Critical Role of CXCL16 in Hypertensive Kidney Injury and Fibrosis

Yunfeng Xia, Mark L. Entman, Yanlin Wang

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Abstract—Recent evidence indicates that inflammation plays a critical role in the initiation and progression of hypertensive kidney disease. However, the signaling mechanisms underlying the induction of inflammation are poorly understood. We found that chemokine (C-X-C motif) ligand 16 (CXCL16) was induced in renal tubular epithelial cells in response to angiotensin II in a nuclear factor-κB–dependent manner. To determine whether CXCL16 plays a role in angiotensin II–induced renal inflammation and fibrosis, wild-type and CXCL16 knockout mice were infused with angiotensin II at 1500 ng/kg per minute for up to 4 weeks. Wild-type and CXCL16 knockout mice had comparable blood pressure at baseline. Angiotensin II treatment led to an increase in blood pressure that was similar between wild-type and CXCL16 knockout mice. CXCL16 knockout mice were protected from angiotensin II–induced renal dysfunction, proteinuria, and fibrosis. CXCL16 deficiency suppressed bone marrow–derived fibroblast accumulation and myofibroblast formation in the kidneys of angiotensin II–treated mice, which was associated with less expression of extracellular matrix proteins. Furthermore, CXCL16 deficiency inhibited infiltration of F4/80+ macrophages and CD3+ T cells in the kidneys of angiotensin II–treated mice compared with wild-type mice. Finally, CXCL16 deficiency reduced angiotensin II–induced proinflammatory cytokine expressions in the kidneys. Taken together, our results indicate that CXCL16 plays a pivotal role in the pathogenesis of angiotensin II–induced renal injury and fibrosis through regulation of macrophage and T cell infiltration and bone marrow–derived fibroblast accumulation. (Hypertension. 2013;62:1129-1137.) • Online Data Supplement

Key Words: angiotensin II • chemokines • fibrosis • inflammation • NF-κB

Hypertension is a major cause of chronic kidney disease (CKD). A prominent pathological feature in patients with hypertensive kidney disease is inflammation, tubular atrophy, and renal fibrosis. The degree of renal fibrosis correlates well with the prognosis of CKD.1 Renal interstitial fibrosis is characterized by substantial fibroblast activation and excessive production and deposition of extracellular matrix (ECM), which leads to the destruction of renal parenchyma and progressive loss of kidney function. The current therapeutic options in the clinical setting for this devastating condition are limited and often ineffective except for dialysis or kidney transplantation, thus making chronic kidney failure one of the most expensive diseases to treat on a per patient basis.2 Despite improvement in the knowledge of diverse aspects of CKD, the initial molecular events leading to chronic renal fibrosis and eventually chronic renal failure remain elusive. Therefore, a better understanding of the cellular and molecular mechanisms underlying the initiation and progression of CKD is essential for developing effective strategies to treat this devastating disorder and prevent its progression.

A large body of evidence indicates that activation of the renin–angiotensin system plays a central role in the pathogenesis of CKD.3 The underlying mechanisms involved in angiotensin II (Ang II)–induced hypertensive kidney disease are incompletely understood. Recent studies have shown that inflammatory and immune cell infiltration and altered chemokine and cytokine production are characteristic for hypertensive kidney damage.4,5 The infiltration of circulating cells into sites of injury is mediated by locally produced chemokines through interaction with their respective receptors.6 Chemokines are divided into 4 subfamilies, CXC, CC, C, and CX3C, according to the number and spacing of conserved cysteine residues in their sequences.6 Chemokine (C-X-C motif) ligand 16 (CXCL16) is a recently discovered cytokine belonging to the CXC chemokine subfamily.7 There are 2 forms of CXCL16. The transmembrane form of CXCL16 functions as an adhesion molecule for CXCR6-expressing cells, whereas the soluble form of CXCL16 mediates infiltration of circulating cells into sites of injury.8,9 In this study, we
investigated the role of CXCL16 in renal injury and fibrosis in Ang II–induced hypertensive kidney disease using CXCL16 knockout (KO) mice.

