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ORIGINAL ARTICLE

AT_2 receptor non-peptide agonist C21 promotes natriuresis in obese Zucker rats

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Previously, we demonstrated that angiotensin II type 2 (AT_2) receptors have a role in natriuresis in obese Zucker rats (OZR). In the present study, we investigated the role of a novel, non-peptide agonist, C21, in natriuresis via AT_2 receptor activation in OZR. Infusion of C21 (1 and 5 $\mu g\,kg^{-1}\,min^{-1}$) into rats under anesthesia caused a dose-dependent increase in urine flow (UF) and urinary Na volume ($U_{Na}V$). These effects of C21 were blocked by pre-infusion of the AT_2 receptor antagonist, PD123319, (50 $\mu g\,kg^{-1}\,min^{-1}$), suggesting involvement of the AT_2 receptor. Infusion of C21 (5 $\mu g\,kg^{-1}\,min^{-1}$) significantly increased the fractional excretion of sodium without changing the glomerular filtration rate or blood pressure, suggesting a tubular effect. Similarly, C21 infusion increased the fractional excretion of lithium, suggesting a proximal tubular effect. Furthermore, we tested the effect of C21 on natriuresis after blocking two main, distal-nephron Na transporters, the epithelial Na channels (ENaC), with amiloride (AM, 3 $mg\,kg^{-1}$ body wt), and the NaCl cotransporters (NCC), with bendroflumethiazide (BFTZ, 7 $mg\,kg^{-1}$ body wt). Infusion of AM + BFTZ caused significant increases in both diuresis and natriuresis, which were further increased by infusion of C21 (5 $\mu g\,kg^{-1}\,min^{-1}$). Natriuresis in response to C21 was associated with increases in urinary NO and cGMP levels. The data indicate that the AT_2 receptor agonist, C21, promotes natriuresis via AT_2 receptor activation and that this effect is potentially based in the proximal tubules and linked to the nitric oxide/cyclic guanosine monophosphate pathway. The natriuretic response to C21 may have therapeutic significance by improving kidney function in obesity.
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Keywords: amiloride; angiotensin II type 2 receptor; bendroflumethiazide; kidney; obese Zucker rats

INTRODUCTION

A considerable number of studies in the literature support the potential role of the angiotensin II type 2 (AT_2) receptor in regulating renal–cardiovascular functions (such as natriuresis and vasodilatation), blood pressure (BP), inflammation and other cellular functions, such as cell differentiation.[1–3] The functions associated with the AT_2 receptor contrast with those of the AT_1 receptor, which include antinatriuresis, vasoconstriction, increase in BP and cellular growth. Although AT_2 receptor expression is lower than that of AT_1 receptors, the presence of AT_2 receptors has been demonstrated in a variety of tissues, including the brain, heart, vasculature and kidney.[4–6] Within the kidneys, AT_2 receptors are expressed in both the afferent and efferent arterioles, the glomerulus and the proximal tubules, as well as the distal tubules and collecting ducts.[7,8] The renal AT_2 receptors have been implicated in natriuresis and regulation of pressure-natriuresis in AT_2 receptor knockout mice and in animals treated with AT_1 receptor blockers.[9–11] Further studies have revealed that the AT_2 receptors in the kidney cortex of obese Zucker rats (OZR) are upregulated[11,12] and may have a role in long-term BP regulation in these animals.[12]

In the isolated proximal tubules of obese rats, we have shown that AT_2 receptor activation inhibits the Na pump via the nitric oxide/cyclic guanosine monophosphate pathway,[13] indicating a potential role of AT_2 receptors in inhibiting proximal Na transport. In addition to their expression in the proximal tubules, AT_2 receptors are also expressed in the distal nephron. However, the site of AT_2 receptor function that contributes to natriuresis is not known. Recently, a novel, orally active, non-peptide AT_2 receptor agonist, C21, has become available.[14] We studied the role of C21 in natriuresis in OZR and investigated the potential contributions of the proximal tubules in C21-induced natriuresis in obese animals.

METHODS

Animals

Experiments were performed in male, OZR (10–11 weeks old), which were purchased from Harlan Laboratories, Indianapolis, IN, USA. After arrival, the rats were housed in the University of Houston animal care facility and allowed 5 days of acclimatization in a room with an ambient temperature of 23 ± 2°C and a 12-h dark/light cycle. The rats were given normal rodent chow and tap water *ad libitum*. The Institutional Animal Care and Use Committee at the University of Houston approved all of the animal experimental protocols.

