Chemokine Receptor 2b Inhibition Provides Renal Protection in Angiotensin II–Salt Hypertension

Ahmed A. Elmarakby, Jeffrey E. Quigley, Jeffrey J. Olearczyk, Aarthi Sridhar, Anthony K. Cook, Edward W. Inscho, David M. Pollock, John D. Imig

Abstract—The present study was designed to determine whether chemokine receptor 2b (CCR2b) contributes to the development of renal injury in salt-sensitive angiotensin II (ANG) hypertension. Rats were infused with ANG and fed a high-salt diet (HS) for 14 days. Rats were divided into 4 groups: HS; HS administered the CCR2b antagonist, RS102895; Ang/HS hypertensive; and Ang/HS hypertensive administered RS102895. CCR2b inhibition slowed the progression of blood pressure elevation during the first week of ANG/HS hypertension; however, it did not alter blood pressure in the HS group. At 2 weeks, arterial pressure was not significantly different between ANG/HS and Ang/HS hypertensive rats administered RS102895. Renal cortical nuclear factor κB activity increased in ANG/HS hypertension compared with the HS group (0.11±0.006 versus 0.08±0.003 ng of activated nuclear factor κB per microgram of protein), and RS102895 treatment lowered nuclear factor κB activity in ANG/HS hypertension (0.08±0.005 ng of activated nuclear factor κB per microgram of protein). Renal tumor necrosis factor-α and intercellular adhesion molecule-1 expression increased, and Cyp2c23 expression decreased in ANG/HS hypertension compared with the HS group, and CCR2b inhibition reduced tumor necrosis factor-α and intercellular adhesion molecule-1 and increased Cyp2c23 expression. Histological immunostaining revealed increased renal monocyte and macrophage infiltration in ANG/HS hypertensive rats with decreased infiltration in rats receiving RS102895 treatment. Albuminuria and cortical collagen staining also increased in ANG/HS hypertensive rats, and RS102895 treatment lowered these effects. Afferent arteriolar autoregulatory responses to increasing renal perfusion pressure were blunted in ANG/HS hypertension, and RS102895 treatment improved this response. These data suggest that CCR2b inhibition protects the kidney in hypertension by reducing inflammation and delaying the progression of hypertension. (Hypertension. 2007;50:1069-1076.)

Key Words: kidney ■ inflammation ■ hypertension ■ angiotensin ■ MCP-1 ■ CCR2b ■ chemokines

Studies suggest that inflammation is involved in the progression of hypertension-induced kidney diseases.1 Cytokines have been identified as components of this inflammation. Chemokines are a class of cytokines that are involved in the proinflammatory response in both normal and pathological conditions.2 The main function of chemokines is to promote leukocyte migration to sites of injury, and this is achieved through ligand/receptor binding with receptors expressed on leukocytes.2 Through chemokine signaling, monocytes infiltrate tissue, differentiate into macrophages, and release additional chemokines perpetuating the inflammatory cycle. Monocyte chemoattractant protein-1 (MCP-1) plays a pivotal role in the development of the inflammatory response.1 MCP-1 expression increases at injury sites to direct macrophage recruitment.2 Mechanistically, MCP-1 binds to the inducible C-C chemokine receptor 2 (CCR2) to promote chemotaxis.3 There are 2 known subtypes of CCR2, CCR2a and CCR2b. CCR2b is 5-fold more potent than CCR2a in inducing macrophage chemotaxis.3,4 Recent research has focused on the role MCP-1 in kidney disease. In human progressive renal disease, MCP-1 is upregulated in the kidney, and urinary MCP-1 excretion also increases.5,6 CCR2 inhibition also reduces interstitial macrophage infiltration and attenuates renal damage in renal diseases.6–8 Furthermore, our laboratory has demonstrated previously that salt-sensitive angiotensin II (ANG) hypertensive rats have increased urinary MCP-1 excretion, as well as increased renal macrophage infiltration.9 These studies clearly suggest a role for MCP-1 in the progression of renal disease. Anti-inflammatory therapies are becoming increasingly popular for the treatment of a variety of diseases, yet most anti-inflammatory therapies only reduce inflammation that is already present. Blocking chemokine ligand/receptor binding and subsequent cell migration and infiltration represents an alternative approach in disrupting the onset of the inflammatory positive-feedback loop. Thus, we hypothesized that MCP-1 activation of the CCR2b receptor is involved in the development of high blood pressure and renal injury in the kidney.
ANG/high-salt diet (HS) model of hypertension via the increase in inflammation and macrophage infiltration.