Materials and Methods
A full description of animals and methods can be found in the online-only Data Supplement.

Results
Ang II Induces CXCL16 Expression
To determine whether Ang II regulates CXCL16 gene expression, mouse kidney tubular epithelial cells were treated with various concentrations of Ang II for different periods of time. Western blot analysis showed that Ang II treatment significantly increased CXCL16 protein expression in vitro in a dose- and time-dependent manner (Figure 1A–1D).

We then determined whether CXCL16 was induced in the kidney in response to Ang II infusion. Quantitative real-time reverse transcriptase polymerase chain reaction showed that mRNA level of CXCL16 was upregulated in the kidneys after Ang II treatment compared with that of control kidneys (Figure 1E). To identify the cell type responsible for CXCL16 production in the kidney, serial kidney sections were stained with an anti-CXCL16 antibody. The results revealed that CXCL16 protein was mainly induced in tubular epithelial cells of Ang II–treated kidneys (Figure 1F).

Because nuclear factor-κB (NF-κB) is a master regulator of inflammation, we next evaluated whether NF-κB mediates Ang II–induced CXCL16 gene expression in vitro. Mouse kidney tubular epithelial cells were pretreated with NF-κB inhibitor SN50 or its inactive control peptide SN50M for 30 minutes and then treated with Ang II at 100 nmol/L or vehicle for 24 hours. Western blot analysis showed that SN50 abolished Ang II–induced CXCL16 gene expression (Figure 2A and 2B). These data indicate that NF-κB plays an essential role in Ang II–induced CXCL16 expression in kidney tubular epithelial cells.

To verify that NF-κB mediates Ang II–induced CXCL16 expression, mouse tubular epithelial cells were transduced with a recombinant adenovirus expressing a dominant negative form of IκB kinase (IKKβ), an upstream signaling of NF-κB. Cells transduced with a recombinant adenovirus expressing eGFP were used as controls. Western blot analysis showed that ectopic expression of dominant negative IKKβ completely blocked Ang II–induced CXCL16 expression (Figure 2C and 2D).

Blood Pressure
To determine the functional significance of CXCL16 induction in the kidney, wild-type (WT) and CXCL16 KO mice were treated with vehicle or Ang II for 4 weeks. There were no significant differences in systolic blood pressure among the 4 groups at baseline. Ang II treatment led to an increase in systolic blood pressure in both WT and CXCL16 KO mice that was comparable between the 2 treatment groups (Figure 3A).

Renal Function
Treatment with Ang II for 4 weeks caused kidney dysfunction in WT mice as reflected by significant elevation of blood urea nitrogen. Kidney function was preserved in CXCL16 KO mice with blood urea nitrogen markedly lower than WT mice (Figure 3B).

Albuminuria
WT mice developed massive albuminuria after Ang II treatment for 4 weeks, whereas Ang II–treated CXCL16 KO mice produced significantly less albuminuria (Figure 3C).

Kidney Injury and Fibrosis
To assess the effect of CXCL16 deficiency on Ang II–induced hypertensive kidney damage, kidney sections were stained with periodic acid-Schiff and scored for histological injury after 4 weeks of saline or Ang II infusion. On a semiquantitative scale that includes glomerulosclerosis, interstitial disease, fibrosis, and vascular injury, the 2 saline-infused groups had minimal kidney damage (Figure 4A; Table). Ang II caused a marked increase in the severity of kidney injury in the WT mice, which was substantially reduced in CXCL16 KO mice (Figure 4A; Table). Sirius red staining showed that Ang II–treated WT mice developed significant collagen deposition in the kidney.