Experimental protocol

Renal function study. On the day of the experiment, we anesthetized the OZR with inactin (150 $mg\,kg^{-1}$ body wt i.p.). After tracheotomy, the right carotid

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artery was cannulated with PE 50 and attached to the data acquisition system (PolyView, Grass, West Warwick, RI, USA) via a Grass PT300 pressure transducer for BP measurement. The jugular vein was cannulated with PE 50 for infusion of normal saline, lithium chloride or drugs. After opening the abdominal cavity, the ureter was catheterized with PE 10 for urine collection.

In the first phase of the experiment, saline was infused through the jugular vein. After 40 min of stabilization, two basal urine samples were collected at 30-min intervals. The AT_2 receptor agonist, C21, was then infused ($1\,\mu g\,kg^{-1}\,min^{-1}$ and $5\,\mu g\,kg^{-1}\,min^{-1}$) through the jugular vein. After 15 min of stabilization, two urine samples were collected, again at 30-min intervals, during each infusion period. Each collection was treated as a separate sample for the measurement of various parameters, as described below. Measurements from the two urine collections were averaged and presented as one basal value or one drug-treatment value. Blood samples of 100 µl each were collected in-between the two-urine collections from the basal or drug treatment rats. A total of three blood samples were collected during the entire experimental period; the volume of blood drawn was replaced by an equal volume of saline. A schematic representation of the protocol used in the study is shown in Figure 1a. The same sequence of basal and post-drug-treatment urine collections, a 15-min stabilization period after drug treatment, and blood sample collection, was followed in the subsequent protocols, 2–4.

In the second phase of the experiment, to determine whether C21 was acting via the AT_2 receptor, we infused PD123319 (AT_2 receptor antagonist, $50\,\mu g\,kg^{-1}\,min^{-1}$) through the jugular vein before the infusion of C21. Two urine samples were collected at 30-min intervals, before and after drug administration. A schematic representation of the protocol used in the study is shown in Figure 1b.

In the third phase of the experiment, lithium chloride ($LiCl_2$, $4\,mmol\,kg^{-1}$ body wt) was infused through the jugular vein. The concentration of $LiCl_2$ used in the study was based on previously published studies.[15] Following stabilization, two urine samples were collected at 30-min intervals, followed by infusion of C21 ($5\,\mu g\,kg^{-1}\,min^{-1}$) and collection of two more urine samples at 30-min intervals. A schematic representation of the protocol used in the study is shown in Figure 1c.

In the final phase of the experiment, we determined the effect of C21 after blocking ENaC and NCC. After stabilization and basal urine collection, we infused a bolus dose of the ENaC blocker, amiloride (AM, $3\,mg\,kg^{-1}$ body wt),[16] and the NCC blocker, bendroflumethiazide (BFTZ, $7\,mg\,kg^{-1}$ body wt),[17,18] followed by infusion of C21 ($5\,\mu g\,kg^{-1}\,min^{-1}$). Two urine samples were collected at 30-min intervals during each drug infusion period. A schematic representation of the protocol used in the study is shown in Figure 1d.

The AM and BFTZ concentrations used in the study are based on earlier reports.[16–20] These concentrations are maximally effective in inhibiting ENaC and NCC.[16–18]

Urine and plasma analysis

The Na and Li concentrations in the urine and plasma were measured using the AAnalyst 400 atomic absorption spectrometer (Perkin Elmer, Waltham, MA, USA). To estimate the glomerular filtration rate (GFR), the plasma and urinary creatinine were measured with a creatinine assay kit (Biovision (Cat no. K625), Mountain View, CA, USA).

Evaluation of renal function

Samples from each urine collection were used to measure urine volume and calculate urine flow rate (UF, $\mu l\,min^{-1}$). For each urine collection, the urinary Na