Methods
Animal protocols were approved by the Medical College of Georgia Animal Care and Use Committee. Telemetry transmitters (Data Science Inc) were implanted according to the manufacturer’s specifications into male Sprague-Dawley rats as described previously. After a week of basal blood pressure recording, osmotic minipumps were implanted (SC) to deliver ANG at a dose of 60 mg/min for 2 weeks. Immediately after minipump implantation, normal chow diet was switched to HS (8% NaCl), and rats were divided into 4 groups: HS, HS administered the selective CCR2b antagonist, RS102895 (10 mg/kg per day) in drinking water, ANG/HS, and ANG/HS administered RS102895 in drinking water. RS102895 (Sigma) is a novel member of the siropipiderine class with potent and specific CCR2b antagonist properties, and this class of compounds has been shown to inhibit MCP-1/chemokine receptor 2 signaling in vivo in rodents. In a separate set of experiments, osmotic minipumps were implanted (SC) to deliver ANG at 60 mg/min for 2 weeks. Rats were fed an HS and divided into 3 groups: HS, ANG/HS, and ANG/HS administered RS102895. After 2 weeks of ANG infusion, rats were euthanized, and the renal cortex was collected, subdivided into 3 tubes, and snap frozen in liquid nitrogen for nuclear factor κB (NFκB) assay, real-time PCR, and Western blotting. Urinary albumin excretion was measured using a highly sensitive immunoassay SPI-BIO kit (Cayman Chemical), and urinary MCP-1 excretion was also determined using a commercially available immunoassay kit (BD Biosciences).

NFκB Transcription Factor Assay
Whole-cell lysates were obtained from the kidney cortex from the above-mentioned groups using the nuclear extract kit (Active Motif). Protein concentrations were determined using a bicinchoninic acid protein assay (Pierce). Twenty micrograms of whole-cell extract were used for the determination of NFκB activity using the TransAM NFκB p65 transcription factor assay kit (Active Motif). The amount of activated NFκB was normalized per microgram of cortical protein.

Real-Time PCR
Total RNA was also isolated from 100 mg of kidney cortex using the ultrapure TRIzol method as described previously, and RNA concentration was determined by measuring absorbance at 260 nm. A mixture of oligo(dT) and random hexanucleotide primers was used in the reverse transcription of equal amounts of total RNA (3 μg) using the iScript cDNA synthesis kit (Bio-Rad Laboratories). TaqMan and Molecular Beacon real-time PCR were used to quantify the mRNA expression of intercellular adhesion molecule-1 (ICAM-1) and NFκB relative to control animals as described previously. For a more detailed Methods section, please see http://hyper.ahajournals.org.

Homogenization of Renal Cortex for Protein Expression
Kidney cortex was dissected quickly in ice-cold homogenization buffer in the presence of protease inhibitors and frozen in liquid nitrogen for determination of ICAM-1, tumor necrosis factor (TNF-α), Cyp2c23, and soluble epoxide hydrolase (sEH) protein expression using Western blotting, as described previously. Band intensity was measured densitometrically, and the values were normalized to β-actin. For a more detailed Methods section, please see the data supplement.

