Renin-Angiotensin-Aldosterone System

Angiotensin AT₂ Receptor Stimulation Inhibits Early Renal Inflammation in Renovascular Hypertension

Luis C. Matavelli, Jiqian Huang, Helmy M. Siragy

Abstract—Angiotensin II type 2 receptor (AT₂R) counteracts most effects of angiotensin II type 1 receptor (AT₁R). We hypothesized that direct AT₂R stimulation reduces renal production of the inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and transforming growth factor-β1 (TGF-β1) and enhances the production of nitric oxide (NO) and cyclic guanosine 3’5’-monophosphate (cGMP) in the clipped kidney of 2-kidney, 1-clip (2K1C) hypertension rat model. We used Sprague-Dawley rats to evaluate changes in renal interstitial fluid recovery levels of TNF-α, IL-6, NO, and cGMP; renal expression of AT₁R, AT₂R, TGF-β1, TNF-α, and IL-6 in sham and 2K1C rats treated for 4 days with vehicle, AT₂R agonist compound 21 (C21), or AT₂R antagonist PD123319 (PD), alone and combined (n=6 each group). Systolic blood pressure increased significantly in 2K1C and was not influenced by any treatment. Clipped kidneys showed significant increases in renal expression of AT₁R, AT₂R, TGF-β1, IL-6, TGF-β1 and decreases in NO and cGMP levels. These factors were not influenced by PD treatment. In contrast, C21 caused significant decrease in renal TNF-α, IL-6, TGF-β1 and an increase in NO and cGMP levels. Combined C21 and PD treatment partially reversed the observed C21 effects. Compared to sham, there were no significant changes in TNF-α, IL-6, TGF-β1, NO, or cGMP in the nonclipped kidneys of 2K1C animals. We conclude that direct AT₂R stimulation reduces early renal inflammatory responses and improves production of NO and cGMP in renovascular hypertension independent of blood pressure reduction. (Hypertension. 2011;57:308-313.)

Key Words: angiotensin receptor ■ cytokines ■ inflammation ■ kidney ■ renin-angiotensin system ■ renovascular hypertension

Renal production of angiotensin (Ang) II is increased in renovascular hypertension. The majority of the pathophysiologic effects of Ang II are mediated by the Ang II subtype 1 receptor (AT₁R). Activation of Ang II subtype 2 receptor (AT₂R) is believed to counteract the effects of AT₁R through inhibiting cellular proliferation and differentiation, enhancing vasodilation and natriuresis. In the kidney, AT₂R is localized to renal vessels, glomeruli, and tubules. However, the role AT₂R plays in the kidney and cardiovascular diseases is not fully elucidated.

Previous studies demonstrated increased tissue inflammation with activation of the renin-angiotensin system. Activation of AT₁R stimulated the production of interleukin (IL)-6, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β1 and induction of oxidative stress. Blockade of AT₂R reduced renal production of nitric oxide (NO) and cyclic guanosine 3’5’-monophosphate (cGMP). These events suggested a cross-talk between AT₁R and AT₂R that could influence development of organ disease.

Elucidating the beneficial effects of the AT₂R activity was limited because of lack of available specific agonists for this receptor. Previous studies evaluated the role of AT₂R by manipulating its expression or blockade. Recently, a novel AT₂R agonist, compound 21 (C21), was developed. C21 is a nonpeptide, orally active, specific, and highly selective agonist for AT₂R. This compound was recently tested in vivo and initial studies suggested that it could reduce tissue inflammation and fibrosis. The present study was conducted to examine the role of direct AT₂R stimulation on early changes in systolic blood pressure (SBP), renal inflammation, and production of NO and cGMP in 2-kidney, 1-clip (2K1C) Goldblatt hypertension rat model.

Subjects and Methods

Animal Preparation

Experiments were approved by the University of Virginia Animal Care and Use Committee and conducted in male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 230 to 260 grams. Animals were fed a normal-sodium diet and tap water ad libitum for the whole experiment and a minimum of 1 week was allowed to adjust to our animal care facility. Rats were randomly allocated to different groups, sham (n=6) and 2K1C-treated for 4 days (n=6 each treatment) with vehicle (5% dextrose in water, intraperitoneal), C21 (0.3 mg/kg/d, intraperitoneal; Vicore), PD123319 (PD), or C21 combined with PD. Treatments were started at the same time and administered throughout the 4-day duration of the study. PD (Sigma Aldrich) was infused at a dose of 10 mg/kg per...
day using an osmotic minipump (model 2001; Alzet). Details of surgical procedures are available in the online Data Supplement (available online at http://hyper.ahajournals.org).