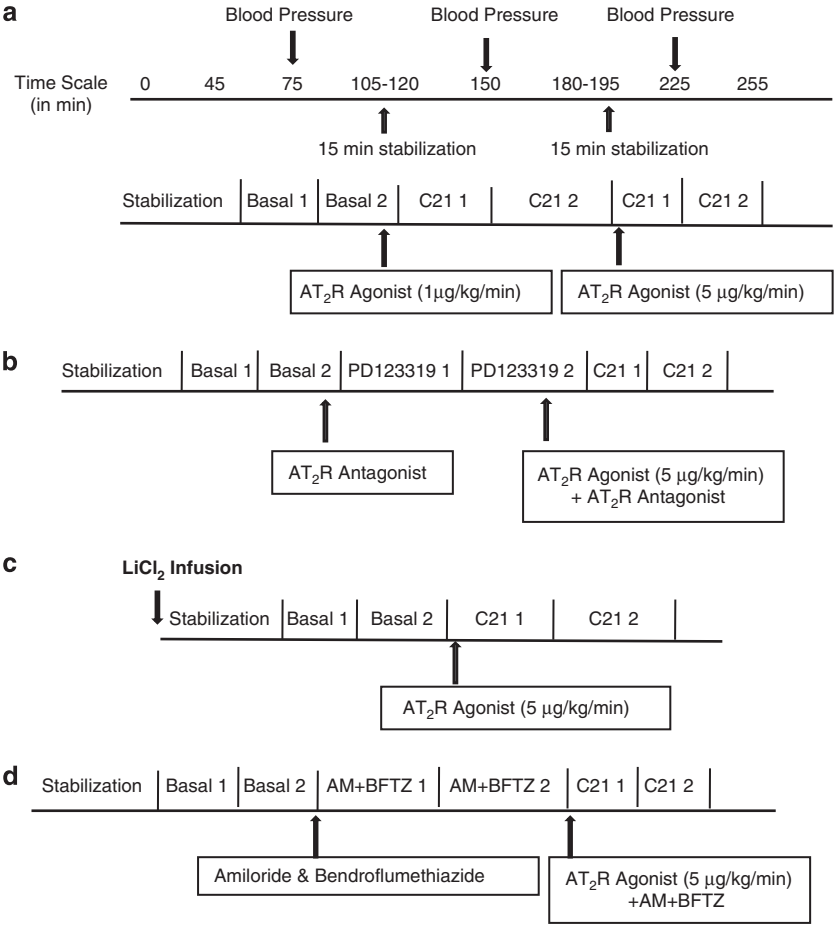

Figure 1 Schematic representation of protocols used in the study. (**a**) To determine the dose-dependent effect of C21 on natriuresis/diuresis. (**b**) To determine whether C21 is acting via AT_2 receptor. (**c**) To determine FE_{Li}. (**d**)To determine the effect of C21 after blocking ENaC and NCC.

and Li (volume) excretion rates ($U_{Na}V$ and $U_{Li}V$, $\mu mol\,min^{-1}$) were calculated as UF×urinary Na/Li concentration ($\mu mol\,\mu l^{-1}$). The GFR ($\mu l\,min^{-1}$) was calculated on the basis of creatinine clearance. To calculate the fractional excretion of sodium or lithium in urine (FE_{Na} or FE_{Li}%), the $U_{Na}V$ or $U_{Li}V$ ($\mu mol\,min^{-1}$) was divided by plasma Na or Li concentration ($mg\,dl^{-1}$) × GFR ($\mu l\,min^{-1}$).

Chemicals

PD123319 was a generous gift from Pfizer (Groton, CT, USA). Compound C21, 97% pure, was custom synthesized using a synthesis scheme previously published.[14] The creatinine assay kit was purchased from Biovision (Cat no. K625). The NO and cGMP EIA kits were purchased from R&D System, Minneapolis, MN, USA. The AM and BFTZ were purchased from Sigma Aldrich, St Louis, MO, USA.

Statistical analysis

The results are expressed as the mean ± s.e.m. Data were subjected to statistical analysis using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA, USA). One-way analysis of variance followed by the Newman–Keuls post-hoc test were performed to determine variation within the groups, and the Student's *t*-test was performed to compare variation between the groups. A value of $P<0.05$ was considered statistically significant.

RESULTS

General parameters

The average body weight of the rats was 472 ± 12 g and the average kidney weight was 2.6 ± 0.2 g.

