Proximal Tubule Angiotensin AT₂ Receptors Mediate an Anti-Inflammatory Response via Interleukin-10
Role in Renoprotection in Obese Rats

Isha Dhande, Quaisar Ali, Tahir Hussain

Abstract—The angiotensin type 2 receptor (AT₂R) has been shown to lower inflammation in the kidney. However, the role of the anti-inflammatory cytokine interleukin (IL)-10 in AT₂R-mediated attenuation of inflammation has not been elucidated. We hypothesized that AT₂R activation is renoprotective by directly increasing the levels of anti-inflammatory cytokine IL-10 in the kidney via nitric oxide (NO) signaling. For in vitro studies, the human proximal tubule epithelial cell-line (human kidney-2 [HK-2]) was activated with lipopolysaccharide (10 μg/mL) and AT₂R agonist C21 (1 μmol/L) for 24 hours, and media cytokine levels were assessed. Lipopolysaccharide modestly downregulated AT₂R expression. Treatment with C21 lowered lipopolysaccharide-induced levels of both tumor necrosis factor-α and IL-6, but increased IL-10 levels. Treatment with neutralizing IL-10 antibody (1 μg/mL) or NO synthase inhibitor L-NAME (1 mmol/L) abolished this effect. For in vivo studies, prehypertensive obese Zucker rats and age-matched lean Zucker rats were treated for 2 weeks with C21 (300 μg/kg per day, IP) and AT₂R antagonist (PD123319; 50 μg/kg per minute, SC infusion). Compared with lean Zucker rats, obese Zucker rats had higher levels of renal AT₂R expression, tumor necrosis factor-α, and IL-6. C21 treatment decreased levels of tumor necrosis factor-α by 75% and IL-6 by 60%. Conversely, PD treatment lowered the renal IL-10 levels in obese Zucker rats by ≈60%. Renal morphometry revealed increased mesangial matrix expansion and glomerular macrophage infiltration, which was improved by C21 treatment in obese Zucker rats. Our findings suggest that proximal tubule AT₂R activation is anti-inflammatory by increasing IL-10 production, which is largely NO dependent and thus offers renoprotection by preventing early inflammation–induced renal injury in obesity. (Hypertension. 2013;61:1218-1226.) • Online Data Supplement

Key Words: anti-inflammatory ■ AT₂ Receptor ■ C21 ■ interleukin-10 ■ nitric oxide ■ renoprotection

Obesity, insulin resistance, hypertension, and renal injury are all associated with underlying low-grade inflammation that results from the proinflammatory cytokines secreted by the adipose tissue. High levels of circulating proinflammatory cytokines result in immune cell infiltration and activation of resident macrophages in the kidney, which is susceptible to injury attributable to obesity-related excessive excretory load, hyperinsulinemia, and renal lipotoxicity. Activated resident macrophages then themselves produce a host of cytokines locally, setting up a proinflammatory milieu, thereby accelerating the renal injury mechanisms. In addition to immune cells, the proximal tubule epithelial cells (PTECs) are known to play an important role in renal inflammation by producing an array of chemokines and cytokines. Among these, tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and IL-10 are key players in mediating renal inflammation and injury.1 Typically, the anti-inflammatory cytokine IL-10 negatively regulates proinflammatory cytokine signaling to maintain homeostasis.2 This self-regulatory system is rendered dysfunctional in obesity/diabetes mellitus3 because of a skewing of macrophages to the proinflammatory phenotype. This unresolved inflammation makes the kidney more susceptible to obesity-related glomerular hyperfiltration, glomerular cell proliferation, matrix accumulation, basement membrane thickening, and ultimately glomerulosclerosis and tubular fibrosis culminating in nephron loss.

In addition to creating a proinflammatory environment, obesity also leads to an abnormally activated renin-angiotensin system, an important hormonal system involved in regulating renal structure and function. Earlier, we and others have reported in obese Zucker rats (OZR), an increased function of the renal angiotensin type 1 receptor (AT₁R)4-6 which mediates most of the deleterious effects of angiotensin II, including the proinflammatory functions.7-12 In addition, there is an increase in the renal angiotensin type 2 receptor (AT₂R) expression in these animals. The AT₂R is believed to be protective by functionally antagonizing the proinflammatory actions of angiotensin II mediated by AT₁R, via multiple
signaling pathways, such as NO/cGMP and activation of tyrosine phosphatases. Recently, it has been demonstrated that acute AT,R stimulation is renoprotective by lowering the renal levels of TNF-α and IL-6 in a rat model of renovascular hypertension. Consistent with this report, we also have previously shown that chronic AT,R activation in hypertensive OZR lowers blood pressure as well as circulating and renal levels of proinflammatory cytokines TNF-α, IL-6, and MCP-1 (monocyte chemoattractant protein). Furthermore, Curato et al have recently identified a population of noncytotoxic AT,R-expressing CD8+ T-cells with a unique phenotype of increased IL-10 expression, suggesting that the AT,R may elicit its anti-inflammatory response by promoting IL-10 production. However, the role of AT,R in renoprotection in obesity independent of blood pressure change and the involvement of proximal tubule AT,Rs in mediating anti-inflammation are not known. We hypothesized that AT,R activation is renoprotective by increasing the levels of the anti-inflammatory cytokine IL-10 in the kidney via nitric oxide (NO) signaling. Here, we demonstrate that AT2R activation is renoprotective by increasing IL-10 production, which is largely NO dependent. Activation of the AT,R is therefore renoprotective because this prevents early inflammation–induced renal injury in obesity.