Evaluation of Cortical ED-1 and Collagen Staining
Kidneys from HS-, ANG/HS hypertension-, and ANG/HS hypertension-administered RS102895 groups were perfused with 10% formalin solution and were then paraffin embedded and cut into 4- to 5-μm sections. Kidney sections were used for immunohistochemical evaluation of ED-1 staining for monocyte/macrophage infiltration, as described previously. Ten microscopic images of kidney cortex per rat were randomly taken (×400), and CD68-positive cells were counted by a blinded reviewer experienced in ED-1 staining analysis. The number of positive cells per millimeter squared was calculated and averaged for each group. Additional kidney sections were stained with Masson’s trichrome and picrosirius red to assess the amount of collagen within the kidney cortex of the 3 rat groups. Ten randomly selected microscopic images of the kidney cortex were studied per each rat (×100). Scoring of slides was performed blindly and graded on a scale of 1 to 10.

Renal Autoregulation Study
Rats were classified into 3 groups (HS-, ANG/HS-, and ANG/HS-administered RS102895), and experiments were conducted using the blood-perfused juxtedudillary nephron technique after 2 weeks of ANG infusion, as described previously. Fifteen rats were used for kidney microdissection, and 15 rats were used as blood donors. Perfusion pressure was initially set at 100 mm Hg for control measurements and was decreased to 65 mm Hg, where afferent arteriolar diameter was measured continuously as perfusion pressure was increased in 15-mm Hg increments from 65 to 170 mm Hg. Afferent arteriolar responses to changes in renal perfusion pressure were determined as described previously. For a more detailed Method section, please see the data supplement.

Statistical Analysis
All of the data are presented as mean±SEM. Data were analyzed using 1-way ANOVA followed by Tukey’s posthoc test for multiple-group comparisons. Differences were considered statistically significant, with P<0.05 versus the control. Analyses were performed using GraphPad Prism version 4.0 software.

Results
Male Sprague-Dawley rats fed an HS exhibited a modest elevation in mean arterial pressure (MAP), and the same trend was apparent in RS102895-treatment rats (Figure 1A). MAP increased significantly in rats receiving an ANG infusion and fed an HS for 2 weeks. RS102895 reduced the elevation in MAP in the first week of ANG/HS hypertension; however, it did not exhibit any significant effect on MAP at the end of the 2 weeks (Figure 1A). Heart rate was initially reduced but then increased after a few days in ANG/HS-hypertensive rats (Figure 1B). This initial decrease in heart rate was greater in ANG/HS-hypertensive rats treated with RS102895 and was not restored to the same level as that of the ANG/HS-hypertensive group (Figure 1B).

NFκB, ICAM-1, MCP-1 excretion, albuminuria, monocye/macrophage infiltration, and collagen deposition were used as indicators of inflammation in the kidney. NFκB mRNA expression was increased 2-fold in the renal cortex of ANG/HS-hypertensive rats compared with HS rats. RS102895 treatment decreased renal cortical NFκB mRNA expression in ANG/HS hypertension to the same range as that in HS rats (Figure 2A). Consistent with these data, renal cortical NFκB activity was significantly higher in ANG/HS-hypertensive rats compared with HS rats, and CCR2b inhibition reduced NFκB activity in ANG/HS hypertension (Figure 2B). Renal cortical ICAM-1 mRNA and protein expression also increased in ANG/HS hypertension, and this increase was attenuated by RS102895 treatment (Figure 3). MCP-1 excretion and renal cortical MCP-1 expression increased in ANG/HS-hypertensive rats, and CCR2b inhibition did not statistically affect these changes (please see Figures S1 and S2).
Renal monocyte/macrophage infiltration was also determined immunohistochemically in rat kidney sections. ANG/HS-hypertensive rats showed a significant increase in cortical CD68-positive staining compared with HS-fed rats. Blocking CCR2b with RS102895 treatment lowered cortical macrophage infiltration in ANG/HS hypertension (Figure 4A).

Albuminuria was increased in ANG/HS hypertension compared with HS rats (125 ± 51 versus 51 ± 2 mg/d), and CCR2b inhibition lowered urinary albumin excretion to 51 ± 20 mg/d in ANG/HS hypertension (Figure 4C). Renal cortical collagen staining was also increased in ANG/HS-hypertensive rats compared with that in the HS group, and RS102895 reduced collagen staining in ANG/HS-hypertensive rats (Figure 5).