**Body Weight and SBP Monitoring**

Body weight and SBP were obtained before surgery and at the end of the study. SBP was assessed by tail-cuff plethysmography (model SC-1000; Hatteras Instruments).

**In Vivo Renal Interstitial Fluid Collections**

The in vivo renal interstitial fluid (RIF) recovery levels of TNF-α, IL-6, NO, and cGMP were determined as previously described.9,10 Please see the online Data Supplement.

**RIF Storage and Assays**

RIF collections were stored at −80°C until assayed. Please see the online Data Supplement.

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**Figure 1.** Renal angiotensin II type 1 (AT1R) and type 2 (AT2R) receptor expressions in right (R) and left (L) kidneys of sham and nonclipped (open bars) and clipped (solid bars) kidneys of 2-kidney, 1-clip (2K1C) hypertension rats treated with vehicle, compound 21 (C21), PD123319 (PD), or C21 plus PD. A and C, mRNA levels of AT1R and AT2R, respectively. B and D, Western blot analyzes of AT1R and AT2R. Top, Representative blots. Bottom, Quantitative results normalized to β-actin. n=5, each group. Data are mean±SEM. *P<0.05 vs sham and corresponding nonclipped kidneys. †P<0.05 vs 2K1C vehicle-treated. ‡P<0.05 vs 2K1C treated with PD.

<table>
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<th>PD</th>
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**Figure 2.** Renal mRNA expression and renal interstitial fluid (RIF) levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in right (R) and left (L) kidneys of sham and nonclipped (open bars) and clipped (solid bars) kidneys of 2-kidney, 1-clip (2K1C) hypertension rats treated with vehicle, compound 21 (C21), PD123319 (PD), or C21 plus PD. A and C, mRNA levels of TNF-α and IL-6, respectively (n=5). B and D, RIF levels of TNF-α and IL-6 (n=6). Data are mean±SEM. *P<0.05 vs sham and corresponding nonclipped kidneys. †P<0.05 vs 2K1C vehicle-treated. ‡P<0.05 vs 2K1C treated with PD.
Determination of mRNA Expression

The procedures for mRNA measurements of TNF-α, IL-6, TGF-β1, AT₁R, and AT₂R were performed as previously described. Please see the online Data Supplement.

Western Blot Analysis

Preparations of kidney tissue lysate and protein quantitation of AT₁R, AT₂R, and TGF-β1 were performed as previously described. Please see the online Data Supplement.

Renal Morphology

Renal tissues obtained from sham and 2K1C rats were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin per standard techniques. Sections were cut at a thickness of 4 μm and stained with hematoxylin and eosin. Renal tissue was examined under light microscopy.

Statistical Analysis

Comparisons among different treatment groups were assessed by ANOVA followed by a Tukey test for post hoc comparisons. Data are expressed as mean±SE. P<0.05 is considered statistically significant.

Results

Body and Kidney Weights

As shown in Supplemental Table S1 (available online at http://hyper.ahajournals.org), body weights were not different between groups. Clipped kidney weight was significantly greater compared to that of contralateral kidneys of vehicle-treated 2K1C group or kidneys of the sham group (P<0.05). C21 treatment reduced the increase in clipped kidney weight (P<0.05). Compared to vehicle treatment, PD treatment did not influence clipped kidney weight. Combined C21 and PD treatment reversed the C21 effect on clipped kidney weight (P<0.05). There were no significant differences between sham and nonclipped kidney weights of the 2K1C groups.

SBP

There were no significant differences in baseline SBP between all groups (Table S1). SBP was significantly elevated in vehicle-treated 2K1C rats compared to sham control (P<0.05). C21 or PD alone or combined did not influence the elevated SBP.