Methods

Cell Culture

Human kidney (HK-2) PTECs were cultured according to standard protocols. For detailed protocols, please refer to the online-only Data Supplement.

In Vitro Experimental Protocols

TNF-α, IL-6, and IL-10 were quantified in the media from HK-2 cells 24 hours after stimulation with lipopolysaccharide (LPS; 10 μg/mL) and C21 (1 μmol/L). For detailed protocols, please refer to the online-only Data Supplement.

Animal Model

Male 5-week-old lean Zucker rats (LZR) and prehypertensive obese Zucker rats (OZR) were used (n=6–7). These animals were treated with AT,R agonist (C21, 300 μg/kg per day IP daily) and antagonist (PD123319, 50 μg/kg per minute SC infusion) for 2 weeks to determine the effect of AT,R activation or blockade on obesity-linked renal inflammation and injury. Renal injury was assessed using periodic acid Schiff-stained sections of the kidney according to the method described by Raij et al. Macrophage infiltration was determined by immunostaining for CD68, a monocye/macrophage marker. For detailed protocols, please refer to the online-only Data Supplement.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cytokines in cell-culture supernates and kidney cortex homogenates were assessed by kit-based ELISA according to the manufacturer instructions. For detailed protocols, please refer to the online-only Data Supplement.

Immunoblotting

Western blotting for plasma cytokines (TNF-α, IL-6, and IL-10) and angiotensin receptors (AT,R) in kidney cortex homogenates and HK-2 cells was carried out according to standard techniques. Amount of protein loaded for rat samples was 20 μg AT,R and 60 μg for AT,R expression. For detailed protocols, please refer to the online-only Data Supplement.

Statistical Analyses

Data are expressed as mean±SEM with n≥5 in each group. Student t test was used. One-way ANOVA with post hoc (Newman-Keuls) tests was used to compare variations within groups. A value of P<0.05 was considered statistically significant.

Results

In Vitro Studies

Effect of AT,R Agonist C21 on Cytokine Production by Activated Proximal Tubule Epithelial Cells

HK-2 cells were treated with bacterial LPS (10 μg/mL) for 24 hours to induce cytokine production in PTECs. Another set of cells were treated with AT,R agonist C21 (1 μmol/L) along with LPS to determine the effect of AT,R stimulation on cytokine production by activated PTECs. Treatment with LPS downregulated AT,R expression (see Results in the online-only Data Supplement), which is consistent with reports in other tissues. Furthermore, LPS treatment alone resulted in a 50-fold increase in TNF-α and 10-fold increase in IL-6 concentration in the media. Concurrent treatment with C21 lowered TNF-α concentration by 70% and IL-6 concentration by 60% (Figure IA and 1B). In addition to LPS, in a separate set of experiments, PTECs were activated using TNF-α (10 ng/mL) for 24 hours, and IL-6 production in the media was determined. Similar to LPS, TNF-α aggravated the production of IL-6 by 10-fold, which was lowered by 50% with concurrent treatment C21. Predictably, treatment with LPS and C21 together resulted in greater IL-10 levels in the media compared with LPS treatment. However, this was not significantly different from the IL-10 production by C21 treatment alone (Figure 1C). Activation of PTECs with TNF-α with and without C21 followed a pattern of IL-10 production similar to that observed with LPS treatment (see Results in the online-only Data Supplement; Figure S3 in the online-only Data Supplement). C21 treatment alone did not alter proinflammatory cytokines, TNF-α, and IL-6 production by PTECs. On the contrary, C21 treatment alone dose dependently (0.1–10 μmol/L) increased the production of IL-10 in PTECs, even in the absence of LPS activation (see Results in the online-only Data Supplement; Figure S4).

Effect of Neutralizing IL-10 Antibody on Cytokine Production by Activated PTECs

HK-2 cells were treated with neutralizing antibody to IL-10, which binds to IL-10 produced by these cells and prevents it from interacting with its receptor. Before treatment with LPS and C21, the cells were preincubated for 30 minutes with different doses (0.25, 0.5, 1, and 5 μg/mL) of the neutralizing IL-10 antibody. The IL-10 antibody was able to dose dependently abolish the ability of the AT,R agonist to lower TNF-α and IL-6 (Figure 2A and 2B).