Renal cortical Cyp2c23 protein expression was decreased in ANG/HS-hypertensive rats compared with HS rats (Figure 6A). Blocking CCR2b increased Cyp2c23 protein expression to levels similar to HS rats. Renal cortical sEH protein expression was not significantly altered in ANG/HS-hypertensive rats with or without RS102895 treatment (Figure 6B).

Renal cortical TNF-α protein expression also significantly increased in ANG/HS-hypertensive rats, and this effect was reduced with CCR2b inhibition (Figure 7B). We also determined the effect of blocking CCR2b in pressure-mediated renal autoregulatory responses in ANG/HS hypertension (Figure 7A). Afferent arteriolar diameter averaged 13.7 ± 1.2 μm at 100-mm Hg renal perfusion pressure and decreased by 13% and 18% when renal perfusion pressure was increased to 140 and 170 mm Hg, respectively, in rats fed an HS. In ANG/HS-hypertensive rats, renal autoregulatory responses were impaired compared with HS rats. Afferent arteriolar diameter averaged 13.9 ± 0.4 μm and decreased by 2% and 7% when renal perfusion pressure was increased to 140 and 170 mm Hg, respectively in ANG/HS hypertension. RS102895 treatment improved the afferent arteriolar autoregulatory response in ANG/HS hypertension. Afferent arteriolar diameter in ANG/HS/RS102895 rats averaged 14 ± 0.2 μm and decreased by 14% and 20% when renal perfusion pressure was increased to 140 and 170 mm Hg, respectively.

Discussion

Macrophage infiltration into the kidney is thought to mediate renal injury via the production of proinflammatory cytokines. The chemokine MCP-1 is a potent macrophage chemoattractant involved in macrophage recruitment and infiltration in renal disease. MCP-1 promotes monocyte chemotaxis and macrophage infiltration via 2 receptor subtypes, CCR2a and CCR2b, with MCP-1’s affinity to the latter being 5 times that of the former. To study the effects of CCR2b inhibition in vivo in hypertension, we treated ANG/HS...
neointimal hyperplasia. Studies have also shown that the migration of vascular smooth muscle cells, resulting in increases the production of adhesion molecules and stimulates inflammation and arteriosclerosis in rats made hypertensive. However, the blood pressure–lowering effect of CCR2b inhibition was somewhat unexpected. This could be related to the anti-inflammatory effects of RS102895, eg, reduced renal NFκB activation and reduced renal TNF-α and ICAM-1 expression. Rodriguez-Iturbe et al reported previously that NFκB inhibition prevents hypertension in spontaneously hypertensive rats. Although there is evidence that MCP-1 is downstream from NFκB, MCP-1/CCR2 signaling is also believed to feedback and enhance NFκB activation. Thus, one possible mechanism for the slowing of blood pressure elevation in the present study is the anti-inflammatory effect, possibly via inhibition of MCP-1–driven NFκB activation. A nonspecific blood pressure effect is unlikely, because the CCR2b antagonist did not alter blood pressure in normotensive rats. RS102895-treated hypertensive rats also experienced a decreased heart rate, but again this did not occur in treated normotensive controls. MCP-1 inhibition has been shown to improve cardiac function in a rabbit model of ischemia-reperfusion injury and to reduce left ventricular dysfunction with a modest decrease in heart rate in a murine model of postmyocardial infarction heart failure. Therefore, heart rate and blood pressure changes are due to the effects of RS102895 in hypertensive conditions. Our data suggest that a chemokine-mediated inflammatory component is involved in the onset of blood pressure in ANG salt-sensitive hypertension, although it is not necessary for the maintenance of high blood pressure.