Renal Expressions of AT₁ and AT₂ Receptors

Renal AT₁R mRNA and protein expressions (Figure 1A, B) were significantly increased (P<0.05) in clipped kidneys of vehicle-treated and PD-treated groups. C21 treatment significantly (P<0.05) reduced AT₁R protein but not mRNA expression in the clipped kidneys. In contrast, PD treatment did not cause significant changes in this receptor mRNA or protein expression. Combined C21 and PD reversed the reduction in AT₁R protein expression that was seen with C21 treatment alone in the clipped kidneys. AT₂R mRNA and protein expressions (Figure 1C, D) were significantly increased (P<0.05) in the vehicle-treated clipped kidneys group. AT₂R mRNA increased further (P<0.05) in clipped kidneys of rats treated with C21. In contrast, PD treatment caused significant reduction in AT₂R mRNA and protein (P<0.05) in clipped kidneys. Similarly, PD reversed the effects of C21 treatment on AT₂R mRNA and protein expression (P<0.05). In nonclipped kidneys, there were no significant changes in AT₁R or AT₂R mRNA and protein expressions.

Figure 3. Renal expression of transforming growth factor-β1 (TGF-β1) in right (R) and left (L) kidneys of sham and nonclipped (open bars) and clipped (solid bars) kidneys of 2-kidney, 1-clip (2K1C) hypertension rats treated with vehicle, compound 21 (C21), PD123319 (PD), or C21 plus PD. A, mRNA levels of TGF-β1. B, Western blot analyzes of TGF-β1. Top, Representative blots. Bottom, Quantitative results normalized to β-actin. n=5, each group. Data are mean±SEM. *P<0.05 vs sham and corresponding nonclipped kidneys. †P<0.05 vs 2K1C treated with PD.

Markers of Inflammation

There were no significant differences in TNF-α mRNA, IL-6 mRNA, and their RIF protein recovery rates between sham group and nonclipped kidneys of 2K1C groups with any treatment (Figure 2). Compared to sham group, TNF-α and IL-6 mRNA expressions and their RIF recovery rates were significantly increased (P<0.05) in clipped kidneys of the vehicle-treated group (Figure 2). In clipped kidneys, C21 treatment caused significant reduction (P<0.05) in TNF-α and IL-6 mRNA expressions and their RIF recovery rates. PD treatment did not influence these inflammatory factors. Compared to vehicle-treated rats, TNF-α mRNA, IL-6 mRNA, and IL-6 RIF protein recovery rates were reduced (P<0.05) in the clipped kidneys of the combined C21 and PD treatment group. Similarly, renal mRNA and protein expressions of TGF-β1 (Figure 3) were significantly increased (P<0.05) in the clipped kidneys of 2K1C vehicle-treated and PD-treated rats. In the clipped kidneys, renal TGF-β1 mRNA and protein expressions were significantly reduced (P<0.05) in animals treated with C21 alone and C21 combined with PD.

NO and cGMP

There were no significant differences in RIF NO (Figure 4A) and cGMP (Figure 4B) recovery rates between sham and nonclipped kidneys of 2K1C-treated groups. Compared to sham, these recovery rates were significantly reduced in clipped kidneys of vehicle-treated rats (P<0.05). In clipped kidneys, C21 treatment caused significant increase in RIF NO and cGMP recovery rates (P<0.05). In contrast, PD treatment...
Renovascular hypertension is associated with increased activity of the renin-angiotensin system and elevated blood pressure.\textsuperscript{20} In the experimental model of this disease, 2K1C Goldblatt hypertension, development of inflammation, and generation of reactive oxygen species are prominent in the ischemic kidney, followed by progressive kidney fibrosis.\textsuperscript{5,21} Because most studies in this hypertension model were conducted several weeks after renal artery clipping, the initial mechanisms that are involved in the pathological process and lead to renal loss are not well-elucidated. In this study, we aimed at evaluating early renal inflammatory process after renal clipping and its response to AT\textsubscript{2}R stimulation. Our study demonstrated an increase in the production of the inflammatory factors TNF-\textalpha, IL-6, and TGF-\textbeta1 and reduction of NO and cGMP in the ischemic kidneys 4 days after clipping of the renal artery while nonclipped kidneys were not affected. Direct pharmacological AT\textsubscript{2}R stimulation reversed this process and promoted reduction of the inflammatory markers TNF-\textalpha, IL-6, and TGF-\textbeta1 and enhanced production of NO and cGMP. These effects were independent of changes in blood pressure.