Effect of L-NAME on Cytokine Production by PTECs

HK-2 cells were preincubated for 15 minutes with NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; 1 mmol/L) before treatment with LPS and C21. Incubation with L-NAME alone led to a 3-fold increase in the levels of IL-6 released in the medium. In cells preincubated with L-NAME, treatment with
C21 also led to a similar increase in IL-6 production compared with control C21-treated cells. In the presence of L-NAME + LPS-treated cells, there was no significant difference in the IL-6 production compared with control LPS–activated cells. However, the attenuation of IL-6 levels by C21 in LPS-activated PTECs was lost in the cells where L-NAME was added (Figure 3A). On the contrary, L-NAME alone significantly lowered the IL-10 production in HK-2 cells. There was also a significant inhibition in the C21 as well as LPS-mediated IL-10 production in the presence of L-NAME. Furthermore, the increase in IL-10 levels in C21-treated LPS-activated PTECs was completely abrogated in the presence of nitric oxide synthase inhibitor (Figure 3B). Taken together, these data indicate that the anti-inflammatory response to AT2R activation is largely dependent on AT2R-mediated NO production.

In Vivo Studies

Body Weight and Blood Pressure
At age 7 weeks, the OZRs weighed significantly more than LZRs, and drug treatment did not alter body weight.
in any of the experimental groups (Lean: Veh 202±3 g, PD 210±4.5 g, C21 204±6 g, PD+C21 199±8 g versus Obese: Veh 348±10.5 g, PD 348±9.6 g, C21 345±9 g, PD+C21 363±11 g). Blood pressure was measured in conscious animals weekly by tail cuff plethysmography (CODA Non-invasive Blood Pressure System, Kent Scientific, Torrington, CT). The systolic blood pressure in OZR was comparable with lean controls throughout the duration of the study, and drug treatment did not affect the blood pressure in any of the experimental groups (Lean: Veh 105±1.7 mm Hg versus Obese: Veh 106±1.5 mm Hg, PD 104±2.1 mm Hg, C21 103±0.5 mm Hg 102±1.5 mm Hg).

**AT₁ and AT₂ Receptor Expression in Renal Cortex**

A distinct band was detected for AT₁R at ≈41 kDa by Western blotting. Densitometric analysis revealed that AT₁R expression was not significantly different in the renal cortex of control OZR compared with control LZR. Treatments did not affect the expression levels of the AT₁R in either strain of rats (Figure 4A). Two bands were detected for AT₂R at ≈44 and 39 kDa in the renal cortex, most likely because of varying degrees of glycosylation. We have previously reported that treatment of the cortical membranes with the deglycosylating enzyme N-glycanase shifted AT₂R multiple bands toward a single band at ≈30 kDa. AT₂R expression was ≈45% higher in OZR compared with control LZR. Drug treatments did not cause significant changes in AT₂R expression in any of the experimental groups (Figure 4B).

**Proinflammatory Cytokine Production in Response to PD and C21**

Compared with control LZR, the levels of proinflammatory cytokines TNF-α and IL-6 were higher in plasma as well as in renal cortex in control OZR. These levels were significantly reduced with AT₂R agonist treatment for 2 weeks. PD treatment itself did not alter the proinflammatory TNF-α and IL-6 cytokine levels; however, it was able to prevent the C21–mediated lowering of proinflammatory cytokines (Figure 5A–5D), indicating that the anti-inflammatory effect of C21 was indeed via the AT₂R. Drug treatment did not have any effect on the cytokine levels in LZR.

**Anti-Inflammatory Cytokine Production in Response to PD and C21**

Compared with control LZR, OZR had higher IL-10 levels in both plasma and kidney cortex, and this was not altered by C21 treatment. On the contrary, PD treatment resulted in ≈45% lowering of circulating and ≈60% reduction in the kidney IL-10 levels in OZR. Treatment with PD had no effect on IL-10 levels in LZR, however C21 treatment in LZR led to a 3-fold increase in IL-10 levels in the plasma and renal cortex (Figure 6A and 6B). Taken together, these data point toward a role of AT₂R in IL-10 production.

**Renal Morphological Analysis**

LZRs exhibited normal renal morphology, and this was not affected by drug treatments (Figure 7A). Control OZR had higher (1.3±0.2) mesangial matrix expansion (MME) scores compared with LZR, which had an MME score below 0.5, indicating intact renal structure. This was worsened by PD treatment; these animals had MME scores of ≈2, which is indicative of damage involving 25% to 50% of the affected glomerulus. OZRs treated with C21 were protected from this early event associated with obesity-linked renal pathology and had near normal MME scores (Figure 7B).