Effect of C21 on diuresis and natriuresis

We determined the effect of the AT_2 receptor agonist, C21 (1 and 5 $\mu g\,kg^{-1}\,min^{-1}$ i.v.), on diuresis and natriuresis in obese rats. Infusion of C21 caused dose-dependent increases in UF and $U_{Na}V$ relative to basal rates, and these increases were highly significant with the 5 $\mu g\,kg^{-1}\,min^{-1}$ dose (Figure 2), which was used in subsequent sets of the experiment. To demonstrate that C21-induced Na excretion is mediated via the AT_2 receptor, we infused the AT_2 receptor antagonist, PD123319 (50 $\mu g\,kg^{-1}\,min^{-1}$ i.v.), before the infusion of C21. Although PD123319 alone did not affect UF or $U_{Na}V$, when compared with basal rates, it completely abolished increases in UF and $U_{Na}V$ in response to C21 (Figure 3), suggesting the involvement of the AT_2 receptor.

Effect of C21 on FE_{Na} and FE_{Li}

Infusion of C21 (5 $\mu g\,kg^{-1}\,min^{-1}$) caused a significant increase in FE_{Na} (Basal: 3.033 ± 0.9%, C21: 14.63 ± 2.3%, $P<0.05$), suggesting a tubular effect of the drug (Figure 4a). Similarly, C21 infusion (5 $\mu g\,kg^{-1}\,min^{-1}$) caused a significant increase in FE_{Li} (Basal: 24.6 ± 2.9, C21: 56.8 ± 5.5 %, $P<0.05$) (Figure 4b).

Effect of C21 on natriuresis after blocking distal nephron Na-transporters

As shown in Figure 5, the blockade of ENaC and NCC in the distal tubules with AM and BFTZ significantly increased $U_{Na}V$ (Basal: 0.27 ± 0.04 AM+BFTZ: 1.4 ± 0.4 $\mu mol\,min^{-1}$, $P<0.05$) and UF (Basal: 3.9 ± 0.6 AM+BFTZ: 8.9 ± 0.8 $\mu l\,min^{-1}$, $P<0.05$). The C21 infusion in AM + BFTZ-infused rats caused a further increase in both the UF and the $U_{Na}V$.

Effect of AM + BFTZ and C21 on urinary nitrates/nitrites and urine cGMP

Levels of urinary nitrates/nitrites and urine cGMP in obese rats did not change after treatment with AM + BFTZ. Conversely, treatment with C21 significantly increased levels of urinary nitrates/nitrites and

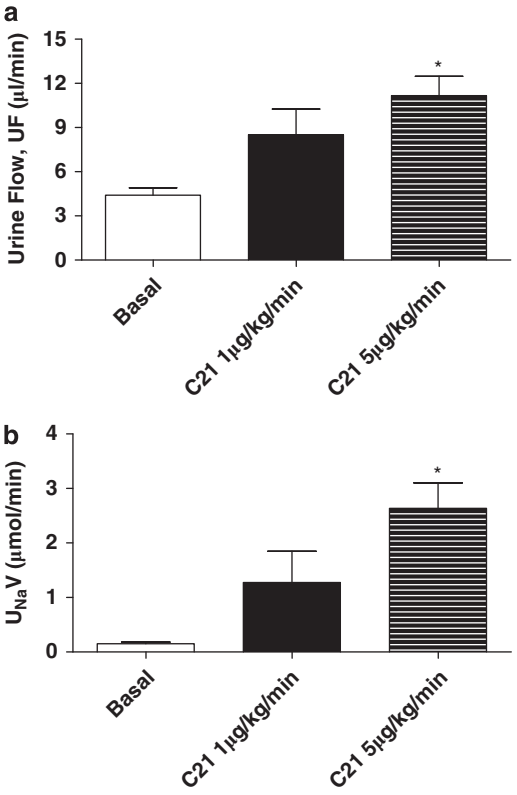

Figure 2 Effect of C21 (1 and 5 $\mu g\,kg^{-1}\,min^{-1}$) on (**a**) urine flow (UF) and (**b**) urinary Na volume ($U_{Na}V$) in obese Zucker rats. Values are represented as mean ± s.e.m.; One-way analysis of variance followed by Neuman–Keuls test, *significantly different from basal, $P<0.05$; N=5–6 rats.

