metalloproteinases, resulting in increased metalloproteinase activity, as seen in diabetic ApoE⁻/⁻ mouse model.

Kidney damage was also assessed by functional changes. HSD-fed OZR exhibited primarily glomerular proteinuria, uAcr>uPcr, which might have originated from glomerular leakage, defective retrieval of tubular protein, or both. HSD intake caused additional tubular proteinuria without an increase in albuminuria as determined by decreased urinary albumin-to-protein ratio in HSD-fed OZR in our study and Dahl S rats. HSD-induced tubular proteinuria may have been because of (1) altered retrieval of excess filtered albumin by endocytosis as demonstrated by loss of megalin-cubilin and (2) abnormal protein degradation by lysosomes and urine proteases, which return nonimmunoreactive peptide fragments of albumin to tubular lumen for excretion that remained undetected by antibodies specific to intact albumin. Other tubular protein retrieval mechanisms exist, but how exactly HSD intake in obesity affects these mechanisms is not known. AT₄R agonist C21 reduced proteinuric indices independent of protein recycling receptors (megalin–cubilin), which suggest that C21 improved either glomerular protein permeability or lysosomal degradation of protein, and thus reduced secretion of nephritic proteins into the tubular lumen. This finding is in accordance with that of Gelosa et al who have shown that

Table 2. Qualitative Assessment of Glomerular, Tubular, and Interstitial Injury in C21-Obese Rats Fed With Either Normal or HSD

<table>
<thead>
<tr>
<th>Morphological Changes</th>
<th>CT</th>
<th>C21</th>
<th>HSD</th>
<th>HSD+C21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular changes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Focal segmental cellularity and mesangial cell expansion</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Thickening of basement membrane</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Narrowing of Bowman space</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Crescent formation</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Focal segmental collapse of glomerular tuft</td>
<td>+</td>
<td>–</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Obliterated capillaries</td>
<td>+</td>
<td>–</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Loss of nephron elements</td>
<td>+</td>
<td>–</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Tubular changes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellularity</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Dilatation</td>
<td>–</td>
<td>–</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Atrophy</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Lipid deposits</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Cast formation and narrowing of lumen</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Vasculization</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Sloughing of brush border and basement membrane</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Visceral epithelial cell hypertrophy in urinary space</td>
<td>+</td>
<td>–</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Interstitial changes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infiltration</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

Several morphological indices were highlighted as bar graph in Figure 4. Intensity was graded as mild (+), moderate (++), and severe (+++). HSD indicates high-sodium diet.
C21 (10 mg/kg per day, for 7 weeks) treatment significantly delayed the time of proteinuria development in stroke-prone spontaneously hypertensive rats. This conclusion is further supported by reduced urinary marker of tubular lysosomal dysfunction (NAG activity) in C21-treated HSD-fed rats.

Despite resolved microscopic changes in AT1R agonist C21-treated HSD-fed OZR, improvements in uAcr and eGFR did not achieve statistical significance. This may have been because of methodology. Light microscopy is a semiquantitative method, whereas pyrogallol red-molybdate method of urinary protein estimation and eGFR is quantitative. Proteinuria itself is a variable index of renal damage and is neither always correlated with renal structural changes nor it predicts renal pathology and progression. The difference in uAcr between HSD and HSD+C21 has 91.1% statistical power. Similarly, it should be noted that eGFR of C21-treated rats was neither statistically different from control nor HSD-fed rats. We calculated statistical power of 77.3% to assess any significant difference in eGFR between HSD and HSD+C21 group. C21 would show improvement in eGFR and reduction in uAcr, perhaps with higher sample size (for eGFR, n=12, beta error level 10%, 95% confidence interval; for uAcr, n=12, beta error level 10%, 99% confidence interval) or longer treatments. As established earlier and seen clinically, HSD-induced decline
in GFR occurred in parallel with decreasing urinary concentrating ability as demonstrated by significantly reduced UPcr and elevated FEUN in HSD-fed OZR, which were appeared to be not significantly improved by C21 treatment (Figure 4). This may have been because of wide range of values that are possible for abnormal concentration of UN and Cr in plasma and urine.\(^\text{61}\) Overall, this study provides evidence suggesting that AT\(_2\)R activation with C21 exerts renoprotection during high-salt intake in obesity.