**Effect of C21 and PD Treatment on Renal Macrophage Infiltration**

LZR exhibited almost no macrophage infiltration (Figure 8A) irrespective of drug treatments. Compared with LZR, control OZR demonstrated increased CD68+ cells in the glomeruli (0.3±0.2 versus 10±0.7), and this was attenuated (10±0.7 versus 4.7±0.5) by C21 treatment (Figure 8B).

**Discussion**

Nonresolving renal inflammation, which begins with infiltration of mononuclear cells in the kidney, is one of the earliest events in progressive renal injury that finally culminates in glomerulosclerosis and tubulointerstitial fibrosis. In addition to the immune cells, the PTECs have recently been shown to be protective and can inhibit the activation of macrophages. Also, PTECs have been shown to express the AT₂R. In the present study, LPS-activated HK-2 cells produced significant amounts of proinflammatory...
cytokines TNF-α and IL-6, which led to a compensatory increase in IL-10 production. Treatment with selective AT₂R agonist C21 resulted in lower levels of TNF-α and IL-6 in the media collected from LPS-activated HK-2 cells, which is in agreement with previous reports in dermal fibroblasts. However, here we also observed a marked increase in the IL-10 levels when LPS-activated cells were treated with C21. Interestingly, although treatment with C21 alone did not alter the production of proinflammatory cytokines, there was a significant increase in IL-10 levels with C21 even in the absence of LPS. Furthermore, this increase was greater than what was observed with LPS-activation, indicating that AT₂R activation directly drives the production of IL-10, possibly independent of classical toll-like receptor–mediated pathways. Moreover, the lowering of proinflammatory cytokines by C21 was completely lost in the presence of a neutralizing antibody.
to IL-10, implying that even a modest increase in IL-10 is essential for the lowering of proinflammatory cytokine levels by AT₂R agonist. Overall, these results suggest that IL-10 is the dominant cytokine responsible for mediating the response to AT₂R agonist. In addition to using LPS to activate PTECs, in another set of experiments, TNF-α also was used to stimulate the production of cytokines (see the online-only Data Supplement). This ensured that the anti-inflammatory response to AT₂R agonist was not specific to toll-like receptor signaling. Moreover, in obesity-associated renal inflammation, TNF-α from the plasma as well as infiltrating macrophages is likely to activate PTECs. However, because the results obtained by both activating agents were similar, the subsequent experiments were conducted using LPS where the outcomes were more remarkable.

Although the anti-inflammatory role of AT₂R stimulation has recently been ascertained by multiple groups, the precise molecular mechanisms that produce this response are still under investigation. A few signaling pathways by which the AT₂R may potentially lower the levels of proinflammatory cytokines have been described, including inhibition of NFκB (nuclear factor κB), activation of protein phosphatases, increase in epoxyeicosatrienoic acid synthesis, and inhibition of STAT3 phosphorylation. NO is known to be a key second messenger in renal AT₂R signaling. Additionally, the anti-inflammatory properties of physiological levels of NO have been described. In the in vitro studies here, it was clear that the nonspecific nitric oxide synthase inhibitor, L-NAME, inhibited the production of IL-10 in LPS- and C21-treated PTECs, while at the same time, the levels of IL-6 were elevated in LPS-activated cells even in the presence of C21. Although the precise link between NO production and IL-10 signaling is still unclear, it can be speculated that the signaling cascades activated by NO, including cGMP-dependent protein kinase activation, may be involved in downstream activation of mitogen-activated protein kinases that are required for IL-10 production.

Resolution of renal inflammation is essential for the control of renal injury initiated by obesity. To demonstrate that AT₂R agonist can attenuate early changes associated with obesity-linked renal inflammation independent of hypertension, we used prehypertensive OZR as the animal model. The OZR is a well-characterized model of metabolic syndrome and develops spontaneous renal injury with increasing age. At age 5 weeks, these animals are obese and hyperinsulinemic.
and develop hyperglycemia and hypertension after 9 weeks of age. These animals also exhibit hyperfiltration, however the relative contribution of the proinflammatory cytokines to this observation is difficult to assess, because hyperfiltration is a cumulative effect of multiple factors, including high body mass index, increased excretory load, hyperinsulinemia, inflammation, and oxidative stress. We have previously shown that 2-week treatment with AT\(_R\) agonist CGP42112A in 12-week-old OZR was able to lower plasma and renal levels of TNF-\(\alpha\), IL-6, and MCP-1\(^{27}\) as well as lower blood pressure. Here, we demonstrate that prehypertensive OZRs also have upregulated renal AT\(_R\) expression and elevated plasma cytokines. Similar to our previous findings, 2 weeks treatment with AT\(_R\) agonist C21 in this age group resulted in marked decline in the TNF-\(\alpha\) and IL-6 levels in the plasma and the kidney cortex, but without any change in blood pressure. This observation, when considered along with the in vitro data, makes it evident that activation of the renal AT\(_R\) has a direct anti-inflammatory effect. Also, in our previous studies, it was reported that there was an increase in TNF-\(\alpha\) in the plasma of lean rats treated with CGP. This was not observed in the present study using C21. This disparity in observations may be a result of different AT\(_R\) agonists used, because CGP has been reported to have some non–AT\(_R\)-mediated effects as well,\(^{38,39}\) whereas C21 is believed to be more AT\(_R\) selective.