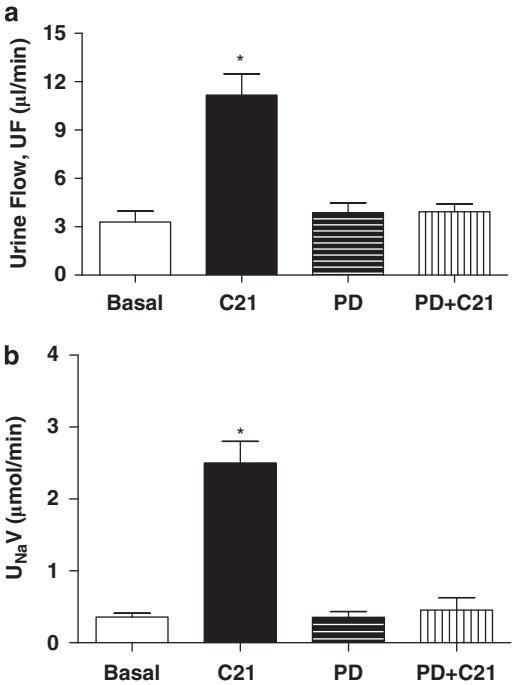

Figure 3 Effect of C21 (5 $\mu g\,kg^{-1}\,min^{-1}$) and PD123319 (50 $\mu g\,kg^{-1}\,min^{-1}$) on (**a**) urine flow (UF) and (**b**) urinary Na volume ($U_{Na}V$) in obese Zucker rats. We have added C21 data from protocol 1 to demonstrate the effect of C21, which is much higher than any basal. Values are represented as mean ± s.e.m.; One-way analysis of variance followed by Neuman–Keuls test, *significantly different from basal, $P<0.05$; N=3 rats.

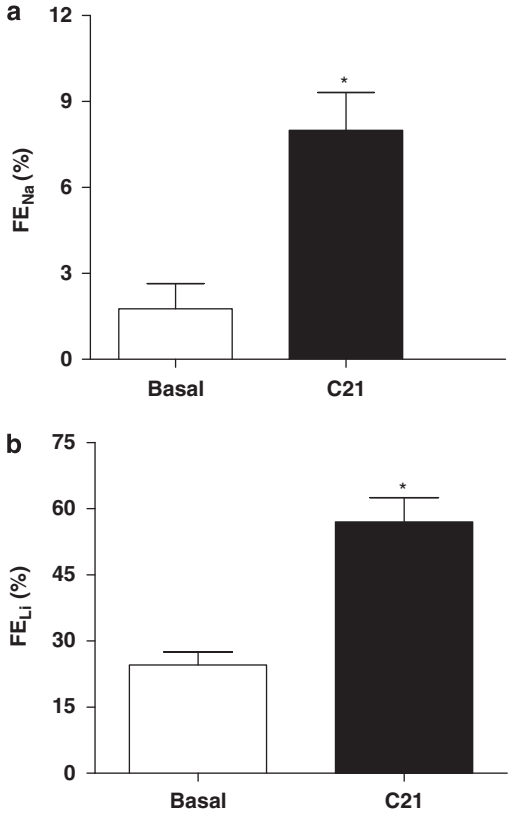

Figure 4 Effect of C21 on (**a**) fractional excretion of sodium (FE_{Na}) and (**b**) fractional excretion of lithium (FE_{Li}) in obese Zucker rats. Values are represented as mean ± s.e.m.; Student's *t*-test, *significantly different from basal, $P<0.05$; N=5–7.

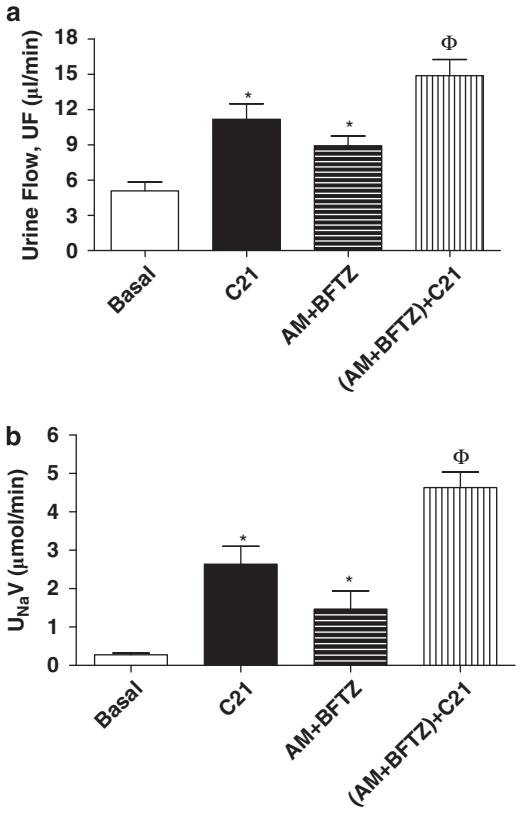

Figure 5 Effect of C21 alone and in combination with amiloride (AM) and bendroflumethiazide (BFTZ) on (**a**) urine flow (UF) and (**b**) urinary Na volume ($U_{Na}V$) in obese Zucker rats. We have added C21 data from protocol 1 to demonstrate the effect of C21, which is higher than any basal. Values are represented as mean ± s.e.m.; One-way analysis of variance followed by Neuman–Keuls test, *significantly different from basal, $^{\Phi}$significantly different from C21 and AM + BFTZ, $P<0.05$; N=7–10 rats.