### Perspectives

Progression of salt-induced proteinuric renal injury occurs via oxidative stress and inflammation, as major pathways that subsequently form a vicious cycle for CKD leading to a faster decline of GFR in obesity. Clinically, angiotensin-converting enzyme-1 inhibitor\(^\text{64,65}\) or AT\(_2\)R blockers\(^\text{66,67}\) help control such pathologies, but they are inconsistent. Our series of experiment highlights AT\(_2\)R stimulation as a therapeutic avenue in managing salt-induced oxidative pathologies, especially in obesity where AT\(_2\)R are upregulated.\(^\text{20}\) More importantly, we show direct evidence that AT\(_2\)R can preserve indices of kidney injury, via protecting plasma nitrites by reducing oxidative stress and limit exposure of nephritic proteins in the tubular lumen by preserving glomerular integrity, tubular protein recycling, or lysosomal processing. AT\(_2\)R agonist can be considered as potentially promising therapy for protecting kidney structure and function in obesity. However, further studies are needed to arrive on more definite conclusion on the use of the AT\(_2\)R agonists as a therapeutic target.

### Sources of Funding

This study was supported by National Institutes of Health R01 grant DK61578.

### Disclosures

None.

### References


### Novelty and Significance

**What Is New?**

- Selective activation of angiotensin II type 2 receptor (AT₂R) by C21 is renoprotective during high-sodium intake in obesity.
- AT₂R agonist C21 reduces exposure of nephrotic proteins to tubular luminal and preserves renal structure and function in high-sodium diet–fed obese rats.
- Superoxide is known to scavenge nitrates with the formation of toxic peroxynitrite. AT₂R agonist C21 reduces nicotinamide adenine dinucleotide phosphate oxidase activity and thereby reduces superoxide formation and thus preserves plasma nitrates.

**What Is Relevant?**

- Obesity, excessive sodium intake, and renal dysfunction are highly implicated in the pathogenesis of hypertension.
- AT₂R activation by C21 during high-sodium diet feeding is renoprotective plausibly by reducing oxidative stress which in turn will have an impact on blood pressure control in obesity.

### Summary

Our study demonstrates that AT₂R agonist treatment protects against high-sodium diet–induced glomerular and tubular damage and preserves kidney function in obesity; these changes in the kidney structure and function are associated with reduced oxidative stress. Moreover, in vitro and vivo studies suggest that AT₂R activation reduces nicotinamide adenine dinucleotide phosphate oxidase activity and thereby reduces superoxide formation and preserves plasma nitrates, which all act in concert to reduce salt-induced kidney injury in obese rats.
Angiotensin II Type 2–Receptor Agonist C21 Reduces Proteinuria and Oxidative Stress in Kidney of High-Salt –Fed Obese Zucker Rats
Sanket N. Patel, Quaisar Ali and Tahir Hussain

Hypertension. 2016;67:906-915; originally published online March 28, 2016; doi: 10.1161/HYPERTENSIONAHA.115.06881

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/67/5/906

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2016/03/28/HYPERTENSIONAHA.115.06881.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ANGIOTENSIN II TYPE 2-RECEPTOR AGONIST C21 REDUCES PROTEINURIA AND OXIDATIVE STRESS IN KIDNEY OF HIGH-SALT FED OBESE ZUCKER RATS.

Sanket N. Patel, Quaisar Ali, Tahir Hussain

Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, TX 77204

Address for correspondence:
Tahir Hussain, Ph.D.
Professor of Pharmacology, 521 Science and Research Building 2, University of Houston, Houston, TX 77204-5037
Email: thussain@central.uh.edu; Ph: 713-743-1276; Fax: 713-743-1229
Chemicals: High sodium diet (HSD, 4%) (TekLad TD.92034) was obtained from Harlan Laboratories, Madison, WI. Human proximal tubule epithelial cells (Human kidney-2, HK-2) were purchased from ATCC. Keratinocyte serum free medium (KSF), fetal bovine serum (FBS), penicillin/streptomycin, epidermal growth factor (EGF) and bovine pituitary extract (BPE) were purchased from Life Technologies (Carlsbad, CA). Dihydroethidium (DHE) (12013), ADHP (10010469), 8-Isoprostane EIA kit (516351), Lucigenin (14872), nitrate reductase enzyme (780010), cofactor preparation (780012) and anti–3-nitrotyrosine antibody (189542) were purchased from Cayman Chemical, Ann Arbor, MI. PD123319 and 4-methylulbelliferyl N-acetyl-β-D-glucosaminide (4MU-NAG) were ordered from Sigma-Aldrich, St. Louis, MO. Antibodies against cubilin (sc-20609), megalin (sc-16478), eNOS (sc-653), and β-actin (sc-47778) were purchased from Santa Cruz Biotechnology, Dallas, TX. PathScan® phospho-eNOS (Ser1177) sandwich ELISA kit (#7980C) was purchased from cell Signaling. Rat albumin ELISA kit (Nephrat II, NR002, Exocell, Philadelphia, PA), QuantiChrom™ Urea Assay Kit (500T, BioAssay Systems, Hayward, CA), and Creatinine Colorimetric/Fluorometric Assay Kit (K625-100, BioVision, Inc., Milpitas, CA) were commercially purchased. All other chemicals used were of standard grade.