Renal morphometry revealed an increase in macrophage infiltration, glomerular hypertrophy, MME, and basement membrane thickening in control OZR compared with lean controls. There was no focal segmental glomerulosclerosis or any extent of significant tubular fibrosis detected, which is not surprising, because at this age, it is unlikely that such drastic nephron damage would occur. Also, OZRs have been documented to have normal renal function at age 6 weeks and only develop proteinuria at 12 to 14 weeks of age and decreased glomerular filtration rate at 32 weeks of age.\(^{35,40}\) Thus, in the age group used in this experiment, no significant changes in renal function were expected. Nevertheless, leukocyte infiltration and MME are still among the earliest pathophysiological changes that are observed in the kidney in obesity-linked renal damage, which predispose to focal segmental glomerulosclerosis and tubulointerstitial fibrosis.\(^{35,37}\)

Treatment with AT\(_R\) antagonist PD worsened the extent of MME and macrophage infiltration, whereas C21 treatment maintained the renal structural integrity in OZRs. Drug treatment in LZR had no effect on renal morphology in any of the experimental groups, emphasizing the fact that AT\(_R\) plays a more significant role in pathophysiological conditions rather than in the normal state. In agreement with our in vitro data, the in vivo studies also revealed that AT\(_R\) is involved in anti-inflammatory cytokine production. However, in animals, we observed that OZRs already had elevated levels of the anti-inflammatory IL-10, which differs from some published reports in humans where obesity was associated with lower circulating levels of IL-10.\(^{41,42}\) It is likely that the levels of IL-10 were increased in the early stages of obesity-linked inflammation in response to the high levels of TNF-\(\alpha\) as a compensatory mechanism. These already high levels of plasma and renal cortical IL-10 were not further increased by C21 treatment, possibly because the IL-10 levels were at the maximal production limit. We did, however, see a marked reduction in the levels of IL-10 when the AT\(_R\) was blocked with PD, making it clear that increase in anti-inflammatory cytokine production is AT\(_R\) mediated. A possible reason for the difference in these findings from those observed in the HK-2 cells might be that in obese animals, the anti-inflammatory cytokines are already at their maximal levels, and hence, the increase caused by AT\(_R\) agonist may not be readily evident. Also, unlike the proinflammatory cytokines, which were unaffected by drug treatments, C21 and PD, both caused significant alterations in the levels of IL-10 in the kidney cortex in lean rats, which suggests that AT\(_R\) activation may have a direct role in increasing IL-10 production.

In this study, we have demonstrated a \(\approx45\%\) increase in renal AT\(_R\) expression in OZR compared with LZR. Although this is a modest increase in receptor expression, the responses to AT\(_R\) agonist and antagonist are quite impressive in comparison. It is important to note that in case of the AT\(_R\), the function of this receptor has been shown to be enhanced in OZR compared with LZR, as we have reported previously.\(^{4,43}\) Furthermore, it has been reported in rat proximal tubules that AT\(_R\) activation itself directly lowers AT\(_R\) function via the NO/cGMP/Sp1 pathway,\(^{44}\) revealing an additional mechanism that could contribute to the physiological response to AT\(_R\) agonist. Thus, receptor expression alone is not sufficient to account for the changes in the response to AT\(_R\) agonist and antagonist, because postsignaling amplification can play a major role in the net response elicited by receptor activation.

Overall, the most noteworthy finding of the in vivo and in vitro studies is the involvement of IL-10 in mediating the anti-inflammatory action of the AT\(_R\), which adds another dimension to the existing paradigm where AT\(_R\) has been shown to merely lower the proinflammatory cytokine levels. Although the effect of C21 on the entire network of cytokines and chemokines has not been investigated in this study, the effects on the key cytokines make it evident that the AT\(_R\) does elicit an anti-inflammatory action, which is independent of its antihypertensive effect. Here, we have shown the beneficial effects of activating the PTECs using HK-2 cells; however, in whole animals, the situation is much more complex, because there are a number of other cell-types, such as the mesangial cells, the podocytes, and the endothelial cells, which express the AT\(_R\) in addition to producing cytokines. So the net anti-inflammatory effect observed in OZRs is most likely a result of all these factors, underscoring the importance of the intrarenal AT\(_R\) in renoprotection. We conclude that chronic AT\(_R\) activation is renoprotective by increasing intrarenal IL-10 production via increased NO signaling.