In the present study, AT\textsubscript{1}R and AT\textsubscript{2}R expressions increased in the clipped kidneys within 4 days of induction of renal ischemia. This finding suggests that in the clipped kidneys, the increased production of Ang II and the associated inflammatory process most likely contributed to the observed increase in renal AT\textsubscript{1}R and AT\textsubscript{2}R expressions. Previous studies demonstrated an increase\textsuperscript{22–25} or a decrease\textsuperscript{26} in the expression of these receptors in response to injury. This controversial information was probably influenced by the differences in the duration of injury, animal model, and/or the involved tissue. AT\textsubscript{2}R expression was shown to be upregulated in experimental models of overt renal damage\textsuperscript{27} and its activation was associated with inhibition of tissue fibrosis.\textsuperscript{27,28} Thus, our observation of early increase in renal AT\textsubscript{2}R expression after renal ischemia suggests a role for this receptor in the injury-healing process.

In our study, AT\textsubscript{2}R stimulation with C21 led to an increase in AT\textsubscript{2}R expression in the clipped kidneys. Previous studies demonstrated increased AT\textsubscript{2}R expression during the stimulation of this receptor with Ang II.\textsuperscript{29,30} This effect was at the AT\textsubscript{2}R gene promoter activity level and inhibited by blockade

Discussion

Renal interstitial fluid (RIF) levels of nitric oxide (NO; A) and cGMP (B) in right (R) and left (L) kidneys of sham and nonclipped (open bars) and clipped (solid bars) kidneys of 2-kidney, 1-clip (2K1C) hypertension rats treated with vehicle, compound 21 (C21), PD123319 (PD), or C21 plus PD. n=6, each group. Data are mean±SEM. *P<0.05 vs sham and corresponding nonclipped kidneys. †P<0.05 vs 2K1C vehicle-treated. ‡P<0.05 vs 2K1C treated with PD.

Renal Morphological Changes

Figure 5 shows hematoxylin and eosin staining of renal cortex (upper panel) and medulla (lower panel) of sham control and 2K1C groups at the end of study. Glomerular, tubular, and interstitial morphology were normal in sham group (Figure 5A). Inflammatory cell infiltration was markedly increased in clipped kidney of vehicle-treated rats (Figure 5B). C21 treatment (Figure 5C) significantly reduced the inflammatory cell infiltration in renal cortex and medulla. Renal inflammatory cells infiltration was not affected by PD treatment (Figure 5D). Combined and PD treatment caused slight reduction in the cell infiltrate of the renal tissue (Figure 5E).
with PD.\textsuperscript{31} Our data are consistent with these studies and confirm that AT\textsubscript{2}R stimulation enhances its gene activity. Thus, AT\textsubscript{2}R stimulation may have positive feedback on its expression. It is not clear why C21 treatment did not affect AT\textsubscript{2}R expression in the nonclipped kidneys. It is possible that exposure of this kidney to systemic hypertension may have prevented the upregulation of AT\textsubscript{2}R.

In the current study, we observed a trend for reduction of AT\textsubscript{1}R mRNA, although it did not reach statistical significance or a significant reduction in this receptor protein with AT\textsubscript{2}R stimulation. A previous study\textsuperscript{22} demonstrated increased AT\textsubscript{1}R expression in absence of AT\textsubscript{2}R expression and activity. Our results confirm this principal of regulation of AT\textsubscript{2}R expression by AT\textsubscript{2}R. Our report of more reduction in AT\textsubscript{1}R protein than its mRNA possibly reflects the influence of AT\textsubscript{2}R on AT\textsubscript{1}R protein synthesis and degradation.

In the present study, we report increased production of IL-6, TNF-\alpha, and TGF-\beta1 and inflammatory cell infiltration in the clipped kidney. Increased intrarenal Ang II is linked to renal inflammatory cell infiltration,\textsuperscript{22} and activation of AT\textsubscript{1}R is known to stimulate the renal production of inflammatory factors, including IL-6, TNF-\alpha, and TGF-\beta1.\textsuperscript{6,7} Enhanced renal inflammation is associated with increased tissue fibrosis and loss of kidney function.\textsuperscript{33} Pharmacological blockade of AT\textsubscript{1}R is well-documented to reduce renal inflammation and to be renoprotective in clinical and experimental settings.\textsuperscript{34,35} In contrast, activation of AT\textsubscript{2}R by C21 was reported to stimulate vasodilation,\textsuperscript{9–11} whereas its blockade was associated with reduction of renal function and production of the vasodilatory factors NO and cGMP.\textsuperscript{8–10} However, the effects of direct AT\textsubscript{2}R stimulation without influencing the AT\textsubscript{1}R in the kidney are unknown. Recently, the in vivo effects of direct AT\textsubscript{2}R stimulation by C21 on tissue inflammation were reported. In these studies, C21 infusion reduced cardiac tissue inflammation in rats after the development of myocardial infarction,\textsuperscript{16} prevented the development of inflammation and fibrosis in kidneys of spontaneously hypertensive stroke-prone rats,\textsuperscript{17} and diminished dermal toxic inflammation in mice.\textsuperscript{18} In the present study, we demonstrated reduction of renal inflammation in ischemic kidneys of 2K1C rats in response to direct AT\textsubscript{2}R stimulation. Interestingly, concomitant administration of C21 and PD treatments did not completely reverse the C21 effects on the monitored inflammatory factors. It is possible that the different affinity of these drugs for AT\textsubscript{2}R and the route of their administration could have influenced their combined effects. C21 has 25,000-times and PD has 3500-times higher affinity for AT\textsubscript{2}R than for AT\textsubscript{1}R.\textsuperscript{15} In addition, the C21 dose was administered daily as a single injection intraperitoneally, whereas the PD dose was administered over the course of 24 hours via osmotic minipump. Thus, it is possible that the AT\textsubscript{2} receptor was exposed to higher concentrations of C21 before PD.

The lack of the effect of C21 on blood pressure despite increased production of NO and cGMP is consistent with previous reports.\textsuperscript{16,17} The hypertension model used in the current study is characterized by increased Ang II production\textsuperscript{20} and increased activation of AT\textsubscript{1}R in the kidney, and systemic circulation contributed to the observed elevation of blood pressure. Another possible mechanism that could contribute to elevated blood pressure in this model is related to inactivation of NO with increased oxidative stress.\textsuperscript{36} Taken together, our results suggest that the beneficial effects of AT\textsubscript{2}R stimulation are most likely related to direct reduction in renal inflammation. Previous studies demonstrated that AT\textsubscript{2}R blockade resulted in reduction of renal NO and cGMP.\textsuperscript{10,37} However, the effects of direct AT\textsubscript{2}R stimulation on renal production of these factors as demonstrated in the current study have not been previously reported. Our current data confirm our previous findings suggesting that AT\textsubscript{2}R activation directly mediates renal vasodilatory mechanisms by stimulating renal production of NO and cGMP signaling pathways.

We conclude that direct pharmacological stimulation of AT\textsubscript{2}R by C21 reduces inflammation and stimulates renal production of NO and cGMP in ischemic kidneys of 2K1C hypertensive rats. These effects were independent of blood pressure changes. Our findings suggest a role of AT\textsubscript{2}R in reducing renovascular inflammatory responses in the 2K1C rat model.

Perspectives

The results of the present study support the concept that AT\textsubscript{2}R stimulation could be an important therapeutic tool in the management of pathological conditions associated with increased production of inflammatory factors. The novel AT\textsubscript{2}R agonist, C21, is a promising therapeutic agent that could help the treatment of a variety of renal and cardiovascular diseases.

Sources of Funding

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Disclosures

None.

References


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Angiotensin AT2 Receptor Stimulation Inhibits Early Renal Inflammation in Renovascular Hypertension
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Running Title: AT2 receptor inhibits renal inflammation

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SUPPLEMENTAL METHODS

Surgical procedures. For minipump implantation and renal artery clipping, rats were anesthetized with ketamine (80 mg/kg, IP) and xylazine (8 mg/kg, IP) and placed on a heating pad throughout the surgery period. The osmotic minipump was implanted subcutaneously in the subscapular region of all rats. Sham and 2K1C vehicle- and C21- treated rats were implanted with osmotic minipumps containing distilled water. After the exposure of the left kidney by a midline laparotomy, left renal artery was isolated and a solid U-shaped silver clip was placed around the vessel. Renal artery clipping was confirmed by a 75%-80% reduction in renal blood flow as determined by laser Doppler probe coupled to a laser Doppler Flowmeter (model ALF21D; Advance Company Ltd, Japan). Rats of sham control group received identical surgical procedure but without renal artery clipping.