urine cGMP, when compared with basal levels or levels following AM + BFTZ infusion (Figure 6).

Mean arterial pressure, heart rate and GFR

Mean arterial pressure and heart rate remained unchanged after infusing the AT_2 receptor agonist, antagonist, or sodium channel blockers (Figure 7a and b). Similarly, the glomerular filtration rate was not changed by the infusion of these drugs (Basal: 175 ± 27 µl min^{-1}, AM + BFTZ: 192 ± 57 µl min^{-1}, AM + BFTZ+C21: 167 ± 14 µl min^{-1}) (Figure 7c).

DISCUSSION

In this study, we demonstrated for the first time that compound 21 (C21), via activation of the AT_2 receptors, promotes natriuresis. This finding was associated with increases in urinary nitrates/nitrites and cGMP levels in OZR. Further studies revealed that C21, without affecting BP or GFR, increases FE_{Na} and FE_{Li}.

Obesity-related hypertension is associated with renal dysfunction, which is believed to be both a cause and a consequence of high BP.[21] OZR have been used as animal models to study the mechanisms of obesity-related renal dysfunction and hypertension.[11–13,22,23] Numerous studies suggest that abnormal regulation of the renin–angiotensin system, including enhanced renal AT_1 receptor function, is one of the major mechanisms contributing to increased renal sodium reabsorption and a shift in pressure natriuresis. This abnormal regulation leads to high BP in obesity, including obesity in Zucker rats.[22–25] Numerous studies have shown that AT_1 receptor blockade by candesartan causes greater natriuresis in obese rats.[11,22] Interestingly, however, the enhanced natriuretic response to candesartan was abolished by the AT_2 receptor antagonist, PD123319, suggesting unopposed action of the AT_2 receptor on natriuresis after AT_1 receptor blockade in obese rats.[11] With the development of the non-peptide, orally active AT_2 receptor agonist compound, C21,[14] there is renewed interest to explore the beneficial role and potential of the AT_2 receptor in various pathophysiological conditions.[26,27] In the present study, we observed that C21 promotes natriuresis via the AT_2 receptor. The C21 dose that was used in this study had no effect on BP or GFR, but it caused an increase in FE_{Na}, suggesting that C21 has tubular effects.

These observations are consistent with our earlier reports showing that the peptide agonist, CGP42112A, caused natriuresis in obese rats without affecting GFR or BP.[11] However, Bosnyak *et al.*[28] showed that 1 µg kg^{-1} of C21 causes an increase in the BP of spontaneously hypertensive rats, which is contrary to our present study showing no effect of C21 (either 1 or 5 µg kg^{-1} min^{-1}) on BP. Interestingly, another study[29] reported that a higher dose (50 µg kg^{-1}) of C21 causes a BP reduction in spontaneously hypertensive rats, suggesting a discrepancy between these two studies in spontaneously hypertensive rats.[28,29] The discrepancy with our study could be based on the use of OZR under anesthesia as opposed to conscious spontaneously hypertensive rats in other studies.[28,29]