**Perspectives**

These in vitro and in vivo studies could help to elucidate the complex AT\(_R\)-mediated anti-inflammatory and renoprotective mechanisms and identify it as a novel therapeutic target for preventing the onset and progression of obesity-associated kidney disease.
Acknowledgements
PD123319 was a kind gift from Pfizer, Inc.

Sources of Funding
This study was supported by National Institutes of Health grant R01-DK61578 to T.H.

Disclosures
None.

References

**Novelty and Significance**

**What Is New?**

- This is the first report demonstrating that the activation of the angiotensin type 2 receptor (AT2R) increases interleukin-10 production in HK-2 proximal tubule epithelial cells and Zucker rats. Moreover, interleukin-10 is the dominant cytokine involved in mediating the anti-inflammatory response of AT2R agonist.
- We demonstrate that chronic AT2R activation exerts an anti-inflammatory response independent of hypertension in obese Zucker rats.
- Treatment with AT2R agonist C21 for 2 weeks protects against the early obesity–linked renal injury.

**What Is Relevant?**

- The AT2R agonist can preserve intact renal structure and function, which may protect against obesity-linked hypertension.

**Summary**

In this study, we demonstrate that activation of the AT2R lowers the levels of proinflammatory cytokines in the kidney, and this anti-inflammatory response is mediated by a NO-dependent increase in interleukin-10 production. Chronic activation of the AT2R for 2 weeks with AT2R agonist, C21, is renoprotective because it prevents the development of early macrophage infiltration and mesangial matrix expansion in obese Zucker rats.
Proximal Tubule Angiotensin AT2 Receptors Mediate an Anti-Inflammatory Response via Interleukin-10: Role in Renoprotection in Obese Rats
Isha Dhande, Quaisar Ali and Tahir Hussain

Hypertension. 2013;61:1218-1226; originally published online April 1, 2013;
doi: 10.1161/HYPERTENSIONAHA.111.00422
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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/61/6/1218

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2013/04/01/HYPERTENSIONAHA.111.00422.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/
ONLINE DATA SUPPLEMENT

PROXIMAL TUBULE ANGIOTENSIN AT₂ RECEPTORS MEDIATE AN ANTI-INFLAMMATORY RESPONSE VIA INTERLEUKIN-10: ROLE IN RENOPROTECTION IN OBESE RATS

Isha Dhande, Quaisar Ali and Tahir Hussain

Heart and Kidney Institute, Department of Pharmacological and Pharmaceutical Sciences, University of Houston, Houston, TX-77204, Department of Pharmacal Sciences, Harrison School of Pharmacy, Auburn University, Auburn, AL-36849

Address for correspondence:
Tahir Hussain, PhD
Professor
Department of Pharmacological and Pharmaceutical Sciences
Rm 521 Science and Research Bldg 2
University of Houston, Houston, TX-77004
E-mail: thussain@central.uh.edu
Ph: 713-743-1276
Fax: 713-743-1884
EXTENDED METHODS

Chemicals and Reagents

Human proximal tubule epithelial cells (HK-2) were purchased from ATCC (Manassas, VA). Keratinocyte serum free medium (K-SFM), fetal bovine serum (FBS), penicillin/streptomycin, epidermal growth factor (EGF) and bovine pituitary extract (BPE) were purchased from Life Technologies (Carlsbad, CA). C21 was custom synthesized according to a previously published scheme (Wan et al., 2004). Polyclonal AT1R antibody was custom raised by Biomolecular Integrations (Little Rock, AR). Polyclonal AT2R antibody was custom raised by EZ Biolab (Carmel, IN). Anti-rat CD68 antibody, L-NAME, TGF-β and IL-10 antibodies, neutralizing IL-10 antibody, total nitrates and cytokine ELISA kits were purchased from R&D Systems (Minneapolis, MN). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture

HK-2 cells were cultured at 37°C, 5% CO2 in K-SFM supplemented with 10% FBS, 1% penicillin/streptomycin, 5ng/ml EGF and 50µg/ml BPE. All experiments were performed on cells between passages 5-12 when the cells were 70-80% confluent. All experiments were performed in triplicate.

In vitro Protocols

HK-2 cells were seeded in 6-well plates and when 70-80% confluent, the media was replaced with in K-SFM without any supplements. These cells were treated for 24 hours with LPS (10 µg/ml), C21 (1 µmol/L or a dose range 0.1-10 µmol/L) or both agents simultaneously for 24 hours. At the end of treatment, media was collected, filtered through 0.2 um filter and analyzed for total nitrates or cytokines by kit-based ELISA (R&D Systems). Total nitrates in the supernates were determined using a kit-based EIA (R&D Systems). To determine whether the effect of C21 on cytokines was indeed mediated by the AT2R, cells were treated with AT2R antagonist PD123319 (10 umol/L) 15 min before the addition of C21. For neutralizing the effect of IL-10, a specific IL-10 antibody (0.25, 0.5, 1.0 and 2.5 µg/ml) was added to the medium 15 min before the addition of LPS and/or C21. For inhibition of nitric oxide synthase, non-specific NOS inhibitor L-NAME (1 mmol/L) was added to the media 15 min before the addition of LPS and/or C21.