Previous studies have shown that ANG stimulates the release of cytokines and growth factors. ANG also increases the expression of chemokines, including MCP-1, that mediates vascular inflammation. High-salt treatment exacerbates ANG-induced elevation in blood pressure and renal injury. ANG can also induce the expression of cell adhesion molecules via the activation of NFκB. In our study, CCR2b inhibition lowered NFκB activity and expression in ANG/HS hypertension, and renal cortical ICAM-1 expression was also reduced. Consistent with our data, Giunti et al have reported that MCP-1 induced ICAM-1 expression in human mesangial cells, and CCR2b inhibition with RS102895 prevented ICAM-1 upregulation. Muller et al have also shown that NFκB inhibition ameliorates renal and cardiac ANG-induced inflammatory damage in rats. NFκB inhibition also prevents hypertension and reduces renal ICAM-1 and MCP-1 inflammatory responses in spontaneously hypertensive rats. Thus, it is possible that reducing NFκB activity by inhibiting CCR2b could account for the delay in the progression of hypertension and decreased ICAM-1 and renal damage in ANG/HS hypertension.

The role of macrophages in the progression of renal injury is well established. Macrophages could mediate renal injury via different mechanisms, including the production of proinflammatory cytokines. Previous studies have shown that MCP-1–deficient mice have a reduction in aortic wall macrophage infiltration and macrophage infiltration during inflammation. We have shown previously that MCP-1 excretion and kidney macrophage infiltration increased in ANG/HS hypertension. In the present study, MCP-1 excretion and renal cortical MCP-1 expression also increased in ANG/HS-hypertensive rats, and CCR2b inhibition did not significantly affect MCP-1 excretion or expression (please see Figures S1 and S2). Macrophage infiltration also significantly decreased in the kidney of the CCR2-deficient mice after ischemia-reperfusion injury compared with wild-type mice. In our study, renal cortical monocyte/macrophage infiltration in-
creased in ANG/HS-hypertensive rats, and this effect was attenuated by CCR2b blockade. We also showed that collagen deposition increased in the kidney of ANG/HS hypertensive rats, and CCR2b inhibition reduced collagen staining. These data further support the concept that MCP-1 activation of CCR2b is involved in the inflammatory response associated with ANG/HS hypertension.

RS102895 treatment slowed the elevation in blood pressure in ANG/HS-hypertensive rats in the first week; however, this effect was not maintained to the end of the 2-week ANG/HS treatment period. This finding indicates that the relative contribution of MCP-1–induced inflammation to blood pressure is short term. Despite being temporary, the blood pressure lowering was adequate to blunt renal damage at the end of the 2-week ANG/HS treatment period. It is likely that the slowing in blood pressure elevation only delays but does not prevent the manifestation of hypertension-induced renal injury. In addition, we also have evidence that TNF-α inhibition reduces renal injury and inflammation despite the lack of blood pressure–lowering effects in desoxicorticosterone acetate-salt hypertensive rats (unpublished data). These data suggest that the decrease in renal injury is mainly because of a reduction in inflammation rather than reducing blood pressure.

An alternative to MCP-1 feedback inhibition as a possible mechanism for reduction in NFκB activity and lowering of blood pressure may be the arachidonic acid–derived epoxygenic acids (EETs). The EETs possess anti-inflammatory along with vasodilatory properties and have the ability to block the induction of NFκB activation.24 Cytokines and, thus, inflammation in general can downregulate the Cyp450 epoxygenases, which catalyze EET production in the kidney.28 The inability to properly upregulate these enzymes is associated with hypertension and end-organ damage in ANG hypertension.9,29 In the present study, we found that renal epoxygenase expression was lower in ANG/HS-hypertensive rats, but expression was appropriate in those that received RS102895 treatment. The EETs can also be metabolized to an inactive molecule by sEH, and studies have shown that sEH inhibitors are effectively antihypertensive and renal protective in ANG hypertension.30 We found that expression of sEH was slightly increased in the ANG/HS-hypertensive rats but not in those treated with RS102895. EETs could, therefore, be a possible link between inflammation and blood pressure in the present study.