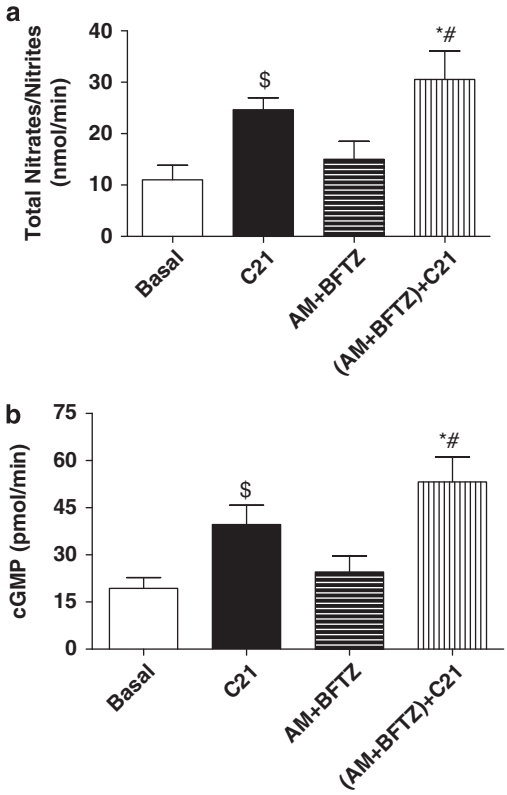

Figure 6 Effect of C21 alone and in combination with amiloride (AM) and bendroflumethiazide (BFTZ) on (**a**) urinary nitrates/nitrites and (**b**) cGMP in obese Zucker rats. We have added C21 data from protocol 1 here, to demonstrate the effect of C21, which is higher than any basal. Values are represented as mean ± s.e.m.; One-way analysis of variance followed by Neuman–Keuls test, *$significantly different from basal, #significantly different from AM + BFTZ, $P<0.05$; N=7–10 rats.

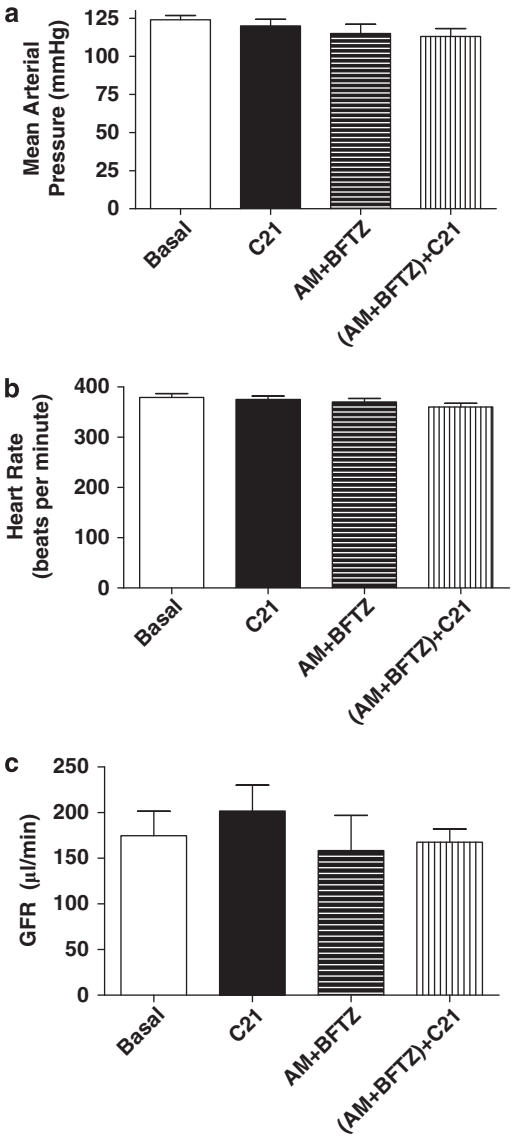

Figure 7 Effect of C21 alone and in combination with amiloride (AM) and bendroflumethiazide (BFTZ) on (**a**) mean arterial pressure, (**b**) heart rate and (**c**) glomerular filtration rate (GFR) in obese Zucker rats. C21 data on MAP, HR and GFR were taken from protocol 1. Values are represented as mean ± s.e.m.; One-way analysis of variance followed by Neuman–Keuls test, $P<0.05$; N=7–10 rats.

A probable signaling mechanism involved in C21-induced natriuresis is the stimulation of the nitric oxide/cyclic guanosine monophosphate pathway.[13] In the present study, natriuresis induced by C21 was associated with increases in urinary NO and cGMP. Previously, we have shown that the stimulation of AT_2 receptors in the isolated proximal tubules of OZR causes an increase in urinary nitrite/nitrate (index of NO production) and cGMP production, thereby inhibiting Na-pump activity.[13] In another study performed with streptozotocin-induced diabetic rats, we observed increased urinary Na excretion that was associated with increased urinary cGMP. These increases in Na excretion and cGMP were abolished by infusion of the AT_2 receptor antagonist, PD123139.[30] In the present study, although it is clear that infusion of C21 causes an increase in both signaling molecules, NO and cGMP, in the urine, the cellular source cannot definitively be attributed to the proximal tubules because of the systemic infusion of C21. The vasculature, in addition to the renal epithelial cells, may contribute to the net increase in urinary levels of nitrite/nitrates and cGMP.[31]