Animal Model

Male lean and obese Zucker rats (5 weeks of age) were purchased from Harlan Laboratories (Madison, WI). The animals were housed in the University of Houston animal care facility and had free access to standard rat chow and tap water. The Institutional Animal Use and Care Committee approved animal experimental protocols. The lean and obese rats (n=5-7) were divided into vehicle, AT1R antagonist (PD123319), AT2R agonist (C21), and AT2R agonist-antagonist (PD123319+C21) treated groups. Vehicle (saline) and C21 (300 µg/kg/day) were injected daily i.p and PD123319 (50 µg/kg/min) was continuously infused for 2 weeks via subcutaneously implanted osmotic pumps (Alzet, Palo Alto, CA).
Immunoblotting for AT1R and AT2R Expression

Treated HK-2 cells were washed with cold phosphate buffered saline and lysed in 0.3 ml of lysis buffer containing 0.5 M Tris base (pH 6.8), 1% SDS, 1 mM EDTA, 1 mM PMSF, and protease inhibitor (aprotinin, calpain inhibitors, leupeptin, pepstatin, and trypsin inhibitor). The cell lysates were used for protein estimation by kit based BCA method. For determining AT1R abundance 10 µg and 50 µg of protein from HK-2 cell lysate were subjected to SDS-PAGE and transferred onto immobilon P (blot). To determine the change in AT2R expression with LPS, 50 µg of protein was loaded and subjected to SDS-PAGE.

For the determination of AT1R abundance in renal cortex homogenates from lean and obese Zucker rats, increasing amounts of protein (5, 15, 30, 50 µg) from the sample were. Similarly, for AT2R abundance increasing amounts of protein (10, 25, 50, 70 µg) of protein were loaded and subjected to western blotting for the protein of interest using respective protein specific polyclonal antibodies. Polyclonal IgG-linked with horseradish peroxidase and the ECL system were used to detect the signal, which were analyzed by FluorChem 8800 (Alpha Innotech Imaging System, San Leandro, CA). β-actin was used as a loading control.

Blood Pressure Measurement

Blood pressure was measured in conscious, restrained rats using CODA System tail-cuff plethysmography (Kent Scientific). Rats were trained for 3 days prior to taking final BP measurements. This system has been clinically validated and the results have been shown to provide 99% correlation with telemetry measurements of BP.

Renal Histology and Morphometry

In order to assess the pathological changes that occur in the kidneys of the lean and obese Zucker rats, mesangial matrix expansion, focal segmental glomerulosclerosis development, tubulo-interstitial fibrosis and macrophage infiltration was assessed. After sacrificing the animals under anesthesia with inactin (150 mg/kg i.p), the kidneys were flushed with cold phosphate buffered saline to remove blood and then be fixed in formalin for 24 hours at 4°C. The formalin-fixed tissue was embedded in paraffin and 4 µm sections were prepared. The slides were further stained with Periodic Acid Schiff reagents.

Periodic Acid Schiff (PAS) Staining: To evaluate the gross tubular and glomerular structural changes, PAS staining was performed on the sections using a kit-based technique (Dako North America, Inc.) according to the manufacturer’s instructions. All tissue samples were evaluated independently by two investigators in a blinded fashion by light microscopy (x400). For each animal, 30 glomeruli and 100 tubules from each of the 3 consecutive sections were assessed. A semi-quantitative scoring method described by Raij et al. (1984) was used to evaluate the degree of damage to the glomeruli or tubules. This was graded according to the severity of the glomerular damage: 0, normal; 1, slight glomerular damage of the mesangial matrix and/or hyalinosis 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: Sclerosis Index = (N1 x 1 + N2 x 2 + N3 x 3 + N4 x 4)/ n; where N1-N4 are number of glomeruli with the respective score, n is the total number of glomeruli.
**Immunostaining for CD68:** CD68 is a cytosolic antigen protein specific for monocytes and macrophages. Immunostaining was carried out according to standard procedures using anti-CD68 (R&D Systems), and biotinylated secondary antibodies (Vector Laboratories) and revealed with avidin-peroxidase (Vectastain Elite; Vector Laboratories Inc.). Slides were mounted with VectaMount (Vector Laboratories) and photographed under a Nikon Eclipse TS100 microscope (Nikon Instruments Inc.) using an Infinity 1 digital camera (Media Cybernetics Inc.). The number of CD-68 positive cells per glomerulus was reported. An average of 30 glomeruli per section and 3-consecutive sections per kidney were analyzed.