Renal function tests: Urinary total protein was estimated by pyrogallol red (PR)-molybdate method as described earlier. Briefly, 3 μL of clear urine sample was allowed to react with 200 μL PR-molybdate reagent for 20 minutes at 37°C. Absorbance was read at 600 nm against the reagent to determine total protein (mg/mL). Urinary albumin was determined by Nephrit II competitive ELISA kit according to manufacturer’s protocol. Urine total protein and albumin were corrected with urine volume (mL/24 hr) and reported as excretion rate in mg/24 hr. Urinary protein-to-albumin ratio (uAPr) was calculated using their mg/24 hr values. Urine and plasma were analyzed for urea nitrogen (QuantiChrom™ Urea Assay Kit) and creatinine (Creatinine Colorimetric/Fluorometric Assay Kit) according to manufacturers’ protocol and reported as mg/dL. Glomerular filtration rate was estimated (eGFR) as described earlier and reported as μL/min.

Nitrite determination: Plasma was analyzed for total nitrites by Griess reagent method utilizing nitrate reductase and cofactor preparation as we have described and reported as μmol/L.

Hydrogen peroxide (H₂O₂) assay: Urine and plasma were analyzed for H₂O₂ by ADHP–horseradish peroxidase (HRP) method. Diluted urine (10 μL, 2X) and plasma (10 μL) were allowed to react with 100 μL (final concentration: 100 μM ADHP, 0.2 U/mL HRP) and fluorescence was recorded using an Ex (540 nm) and Em (590 nm) against reagent and values were reported as mM/mg Cr.

Determination of 8-isoprostanes: Total urinary 8-isoprostane was determined by EIA assay kit. Briefly, urine was hydrolyzed with one volume of 15% potassium hydroxide at 40°C for 60 minutes and neutralized by the addition of ~10 times the original volume of 1 M potassium phosphate buffer, pH 7-7.4. Hydrolyzed samples were used further according to manufacturer’s protocol and values were reported as pg/μg Cr.

Assay of protein carbonyls: Kidney homogenate was precipitated of nucleic acids with 1% streptomycin sulfate to minimize interference. Protein carbonyls were measured using spectrophotometric DNPH assay. Briefly, protein (~200 μg) was precipitated in 10% trichloroacetic acid at 2,000xg for 5 min. Pellet was re-dispersed in derivatization solution (0.5 mL 0.2% DNPH in 2 M HCl). Samples were treated similarly with 0.5 mL 2 M HCl in parallel as negative control. Samples were allowed to stand for 60 minutes with intermittent vortexing every 10 minute. Samples were pelleted at 2,000xg for 5 minute. Extra DNPH was removed with three
successive 1 mL wash with ethanol–ethyl acetate (1:1). Pellet was re-dissolved in 100 μL 6 M guanidine containing 0.5 M KCl, pH 2.5 and centrifuged to remove insolubles. Absorbance of carbonyl was read at 370 nm and corrected for protein content.

**NADPH oxidase (NOX) activity assay:** NOX activity was measured by lucigenin-enhanced chemiluminescence. Briefly, homogenate was allowed to react with NADPH (100 μM) and dark-adapted lucigenin (5 μM). Light intensity was recorded every minute for 5 minute. Specificity was determined by diphenyleneiodonium (25 μM).

**Superoxide dismutase (SOD) activity assay:** The activity of SOD was measured by the spectrophotometric method. This method measures the ability of SOD to prevent the autoxidation of epinephrine by superoxide to a pink adrenochrome that is measured at 480 nm. Hence, the extent of inhibition of autoxidation, as reflected by the dismutation of superoxide by SOD, is taken as measure of catalytic activity. SOD reaction mixture consisted of 20 μL homogenate in potassium phosphate buffer (100 μL), containing 0.125 mM EDTA, pH 6.4, and (±) epinephrine in 100 mM HCl was added to a 15 mM concentration to start the reaction. The rate of increase in absorbance at 480 nm was measured for 10 min. The activity of SOD was reported as U/mg protein/min.