As AT_2 receptors are expressed on proximal and distal tubules and are found in higher density in the proximal tubules,[32] we attempted to examine whether the proximal tubules are involved in AT_2 receptor action, leading to natriuresis. We observed that C21 infusion caused a significant increase in FE_{Li}, which is used as marker of proximal-tubule Na handling.[33] As Li reabsorption occurs only in the proximal tubular segments of the nephron and is associated with reabsorption of Na,[33] change in Li excretion serves an index of proximal tubule activity. In addition to this direct evidence that AT_2 receptor-mediated natriuresis may involve the proximal tubules, we also observed that C21 promoted natriuresis even after blocking NCC and ENaC, two major Na transporters present in the distal nephron segments (which include the convoluted distal tubule, the connecting tubule and the cortical and medullary-collecting duct).[16–20,32] We observed that C21 alone caused a greater degree of natriuresis than AM + BFTZ. The larger effect of C21 could be a result of the action of AT_2R in various parts of the nephron, including the proximal tubules, which are the major site of sodium transport, as opposed to the isolated, distal inhibition of sodium transport by AM + BFTZ. The doses of AM and BFTZ used in the study are maximally effective doses to block ENaC, NCC and Na transport in these nephron segments.[16–18]

These findings suggest that the majority of C21-induced natriuresis originates from the proximal nephron segments; however, the findings do not rule out the possibility of involvement at the loop of Henle. As the loop of Henle does not express AT_2 receptors,[5,34] C21-induced natriuresis, after blocking ENaC and NCC, can be attributed to the proximal tubules alone. Collectively, findings from both sets of experiments, including FE_{Li} and natriuresis following ENaC/NCC blockade, indicate a potential role of the proximal tubule in AT_2 receptor-mediated natriuresis. However, although we have measured lithium clearance in the present study as a marker of proximal tubular handling, several investigators have demonstrated that infusion of lithium has a dose-dependent natriuretic effect.[35] Moreover, a small fraction of lithium may also be reabsorbed in the loop of Henle.[36] These observations highlight the limitation of using FE_{Li} as a marker of proximal tubule involvement in AT_2 receptor function.

As a result of the proximal tubule action of the AT_2 receptor, the increased Na delivery to the macula densa was expected to initiate a tubulo-glomerular feedback response, leading to reduced GFR. Surprisingly, we found no change in GFR. The reason for this anomalous observation is not known. A likely explanation may be based on reports suggesting a disrupted tubulo-glomerular feedback mechanism in obesity and hyperinsulinemia.[37] The OZR is a model of both obesity and hyperinsulinemia, and these two conditions might have disrupted normal functioning of tubulo-glomerular feedback mechanisms in these animals. Although our experiments indicate the potential role of the proximal tubules in AT_2 receptor-mediated natriuresis, the possible role of other nephron segments, which fine-tune Na reabsorption and help maintain Na homeostasis,[19] remains to be determined.

One of the mechanisms contributing to obesity-related hypertension is attributed to enhanced tubular Na reabsorption resulting from an imbalance of pro-natriuretic and anti-natriuretic hormone systems.[38] It is reported that OZR exhibit defective pro-natriuretic dopaminergic[39,40] and atrial natriuretic peptide[41] function, enhanced activity of anti-natriuretic activity in the sympathetic nervous system[38] and renin–angiotensin system, and increased AT_1 receptor function.[11,23,24] In the light of these reports, the natriuretic response of AT_2 receptor activation by this novel C21 compound provides a pharmacological basis to shift renal Na handling. However, whether C21-induced natriuresis might have a role in the long-term regulation of renal function and blood-pressure control in obesity remains to be determined.

In summary, we demonstrated that selective activation of the AT_2 receptor by a novel AT_2 receptor agonist, C21, promoted natriuresis in obese rats, and that the natriuresis was associated with a parallel increase in the production of urinary nitric oxide/cyclic guanosine monophosphate, which might have a role in AT_2 receptor-mediated natriuresis. Moreover, we observed that AT_2 receptor-induced natriuresis might be based on proximal tubule activity; however, establishing the role of other nephron segments in AT_2 receptor-mediated natriuresis needs further investigation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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