Figure 1. Angiotensin II (Ang II) induces chemokine (C-X-C motif) ligand 16 (CXCL16) expression. Ang II time-dependently increases CXCL16 protein expression in kidney tubular epithelial cells (A and B). Ang II dose-dependently increases CXCL16 protein expression in kidney tubular epithelial cells (C and D). *P<0.05 vs vehicle controls (CON). n=4 per group for B and D. Ang II induces CXCL16 mRNA in the kidneys (E). **P<0.01 vs vehicle-treated controls. n=6 per group. Representative photomicrographs of kidney sections stained for CXCL16 (green) and DAPI (blue; F). Scale bar, 25 μm.
compared with saline-treated WT mice (Figure 4B and 4C). These fibrotic responses were significantly reduced in CXCL16 KO mice after 4 weeks of Ang II infusion (Figure 4B and 4C).

**CXCL16 Deficiency Attenuates ECM Protein Expression**

We next examined the effect of CXCL16 deficiency on the expression and accumulation of collagen I and fibronectin, 2 major components of ECM. Immunofluorescence and Western blot analysis demonstrated that CXCL16 deficiency attenuated the upregulation of collagen I and fibronectin in the kidneys after 4 weeks of Ang II infusion (Figure 5). These data indicate that CXCL16 deficiency inhibits Ang II–induced ECM protein expression.

**Myeloid Fibroblasts Accumulation**

Recent evidence indicates that myeloid fibroblasts contribute significantly to the pathogenesis of renal fibrosis. To examine the effect of CXCL16 deficiency on the accumulation of myeloid fibroblasts in the kidney, WT and CXCL16 KO mice were infused with vehicle or Ang II for 2 weeks. Kidney sections were stained for CD45 and procollagen I and examined with a fluorescence microscope. The results showed that the number of bone marrow–derived fibroblasts dual positive for CD45 and procollagen I was significantly reduced in the kidneys of Ang II–treated CXCL16 KO mice compared with WT mice (Figure 6A and 6B). These data indicate that CXCL16 has an important role in recruiting bone marrow–derived fibroblasts into the kidney in response to Ang II.

To determine whether CXCL16 deficiency influences myofibroblast population, kidney sections were stained for α-smooth muscle actin (α-SMA), a marker of myofibroblasts, and examined with a fluorescence microscope. The results revealed that Ang II–treated CXCL16 KO mice exhibited a significant reduction in the number of α-SMA+ myofibroblasts in the kidneys compared with Ang II–treated WT mice (Figure 6C and 6D). Consistent with these findings, Western blot analysis showed that CXCL16 deficiency significantly reduced the protein expression levels of α-SMA in the kidneys after Ang II treatment compared with WT mice (Figure 6E and 6F). These results indicate that CXCL16 deficiency reduces the population of myofibroblasts in the kidney.

**Macrophage and T Cell Infiltration**

To examine whether CXCL16 plays a role in the regulation of inflammatory cell infiltration into the kidney, WT and CXCL16 KO mice were infused with vehicle or Ang II for 2 weeks. Kidney sections were stained for F4/80 and CD3. Significant infiltration of macrophages and T cells was observed in the kidneys of WT mice after Ang II treatment compared with the vehicle-treated control group (Figure 7). In comparison, CXCL16 deficiency significantly inhibited macrophage and T cell infiltration into the kidneys after Ang II treatment (Figure 7). These results indicate that CXCL16 plays a critical role in recruiting inflammatory cell into the kidney in Ang II–induced hypertensive kidney disease.

**Proinflammatory Cytokine Production**

We next examined the effect of CXCL16 deficiency on the expression of known proinflammatory cytokines that are involved in the pathogenesis of kidney injury. The mRNA levels of interleukin (IL)-6, tumor necrosis factor-α, IL-1β, and transforming growth factor-β1 (TGF-β1) were increased significantly in the kidneys of WT mice after Ang II treatment (Figure 8). In contrast, the upregulation of IL-6, tumor necrosis factor-α, IL-1β, and TGF-β1 was greatly attenuated in the kidneys of CXCL16 KO mice after Ang II treatment (Figure 8). These results suggest that CXCL16 regulates proinflammatory cytokine expression in the kidney in response to Ang II.
In this study, we have demonstrated that Ang II induces CXCL16 gene expression in kidney tubular epithelial cells via activation of NF-κB, and deletion of CXCL16 led to renal protection in an experimental model of chronic progressive kidney disease induced by Ang II infusion. In the Ang II–induced hypertension model, genetic deletion of CXCL16 preserves renal function, reduces urinary albumin excretion, and diminishes tubulointerstitial disease, glomerular injury, and vascular damage. It is noteworthy that genetic deletion of CXCL16 has no effect on arterial blood pressure. These findings are significant because renal protection is achieved even in the setting of sustained hypertension.