**Statistical Analysis**

Data are presented as means ±SE. To analyze variations between groups, Student’s t-test was used. One-way ANOVA with post-hoc (Newman-Keuls) tests was used to compare variations within groups. A value of p<0.05 was considered statistically significant, with n=5–7 experiments or rats per group.

**SUPPLEMENTAL RESULTS**

**AT₁R and AT₂R Expression in PTECs:** A single band (~41 kDa) for AT₁R was detected even at 10 µg of protein loading (Fig S1). Western blotting showed the presence of the AT₂R as two distinct bands (~45 and ~40 kDa) in HK-2 cells at 50 µg of protein loading (Fig. S2). No band was detected at 10 µg protein (data not shown). The two bands are likely due to varying degrees of glycosylation as has been previously reported (Hakam and Hussain, 2005, Kornfeld and Kornfeld, 1985). LPS treatment significantly downregulated AT₂R expression in activated PTECs (Fig S2).

![Fig. S1](image1.png)  
**Fig. S1:** AT₁R Expression in HK-2 cells. Representative western blot for AT₁R (~41 kDa) showing a detectable band with both 10 µg and 50 µg of loaded protein.

![Fig. S2](image2.png)  
**Fig. S2:** AT₂R Expression in HK-2 proximal tubule epithelial cells. Representative western blots for AT₂R protein (approx. 40 and 45 kDa) top panel and for β–actin in the lower panel. Data are mean ± SEM (n = 3). * indicates p<0.001 vs control untreated HK-2 cells.
Effect of AT$_2$R agonist C21 on cytokine production by TNF-α activated PTECs: Similar to LPS, TNF-α (10 ng/ml) was able to significantly increase the production of pro-inflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10 (Fig. S3).

**Fig. S3:** (A) Interleukin-6 (IL-6) and (B) interleukin-10 (IL-10) concentration in the media collected from HK-2 cells stimulated by TNF-α (10 ng/ml) and/or C21 (1 umol/L). Data are mean ± SEM (n = 3). * indicates p<0.05 vs control untreated, # indicates p<0.05 vs TNF-α treated and $ indicates p<0.05 vs C21 treated HK-2 cells.

Dose response effect of AT$_2$R agonist on IL-10 production by LPS-activated PTECs: Increasing doses of C21 (0.1-10 µmol/L) led to a corresponding increase in IL-10 levels in the media even in the absence of LPS activation. This effect was blocked by concurrent addition of AT$_2$R antagonist PD123319, confirming that the NO production was indeed AT$_2$R mediated (Fig. S4).

**Fig. S4:** Dose dependent increase in interleukin-10 (IL-10) production by HK-2 proximal tubule epithelial cells following treatment with AT$_2$R agonist (C21; 0.1-10 µmol/L) for 24 hours. AT$_2$R antagonist PD123319 (PD, 10 µmol/L) was able to block the C21-mediated increase in IL-10 concentration. Cytokine concentrations in media were measured by ELISA. Data are represented as mean ± SEM (n=5).

Effect of AT$_2$R agonist C21 on nitric oxide production by PTECs: In this set of experiments, HK-2 cells were treated with increasing doses of C21 and the production of nitrates/nitrites in the medium was determined after 24 hours, as an indicator of NO production. C21 treatment dose (0.1-10µmol/L) dependently increased the level of nitrates/nitrites and this increase was blocked by concurrent addition of AT$_2$R antagonist PD123319, confirming that the NO production was indeed AT$_2$R mediated (Fig. S5).

**Fig. S5:** Dose dependent increase in total nitrates in the media collected from HK-2 proximal tubule epithelial cells
following treatment with AT₂R agonist (C21; 0.1-10 µmol/L) for 24 hours. AT₂R antagonist PD123319 (PD, 10 µmol/L) was able to block the C21-mediated increase in total nitrate production. Data are represented as mean ± SEM (n=5).

**AT₁ and AT₂ receptor abundance in lean and obese Zucker rats:** Increasing amounts of protein were loaded for detection of AT₁R (5, 15, 30, 50 µg) and AT₂R (10, 25, 50, 70 µg) in lean and obese rats. In LZR and OZR a band for AT₁R (~41 kDa) was detected even at 5 µg. In OZR, a pair of faint bands (~44 and 39 kDa) was detected at 10 µg of loaded sample, while a band of similar intensity could be detected in the lean rat only at 50 µg of loaded sample. Taken together, these observations suggest that in both LZR and OZR, AT₁R is more abundant than AT₂R and that AT₂R is up-regulated in OZR compared to LZR (Fig. S6).

![Representative blots depicting the relative abundances of AT₁R and AT₂R in lean and obese Zucker rats.](image)

**Fig. S6:** Representative blots depicting the relative abundances of AT₁R and AT₂R in lean and obese Zucker rats.