**Catalase (CAT) activity assay:** The activity of CAT was measured spectrophotometrically. The method is based on the rate of disappearance of exogenous H₂O₂ added to the sample. An aliquot of homogenate (20 μL) was mixed with potassium phosphate buffer (170 μl, 100 mM, pH 7) and 3% H₂O₂ (10 μL), and a rate of decrease in absorbance was monitored immediately at 240 nm for 3 minutes. The activity of catalase was reported as U/mg of protein/min.

**Immunoblotting:** The expression of 3-nitrotyrosine and eNOS in the kidney was determined semi-quantitatively by western blot analysis. Equal amount of protein was subjected to 4-20% SDS-PAGE, transferred to activated PVDF membrane, and immunoblotted for 3-nitrotyrosine or eNOS. β-Actin was used as loading control. For Dot blot analysis, equal protein (~10 μg) in duplicate, were directly spotted onto PVDF membrane. Membrane was allowed to dry, soaked in 5% milk-TBST (Tris buffered saline containing 0.05% tween-20), immunoprobed with specific anti-cubilin or anti-megalin antibody in 5% milk-TBST overnight at 4°C, washed with TBST (~10 mL, 10 min x 3), immunoprobed with relevant secondary HRP-conjugated antibody for 2 hours at room temperature, washed with TBST (~10 mL, 10 min x 3), and the electrochemiluminescence signal was recorded and analyzed densitometrically by Fluorchem 8800 (Alpha Innotech Imaging System, San Leandro, CA).

**PathScan® phospho-eNOS (Ser¹¹⁷⁷) Sandwich ELISA assay:** Phosphorylation of eNOS at Ser¹¹⁷⁷ was determined quantitatively by PathScan® sandwich ELISA assay a per manufacturer's instructions.

**Periodic Acid-Schiff (PAS) Staining:** Renal structural changes were determined by periodic acid-Schiff staining. All tissue sections (3-5 μ) were evaluated in a blinded manner by light microscopy (X40). Fifty glomeruli and hundred tubules from each of the two consecutive sections were assessed. A semi-quantitative scoring method was employed in a blinded manner to evaluate the degree of glomerular injury according to the severity: 0, normal; 1, minor structural changes of the mesangial matrix with focal adhesion involving <25% of the glomerulus; 2, sclerosis of 25% to <50%; 3, sclerosis of 50% to <75%; and 4, sclerosis >75% of the glomerulus. The glomerulosclerosis index was calculated by averaging the grades assigned to all glomeruli fields using the formula: \[
\frac{(N_1 \times 1) + (N_2 \times 2) + (N_3 \times 3) + (N_4 \times 4)}{n},
\]
where \(N_1-N4\) are number.
of glomeruli with the respective score and \( n \) is the total number of glomeruli. Tubulointerstitial changes were classified according to extension (%) of the damaged area as described earlier.\(^{10}\) 0 = no changes present; 1+ = <10%; 2+ = 10-25%; 3+ = 25-50%; 4+ = 50-75%; and 5+ = 75-100%.

**Urinary N-acetyl-β-D-glucosaminidase (NAG) activity:** NAG activity in urine was determined using sensitive fluorogenic substrate 4MU-NAG. Briefly, an aliquot of 10 μL of clear urine sample (after centrifugation) was incubated with 40 μL 4MU-NAG (2.6 mM) in 150 μL citrate buffer (150 mM, pH 4.5) at 37°C for 30 min. Reaction was stopped by adding 40 μL glycine-NaOH buffer (1M, pH 10.5) and fluorescence was measured at 362 nm (Ex) and 448 nm (Em). Urinary NAG activity values per 30 min were corrected with urinary Cr values and were presented as RFU/30min/μg Cr.

**Superoxide measurement in vitro:** HK-2 cells were cultured at 37°C, 5% CO\(_2\) in KSFM supplemented with 10% FBS, 1% penicillin/streptomycin, 5ng/ml EGF and 50μg/ml BPE. All experiments were performed on cells between passages 3-5 when the cells were 70-80% confluent. HK-2 cells (3X10\(^5\) per well) were seeded in 96 well plates with KSFM and treated for 24 hours with hyperosmolar salt (0.25 M NaCl) solution without or with diphenyliodonium (DPI, 2.5 μM) to detect NOX-specific superoxide formation. After 24 hours, cells were treated with C21 (1 μM) for 30 min. DHE (25 μM), a superoxide sensitive dye, was added and fluorescence was measured at Ex (490 nm) and Em (610 nm). To determine whether the effect of C21 on superoxide formation was AT\(_2R\) dependent, cells were treated with AT\(_2R\) antagonist PD123319 (1 μM) 15 min before the addition of C21. DHE fluorescence was corrected for DPI and values were presented as fold change compared to control.

**References:**