Mounting evidence indicates that activation of renin-angiotensin system plays a central role in the development of CKD. The underlying mechanisms involved in Ang II–induced hypertensive kidney disease are incompletely understood. Recent studies have shown that inflammatory and immune cell infiltration and altered chemokine production are characteristic for hypertensive kidney disease. The infiltration of circulating cells into sites in injury is mediated by locally produced chemokines through interaction with their respective receptors. Chemokines are a group of structurally related cytokines that are classified into 4 major subfamilies, CC, CXC, C, and CX3C, based on amino-terminal cysteine motif. CXCL16 is a newly discovered chemokine that belongs to the CXC chemokine subfamily. There are 2 forms of CXCL16. The transmembrane form of CXCL16 is composed of a CXC chemokine domain, a mucin-like stalk, a transmembrane domain, and a cytoplasmic tail. The soluble form of CXCL16 resulting from cleavage by ADAM10 (a disintegrin and metalloproteinase domain-containing protein 10) at the cell surface is composed of the extracellular stalk and the chemokine domain. The transmembrane form of CXCL16 functions as an adhesion molecule for CXCR6 (the only known receptor for CXCL16) expressing cells, whereas the soluble form of CXCL16 functions as a chemoattractant to promote migration of CXCR6-expressing cells including T cells, monocytes, and myeloid fibroblasts. CXCL16 has been reported to be expressed...
However, its role in the pathogenesis of Ang II–induced renal injury is not known. In the present study, we demonstrate that Ang II induces CXCL16 gene expression in tubular epithelial cells in a time- and dose-dependent fashion. Furthermore, inhibition of NF-κB signaling with a pharmacological inhibitor or dominant negative IKKβ abolishes Ang II–induced CXCL16 gene expression. These data indicate that NF-κB signaling mediates Ang II–induced CXCL16 gene expression in kidney tubular epithelial cells. We provide experimental evidence that CXCL16 is pathologically important in the development of renal injury and fibrosis because deletion of CXCL16 inhibits the recruitment of bone marrow–derived fibroblasts, macrophages, and T cells into the kidney and the development of renal interstitial fibrosis and proteinuria in response to Ang II treatment. These data indicate that CXCL16 signaling plays an important role in the recruitment of bone marrow–derived fibroblasts, macrophages, and T cells into the kidney and pathogenesis of renal injury.

A significant finding of this study is the marked reduction in Ang II–induced renal interstitial fibrosis in CXCL16 KO mice. Renal interstitial fibrosis is a hallmark of CKD, and the degree of interstitial fibrosis strongly predicts the prognosis of CKD. Renal interstitial fibrosis is characterized by substantial fibroblast activation and excessive production of extracellular matrix proteins.

### Table

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Glomerular Injury</th>
<th>Tubulointerstitial Disease</th>
<th>Vascular Damage</th>
<th>Total Injury</th>
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<td>2.37±0.05§</td>
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<td>4.18±0.52§</td>
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</table>

*P<0.01 vs WT-CON.
†P<0.01 vs KO-Ang II.
‡P<0.05 vs KO-Ang II.
§P<0.05 vs WT-Ang II.

\*n=6 per group. Ang II indicates angiotensin II; CON, controls; CXCL16, chemokine (C-X-C motif) ligand 16; KO, knockout; and WT, wild-type.