Previous studies have shown that TNF-α activates the NFκB inflammatory pathway,26,31 and we have shown previously that TNF-α inhibition slowed the progression of hypertension, reduced renal injury, and restored the decrease in Cyp2c23 expression in ANG/HS-hypertensive rats, suggesting a role of TNF-α in blood pressure elevation and renal injury in this model.3 CCR2 knockout mice have been shown to have reduced TNF-α activation in myocardial and cerebral ischemic-reperfusion models compared with wild-type mice.32,33 In our study, CCR2b inhibition prevented the increase in renal cortical TNF-α expression in ANG/HS-hypertensive rats. These data suggest that reducing MCP-1 signaling activation with RS102895 treatment might inhibit positive feedback stimulation of TNF-α, which, in turn, inhibits NFκB activation and subsequent downregulation of Cyp2c23 (please see supplemental Figure S3). We also observed a reversal of afferent arteriolar autoregulatory dysfunction in the kidneys of CCR2b inhibitor–treated hypertensive animals. Under normal physiological conditions, afferent arterioles constrict or relax in response to changes in arterial pressure to maintain the glomerular capillary pressure and glomerular filtration rate at appropriate levels; this is impaired in ANG/HS hypertension, resulting in glomerular
charging. In a previous study, increased blood pressure was sufficient to cause the impairment, because responsiveness is restored with triple antihypertensive therapy treatment and ANG receptor subtype 1 blockade in ANG hypertensive rats. However, physiological changes because of the high blood pressure itself, such as vascular inflammation, cannot be ruled out. Sharma et al. found that the cytokine transforming growth factor-β1 completely blocked afferent arteriolar autoregulatory responsiveness through the generation of reactive oxygen species. In our study, restoration of afferent arteriolar autoregulatory responsiveness with CCR2b antagonist treatment in ANG/HS hypertension may, therefore, be attributed, at least in part, to both the slow in the progression of hypertension and the anti-inflammatory effects.

**Perspectives**

Overall, these data suggest that MCP-1-mediated inflammatory response, specifically through the activation of CCR2b, contributes to the onset of hypertension and renal inflamma-

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**Figure 5.** Effect of CCR2b inhibition on collagen deposition in kidney cortex of ANG/HS-hypertensive rats. A, Masson’s trichrome scoring on a scale of 1 to 10 and representative images (×100) of the blue staining for kidney cortex of HS and ANG/HS-hypertensive rats with or without RS102895 treatment. B, Picrosirius red scoring on a scale of 1 to 10 and representative images (×100) of the picrosirius red staining for the kidney cortex of HS and ANG/HS-hypertensive rats with or without RS102895 treatment. *P < 0.05 vs HS group; #P < 0.05 vs ANG/HS-hypertensive group.

**Figure 6.** Effect of CCR2b inhibition on renal cortical Cyp2c23 and sEH expression in ANG/HS hypertension. Renal Cyp2c23 (A) and sEH protein expression (B) normalized to β-actin in ANG/HS-hypertensive rats with or without RS102895 treatment (n=5 per group). Values are mean ± SEM. *P < 0.05 vs HS group.
tion and leads to impaired afferent arteriolar autoregulation and renal injury in ANG/HS hypertension. These effects may be because of increased renal monocyte/macrophage infiltration, vascular inflammation, enhancement of the NFκB inflammatory signaling, and decreased epoxyenase expression. Although additional studies will be needed to detail the inflammatory signaling, and decreased epoxygenase expression (A) and autoregulatory response of afferent arterioles (B) in ANG/HS-hypertensive rats. The percentage changes of afferent arteriolar diameter to the increase in renal perfusion pressure are shown in kidneys of HS, ANG/HS hypertension, and ANG/HS hypertension administered RS102895 (n = 5 per group). Values are mean ± SEM. *P < 0.05 vs HS group; #P < 0.05 vs ANG/HS-hypertensive group.

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Disclosures
None.

References


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Supplementary Method Section

Real Time PCR probes and primer sequences

Probes and primer sequences that were used for real time PCR are as follows:

NFκB probe 5’-FAM- CGC GAT CAC TAA ATC CAA CAC AGG CAT CAC CCA GAT CGC G-BHQ-3’; NFκB forward 5’- GTA TGG CTT CCC GCA CTA TGG -3’; NFκB reverse 5’- TCG TCA CTC TTG GCA CAA TCT C -3’; ICAM-1 probe 5’- FAM-CCT CCT CCT GAG CCT TCT GTA ACT TGT A-BHQ- 3’; ICAM-1 Forward 5’- GTA CTG ATT GCG GGC TT- 3’; ICAM-1 reverse 5’- GGG GCT TGT ACC TTG AGT TT- 3’ GAPDH probe 5’- FAM- ACT CCA CGA CAT ACT CAG CAC CAG CA- BHQ- 3’; GAPDH forward 5’- CAC GGC AAG TTC AAC GGC- 3’; GAPDH reverse 5’- GGT GGT GAA GAC GCC AGT A- 3’.