In vivo renal interstitial fluid (RIF) collections. To determine the in vivo RIF recovery levels of TNF-α, IL-6, NO, and cGMP we constructed a microdialysis probe. In this technique, substances with molecular mass > 40,000 Da cannot cross the dialysis membrane. This molecular mass cutoff allows free passage of the above mentioned substances. Four days after renal artery clipping, RIF collections were performed under sodium pentobarbital anesthesia (50 mg/kg, IP; Sigma). Following a midline laparotomy, the microdialysis probes were placed in each cortex of both clipped and non-clipped kidneys. In brief, a 30-gauge needle was tunneled approximately 1-2 mm from the outer renal surface for about 0.5 cm before it exited by penetrating the capsule again. The tip of the needle was then inserted into one end of the dialysis probe, and the needle was pulled together with the dialysis tube until the dialysis fiber was situated into the renal cortex. Thereafter, the inflow tube of the dialysis probe was connected to a gas-tight syringe filled with saline and perfused at a rate of 3 μl/min using an infusion pump. After a 60-min period for stabilization following completion of surgical procedures, the effluent from each kidney was collected from the microdialysis outflow tube in nonheparinized plastic tubes placed over ice through five periods of 60-min each. At the end of each experiment, animals were euthanized and kidneys were harvested and weighed. Kidney tissue was immediately frozen in liquid nitrogen and stored at -80°C for further mRNA and protein analysis.

RIF storage and assays. Both RIF TNF-α and IL-6 recovery levels were measured using an enzyme immunoassay kit (R&D Systems, Minneapolis, MN) and is expressed as pg/min. RIF cGMP recovery levels were measured with an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) and presented as fmol/min. RIF nitric oxide recovery levels were measured using a nitrate/nitrite fluorometric assay kit (Cayman) and presented as μmol/min.

Determination of mRNA expression. Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was used to determine changes in renal expression of TNF-α, IL-6, TGF-β1, AT1R, and AT2R mRNAs. The RNA (n=5, each group) was extracted using Trizol (Invitrogen, Carlsbad, CA). Reverse transcription of the RNA was performed by the first strand cDNA synthesis kit (Bio-Rad, Hercules, CA). The PCR was analyzed using SYBR Green Supermix (Bio-Rad). Primer sequences were: 18S rRNA forward, 5'-CGAAAGCATTTGCCAAGAAT-3', reverse, 5'-AGTCGGCATCGTTTATGGTC-3'; TNF-α forward, 5'-ACTCCAGAAAAGCAAGCAA-3', reverse, 5'-
CGAGCAGGAATGAGAAGAGG-3'; IL-6 forward, 5’-CCGGAGAGGAGACTTCACAG-3’, reverse, 5’-ACAGTGCATCATCGCTGTTC-3’; TGF-β1 forward, 5’-ATACGCCTGAGTGCTGTCT-3’, reverse, 5’-TGGAAGACTGATCCCATTGATT-3’; AT1R forward, 5’-CCGTGACTGTGAAATTGCTG-3’, reverse, 5’-CCCCAGAAAGCCATAAACA-3’; and AT2R forward, 5’-TTCTTGAGGAGAAAACAGACC-3’, reverse, 5’-CTGGAAACTGTGCCCAGAAAT-3’. RT-PCR was performed using iCycler (Bio-Rad), and threshold cycle number was determined using iCycler software version 3.0 (Bio-Rad). Reactions were performed in triplicate, and threshold cycle numbers were averaged. The mRNA results for specific target genes were calculated with normalization to 18S rRNA.

**Western blot analysis.** Antibody to AT1R, AT2R, and TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA) were used in the Western blot. Signal detection was carried out by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL). The blots were treated using Restore Western Blot Stripping Buffer (Thermo Fisher) according to the manufacturer’s recommendation and followed by reprobing with a monoclonal antibody against β-actin (Sigma). Densitometry evaluation of the bands was done using ImageMaster TotalLab software version 2.0 (Amersham Pharmacia Biotech, Piscataway, NJ). The bands densities of target proteins were normalized to the corresponding density of β-actin. The arbitrary unit of band densities was represented as the expression level.
Table S1. Body and kidney weights and systolic blood pressure (SBP) of sham control and 2-kidney, 1-clip (2K1C) hypertension rats treated for 4 days with vehicle, Compound 21 (C21) or PD123319 (PD) alone and combined.

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R, right kidney; L, left kidney. Data are mean ± SEM. n=6, each group. *P<0.05 vs. sham control; †P<0.05 vs. 2K1C vehicle treatment.