![Figure 5](http://hyper.ahajournals.org/)

*Figure 5.* Chemokine (C-X-C motif) ligand 16 (CXCL16) deficiency reduces fibronectin (FN) and collagen I (Col I) expression. A, Representative photomicrographs of FN immunofluorescence staining in the kidneys of wild-type (WT) and CXCL16 knockout (KO) mice 4 weeks after angiotensin II (Ang II) or saline treatment. Scale bar, 50 μm. B, Quantitative analysis of FN-positive area in the kidneys of WT and CXCL16 KO mice. **P<0.01 vs WT controls (CON), +P<0.05 vs KO-Ang II, and #P<0.05 vs WT-Ang II. n=6 per group. C, Representative photomicrographs of Col I immunofluorescence staining in the kidneys of WT and CXCL16 KO mice 4 weeks after Ang II or saline treatment. Scale bar, 50 μm. D, Quantitative analysis of Col I positive area in the kidneys of WT and CXCL16 KO mice 4 weeks after Ang II or saline treatment. **P<0.01 vs WT controls; +P<0.05 vs KO-Ang II; and #P<0.05 vs WT-Ang II. n=6 per group. E, Representative Western blots show the protein levels of FN and Col I in the kidneys of WT and CXCL16 KO mice 4 weeks after Ang II or saline treatment. F, Quantitative analysis of FN and Col I protein expression in the kidneys of WT and CXCL16 KO mice. **P<0.01 vs WT controls; +P<0.05 vs KO-Ang II; and #P<0.05 vs WT-Ang II. n=6 per group.
and deposition of ECM, which leads to the destruction of renal parenchyma and progressive loss of kidney function. Because activated fibroblasts are the principal cells responsible for ECM production in the fibrotic kidney, their activation is considered as a key event in the pathogenesis of renal fibrosis. However, the origin of these

Figure 6. Chemokine (C-X-C motif) ligand 16 (CXCL16) deficiency suppresses bone marrow–derived fibroblast accumulation and myofibroblast formation in the kidney. A, Representative photomicrographs of kidney sections from wild-type (WT) and CXCL16 knockout (KO) mice 2 weeks after angiotensin II (Ang II) or saline treatment stained for CD45 (red), procollagen I (green), and DAPI (blue). Scale bar, 50 μm. B, Quantitative analysis of CD45+ and procollagen I+ fibroblasts in kidneys of WT and CXCL16 KO mice 2 weeks after Ang II or saline treatment. **P<0.01 vs WT controls (CON); +P<0.05 vs KO-Ang II; and #P<0.05 vs WT-Ang II. n=6 per group. C, Representative photomicrographs of α-smooth muscle actin (α-SMA) immunofluorescent staining in kidneys of WT and CXCL16 KO mice 4 weeks after Ang II or saline treatment. Scale bar, 50 μm. D, Quantitative analysis of α-SMA protein expression in kidneys of WT and CXCL16 KO mice. **P<0.01 vs WT controls; +P<0.05 vs KO-Ang II; and #P<0.05 vs WT-Ang II. n=6 per group. E, Representative Western blots show the levels of α-SMA protein expression in the kidneys of WT and CXCL16 KO mice 4 weeks after Ang II or saline treatment. F, Quantitative analysis of α-SMA protein expression in the kidneys of WT and CXCL16 KO mice. **P<0.01 vs WT controls; +P<0.05 vs KO-Ang II; and #P<0.05 vs WT-Ang II. n=5 per group. HPF indicates high-power field.

Figure 7. Chemokine (C-X-C motif) ligand 16 (CXCL16) deficiency reduces macrophage and T cell infiltration. A, Representative photomicrographs of kidney sections stained for F4/80 (a macrophage marker; brown) and counterstained with hematoxylin (blue) in wild-type (WT) and CXCL16 knockout (KO) mice 2 weeks after angiotensin II (Ang II) or saline treatment. Scale bar, 50 μm. B, Quantitative analysis of F4/80+ macrophages in the kidneys of WT and CXCL16 KO mice. **P<0.01 vs WT controls (CON); +P<0.05 vs KO-Ang II; and #P<0.05 vs WT-Ang II. n=6 in each group. C, Representative photomicrographs of kidney sections stained for CD3 (a T lymphocyte marker; brown) and counterstained with hematoxylin (blue) in WT and CXCL16 KO mice 2 weeks after Ang II or saline treatment. Scale bar, 50 μm. D, Quantitative analysis of CD3+ T cells in the kidneys of WT and CXCL16 KO mice. **P<0.01 vs WT controls; #P<0.01 vs WT-Ang II; +P<0.05 vs KO-Ang II. n=6 in each group. HPF indicates high-power field.
fibroblasts remains controversial. They are traditionally thought to arise from resident renal fibroblasts. Recent evidence suggests they may originate from bone marrow–derived cells. Bone marrow–derived fibroblast precursors termed fibrocytes are derived from a subpopulation of circulating mononuclear cells. These cells express hematopoietic markers such as CD45 and CD11b and mesenchymal markers such as collagen I and vimentin. We and others have shown that these cells migrate into the kidney in response to obstructive injury and contribute significantly to the development of renal fibrosis. However, the signaling mechanisms underlying the recruitment of these bone marrow–derived fibroblast precursors into the kidney are incompletely understood. In the present study, we showed that CXCL16 is pathologically important in recruiting myeloid fibroblasts into the kidney and the development of renal fibrosis because deletion of CXCL16 inhibits bone marrow–derived fibroblast accumulation and myofibroblast formation in the kidney and the development of renal interstitial fibrosis in response to chronic Ang II treatment. These data indicate that CXCL16 signaling plays a key role in the recruitment of bone marrow–derived fibroblasts into the kidney and the development of renal fibrosis in Ang II–induced hypertensive nephropathy.

Macrophages and T cells have been shown to play an important role in the development of hypertensive kidney disease. Ang II is a key factor in the regulation of inflammatory response in hypertensive end-organ damage. CXCR6, the only known receptor for CXCL16, is expressed in various leukocyte subsets including monocytes and T cells. In the present study, our results demonstrate that Ang II–induced interstitial infiltration of macrophages and T cells into the kidney is significantly attenuated in CXCL16 KO mice. These data indicate that CXCL16 signaling mediates Ang II–induced macrophage and T cell infiltration into the kidney.

In addition to establishing a critical role of CXCL16 in recruiting inflammatory cells into the kidney, our results indicate that absence of CXCL16 influences proinflammatory cytokine expression in the kidney. This is relevant because proinflammatory cytokines, tumor necrosis factor-α, IL-6, and IL-1β have been implicated in the pathogenesis of Ang II–induced target organ damage. A recent experimental study has shown that IL-6 contributes to Ang II–induced hypertension and renal damage. Population-based clinical studies have demonstrated that a significant association between inflammatory markers and renal function support a link between inflammation and the development of CKD. TGF-β1 is a key profibrotic cytokine that contributes to renal damage and tubulointerstitial fibrosis. Studies have demonstrated that TGF-β1 functions as a downstream mediator of Ang II–induced renal fibrosis, and that both factors share several intracellular mechanisms involved in the regulation of ECM protein expression. Although studies have shown that bone marrow–derived fibroblasts significantly contribute to the pathogenesis of renal fibrosis, the molecular mechanisms underlying the differentiation of these cells into activated fibroblasts are not fully understood. Recent studies have demonstrated that TGF-β1 is a critical factor for the migration and differentiation of these myeloid fibroblasts precursors. In the present study, our results show that Ang II induces TGF-β1 gene expression in the kidney of WT mice, and that CXCL16 deficiency significantly attenuates Ang II–induced TGF-β1 gene expression. The decrease in TGF-β1 could be another mechanism by which CXCL16 deficiency suppresses Ang II–induced renal fibrosis.

**Perspectives**

Our studies identify CXCL16 as a critical factor that regulates Ang II–induced renal injury and fibrosis. In response to Ang II, CXCL16 recruits macrophages, T cells, and myeloid fibroblasts into the kidney, leading to renal injury and fibrosis. These findings suggest that inhibition of CXCL16 signaling could constitute a novel therapeutic strategy for hypertensive kidney disease.

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Disclosures
None.

References
What Is New?
- We report that chemokine (C-X-C motif) ligand 16 (CXCL16) is a critical factor that regulates angiotensin II–induced renal injury and fibrosis.

What Is Relevant?
- The role of CXCL16 in the development of hypertensive kidney disease is not known. Our study shows that CXCL16 plays a critical role in the development of hypertensive kidney disease, suggesting that inhibition of CXCL16 signaling represents a novel therapy for hypertensive kidney disease.

Summary
This study demonstrates that CXCL16 regulates hypertensive renal injury and fibrosis via recruitment of macrophages, T cells, and myeloid fibroblasts into the kidney and suggests that inhibition of CXCL16 signaling could be a novel therapeutic target for hypertensive kidney disease.
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Running title: CXCL16 in Hypertensive Renal Injury

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Materials and Methods

Cell Culture
The mouse kidney tubular epithelial cell line (TCMK-1, CCL-139) was obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin in a humidified 5% CO2/95% air incubator at 37°C. Cells were made quiescent by starvation in DMEM with 1% FBS overnight before Ang II treatment.

Animals
Wild-type C57BL/6 mice were purchased from the Jackson Laboratory and CXCL16 knockout mice on a C57BL/6 background were a generous gift from Dr. Shuhua Han at Baylor College of Medicine as described1. Mice were bred and maintained in the animal care facility of Baylor College of Medicine and had access to food and water ad libitum. All animal procedures were in accordance with national and international animal care and ethical guidelines and have been approved by the institutional animal welfare committee.

Ang II-induced Hypertension
Male 8-10-week-old mice were infused with Ang II (1.5 µg/kg/min; Sigma) or vehicle (0.9% NaCl) continuously via subcutaneous osmotic minipumps (Alzet) for 14 or 28 days following uninephrectomy as described2. To accelerate renal injury, Ang II-treated mice were supplied with 1% saline drinking water ad libitum.

Blood Pressure and Heart Rate
Systolic blood pressure (SBP) and heart rate (HR) were measured in conscious mice using a tail cuff system (Visitech Systems) as reported2.

Renal Function
Blood urea nitrogen was determined fluorometrically as described3.

Histopathologic Analysis
At the end of experiments, mice were perfused with PBS to remove the blood. A portion of kidney tissue was fixed in 10% buffered formalin, embedded in paraffin, and cut at 5-µm thickness. After deparaffinization and rehydration, sections were stained with Periodic Acid Schiff (PAS) and Sirius red. The pathological abnormalities in the kidney were graded on the basis of the presence and severity of component abnormalities, including glomerulosclerosis, epithelial reactivity, chronic inflammation, tubular casts, fibrosis, and vascular injury. Grading for each component was performed using a semiquantitative scale, as described4, where 0 represented no abnormality and where 1, 2, 3, and 4 represented mild, moderate, moderately severe, and severe abnormalities, respectively. The total injury score for each kidney was a summation of these component injury scores. The Sirius red-stained sections were scanned using a microscope equipped with a digital camera (Nikon Instruments Inc., Melville, NY), and quantitative evaluation was performed using NIS-Elements Br 3.0 software as described1. The Sirius red-stained area was calculated as a percentage of the total
area. To assess monocyte/macrophage and T lymphocyte infiltration into the kidneys, sections were stained with antibodies against F4/80 (Serotec) and CD3 (Calbiochem) respectively. Interstitial infiltrating F4/80-positive macrophages and CD3-positive T cells were counted in the cortex under x400 magnification observing 10 consecutive non-overlapping fields per animal.

**Immunofluorescence**

Renal tissues were embedded in OCT compound, snap-frozen on dry ice, cut at 5 µm thickness, and mounted. After fixation, nonspecific binding was blocked with protein block (Dako, Carpinteria, CA). Kidney sections were then incubated with rabbit anti-CXCL16 antibody (Torrey Pines Biolabs, Secaucus, NJ) followed by Alexa-488 conjugated donkey anti-rabbit antibody (Invitrogen, Carlsbad, CA), rabbit anti-collagen I antibody (Rockland Immunochemicals, Gilbertsville, PA) followed by Alexa-488 conjugated donkey anti-rabbit antibody (Invitrogen, Carlsbad, CA), rabbit anti-fibronectin antibody (Sigma-Aldrich, St. Louis, MO) followed by Alexa-488 conjugated donkey anti-rabbit antibody (Invitrogen, Carlsbad, CA), or rabbit anti-α-SMA antibody (Abcam, Cambridge, MA) followed by Alexa-488 conjugated donkey anti-rabbit antibody (Invitrogen, Carlsbad, CA). For double immunofluorescence, kidney sections were fixed and stained with rat anti-CD45 (BD Biosciences) and mouse anti-procollagen I (Santa Cruz Biotechnology) followed by appropriate secondary antibodies sequentially. Slides were mounted with medium containing DAPI. Fluorescence intensity was visualized using a microscope equipped with a digital camera (Nikon Instruments Inc., Melville, NY). Quantitative evaluation of sections stained with antibodies to α-SMA, collagen I and fibronectin was performed using NIS-Elements Br 3.0 software. The fluorescence positive area was calculated as a percentage of the total area as described1, 5.

**Albuminuria**

Mice were placed into metabolic cages for urine collection. Albumin and creatinine in the urine were measured using commercially available kits (EXOCELL, Inc).

**Quantitative Real-Time RT-PCR**

Total RNA was extracted from kidney tissues with TRIzol reagent (Invitrogen). Aliquots (1 µg) of total RNA were reverse transcribed using SuperScript II reverse transcriptase. Real-time PCR was performed using IQ SYBR green supermix reagent (Bio-Rad, Hercules, CA) with a Bio-Rad real-time PCR machine according to the manufacturer’s instructions. The specificity of real-time PCR was confirmed via melting-curve analysis. The comparative Ct method (ΔΔCt) was used to quantify gene expression, and the relative quantification was calculated as $2^{-\Delta\Delta Ct}$. The expression levels of the target genes were normalized to GAPDH level in each sample. The primer sequences were:

- **CXCL16**
  - forward 5’- ACCCTTGTCTTCTGCTTCTCCT-3’
  - reverse 5’- ATGTGATCCAAAGTACCTGCGGT-3’

- **IL-6**
  - forward, 5’- GAGGATACCACTCCCAACAGACC-3’
  - reverse, 5’- AAGTCATCATCGTTGCTCATAA-3’

- **TNF-α**
  - forward, 5’- CATGAGACAGCAAACAGATCCG-3’
  - reverse, 5’- AAGCAGAATGAGAGGCTGAGG-3’

- **IL-1β**
  - forward, 5’- CTTCAGGCAGGCAGTATCAGCTC-3’
  - reverse, 5’-
Western Blot Analysis
Protein was extracted using the RIPA buffer containing a cocktail of proteinase inhibitors (Thermo Fisher Scientific Inc., Rockford, IL) and quantified with Bio-Rad protein assay. Equal amounts of protein were separated on SDS–polyacrylamide gels in a tris/glycine buffer system, transferred onto nitrocellulose membranes, and blotted according to standard procedures with primary antibodies (CXCL16, collagen I, fibronectin, and α-SMA) followed by appropriate secondary antibodies as described5. Membranes were reblotted with anti-GAPDH antibody (Millipore, Billerica, CA). The specific bands of target proteins were analyzed using an Odyssey IR scanner (LI-COR Bioscience, Lincoln, NE) and band intensities were quantified using NIH Image/J.

Statistical Analysis
All data were expressed as mean ± SEM. Two group comparisons were performed by Student’s t test. Multiple group comparisons were performed by one-way ANOVA followed by the Bonferroni procedure for comparison of means. A P value < 0.05 was considered statistically significant.

References