Western blotting antibodies

The primary antibodies used were goat anti-rat ICAM-1 (1:500; R&D, Minneapolis, MN), mouse anti-rat TNF-α (1:500; R&D, Minneapolis, MN), rabbit anti-rat Cyp2c23 (1:2000; Dr. Capdevila, Nashville, TN), and rabbit anti-mouse sEH (1:2000; Dr. Hammock, Davis, CA), respectively. Donkey anti-goat, goat anti-mouse, and goat anti-rabbit (Santa Cruz, CA) were used as secondary antibodies for ICAM-1, TNF-α, and Cyp2c23 & sEH, respectively.

Evaluation of cortical ED-1 staining

Kidney sections were incubated overnight at room temperature with mouse anti-rat CD-68 primary antibody 1:100 (Serotec, Raleigh, NC) followed by the secondary antibody goat anti-mouse IgG HRP 1:50 (Serotec, Raleigh, NC) for 1 hour. Slides were then incubated
with AEC substrate chromogen (DAKO, Carpinteria, CA) for 20 minutes, rinsed, and counterstained with Mayer’s Hematoxylin for 30 seconds.

Renal autoregulation study

Rats were classified into three groups (HS, ANG/HS, ANG/HS administered RS102895) and experiments were conducted using the blood-perfused juxtamedullary nephron technique after 2 weeks of ANG infusion as previously described.¹ Fifteen rats were used for kidney micro-dissection and fifteen rats were used as blood donors. After the micro-dissection procedures, the kidneys were perfused with blood collected and prepared from donor rats. The blood perfusate was stirred continuously in a closed reservoir while being oxygenated with a 95% O2-5% CO2 gas mixture. Perfusion pressure was continuously monitored using a pressure cannula positioned in the tip of a double-barreled perfusion cannula in the renal artery. The pressure cannula was connected to a calibrated pressure transducer linked to a polygraph recorder. The inner cortical surface of the kidney was continuously superfused with warmed (37°C) Tyrode's buffer containing 10.0 g/l BSA, and the kidney was allowed to equilibrate for at least 15 min. Perfusion pressure was initially set at 100 mmHg for control measurements and was decreased to 65 mmHg where afferent arteriolar diameter was measured continuously as perfusion pressure was increased in 15 mmHg increments from 65-170 mmHg. Afferent arteriolar responses to changes in renal perfusion pressure were determined as previously described.¹
Reference


Figure Legends

Figure S1: Urinary MCP-1 excretion in ANG/HS hypertensive rats with or without RS102895 treatment (n=5-6 per group). Values are means±SEM. * P< 0.05 vs. HS group.

Figure S2: Renal cortical MCP-1 expression normalized to ß-actin in ANG/HS hypertensive rats with or without RS102895 treatment (n=4-5 per group). Values are means±SEM. * P< 0.05 vs. HS group.

Figure S3: Schematic diagram of the proposed inflammatory cytokines signaling pathway in ANG/HS hypertension.
Figure S1:

Comparison of MCP-1 excretion (ng/day) across different conditions: HS, ANG/HS, and ANG/HS/RS102895. The ANG/HS condition shows a significantly higher MCP-1 excretion compared to HS and ANG/HS/RS102895 (indicated by the asterisk).
Figure S2

The diagram shows a Western blot analysis of MCP-1 and β-actin expression under different conditions: HS, ANG/HS, and ANG/HS/RS102895. The bar graph indicates a significant increase in MCP-1 (DU) expression in the ANG/HS group compared to the other conditions, as marked by an asterisk (*).
Figure S3: