Role of Inflammation in the Development of Renal Damage and Dysfunction in Angiotensin II–Induced Hypertension

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Abstract—Angiotensin II (Ang II)–induced hypertension is associated with an inflammatory response that may contribute to the development of target organ damage. We tested the hypothesis that, in Ang II–induced hypertension, CC chemokine receptor 2 (CCR2) activation plays an important role in the development of renal fibrosis, damage, and dysfunction by causing oxidative stress, macrophage infiltration, and cell proliferation. To test this hypothesis, we used CCR2 knockout mice (CCR2<sup>−/−</sup>). The natural ligand of CCR2 is monocyte chemotactic protein-1, a chemokine important for macrophage recruitment and activation. CCR2<sup>−/−</sup> and age-matched wild-type (CCR2<sup>+/+</sup>) C57BL/6J mice were infused continuously with either Ang II (5.2 ng/10 g per minute) or vehicle via osmotic minipumps for 2 or 4 weeks. Ang II infusion caused similar increases in systolic blood pressure and left ventricular hypertrophy in both strains of mice. However, in CCR2<sup>−/−</sup> mice with Ang II–induced hypertension, oxidative stress, macrophage infiltration, albuminuria, and renal damage were significantly decreased, and glomerular filtration rate was significantly higher than in CCR2<sup>+/+</sup> mice. We concluded that, in Ang II–induced hypertension, CCR2 activation plays an important role in the development of hypertensive nephropathy via increased oxidative stress and inflammation. (Hypertension. 2008;52:1-8.)

Key Words: inflammation ■ chemokine receptors ■ macrophage ■ reactive oxygen species ■ albuminuria ■ fibrosis

Hypertension is a major risk factor for renal nephrosclerosis; however, the mechanisms by which high blood pressure causes renal damage are not completely understood. Angiotensin II (Ang II), in addition to causing vasoconstriction, aldosterone release, and Na reabsorption by the nephron, also causes oxidative stress, inflammation, cell proliferation, and, as a consequence, interstitial matrix accumulation and target organ damage. In the kidney, Ang II causes renal inflammation by stimulating superoxide formation and increasing chemokine release.1–3 Chemokines are a family of low–molecular-weight cytokines that induce activation and migration of inflammatory cells and modulate functions of these cells. Monocyte chemotactant protein (MCP-1) is one of the most prominent chemokines that regulates monocyte-macrophage infiltration. MCP-1 acts via its receptor, the CC chemokine receptor 2 (CCR2).4,5 In mice lacking CCR2 (CCR2<sup>−/−</sup>), Ang II–induced vascular inflammation and remodeling are significantly reduced.6 In a unilateral ureteral obstruction model of hypertension, MCP-1/CCR2 activation, via macrophage infiltration, plays a crucial role in the development of vascular and renal damage, it is unknown whether it contributes to renal damage and dysfunction in hypertension. Here we test the hypothesis that, in Ang II–induced hypertension, CCR2 activation plays an important role in the development of renal fibrosis, damage, and dysfunction by causing oxidative stress, macrophage infiltration, and cell proliferation. To test this hypothesis we used CCR2 knockout mice (CCR2<sup>−/−</sup>) with Ang II–induced hypertension.

Methods

Male 12- to 14-week-old homozygous CCR2<sup>−/−</sup> mice with C57BL/6J genetic background (maintained in our mutant core facility) and matched male C57BL/6J CCR2<sup>+/+</sup> mice (Jackson Laboratory, Bar Harbor, Maine) were used in this study. All of the animal procedures, care, and housing were in accordance with guidelines of the Institutional Animal Care and Use Committee of Henry Ford Hospital.

Ang II–Induced Hypertension

Hypertension was induced by Ang II infusion via osmotic minipump (Alzet). Briefly, mice were anesthetized with sodium pentobarbital (50 mg/kg IP). Ang II was dissolved in 0.01 N acetic acid saline solution to prevent it from attaching to the pump wall. Using a sterile technique, minipumps were placed subcutaneously in the intrascapular area to deliver Ang II at a dose of 5.2 ng/10 g per minute. Vehicle groups were given 0.01 N acetic acid saline solution. Before minipump implantation, mice were trained daily for 7 days to have systolic blood pressure (SBP) determined with a computerized tail-cuff system (BP 2000, Visitech). SBP was measured weekly. Three sets of 10 measurements were made for each recording.

Received February 27, 2008; first decision March 24, 2008; revision accepted May 15, 2008.
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Hypertension is available at http://hypertension.ahajournals.org
DOI: 10.1161/HYPERTENSIONAHA.108.112706
Experimental Groups

CCR2−/− and age-matched CCR2+/+ mice were randomly divided into 4 groups (11 to 15 mice per group): (1) CCR2−/− plus vehicle; (2) CCR2−/− plus vehicle; (3) CCR2+/+ and Ang II; and (4) CCR2−/− plus Ang II. After 4 weeks of Ang II or vehicle infusion, the mice were placed in metabolic cages for 24-hour urine collection. Volume was recorded and albumin measured using a commercially available ELISA (Alpha Diagnostic International). Small groups of mice (n = 5 per group) were studied after 2 weeks of Ang II infusion. Because they did not yet have a significant renal disease, they were only used to determine macrophage infiltration and cell proliferation.

Glomerular Filtration Rate

Glomerular filtration rate (GFR) was measured as described previously using fluorescein isothiocyanate-labeled inulin (Sigma).10 Briefly, fluorescein isothiocyanate-labeled inulin was injected as a bolus at 3 μL/g of body weight and followed immediately by constant infusion of 0.15 μL/min per gram of body weight. After a 30-minute stabilization period, urine was collected for 30 minutes with a 100-μL blood sample taken before and after urinary collection. Positive-staining cells were recognized as macrophages, and those plasma, and diluted urine were individually transferred to a 96-well black microplate in triplicate and mixed with 10 mmol/L of HEPES buffer (pH 7.4). Plates were read with a microplate fluorosence reader (Labsystems Fluoroskan II) at excitation 485 nm and emission 538 nm. GFR was calculated using the following formula:

GFR = (urine fluorescence value × urine volume/blood fluorescence value)/collection time.

Glomerular Matrix

The glomerular matrix was evaluated by periodic acid-Schiff staining (Sigma), as described previously.13 Dark purple color in the glomerulus was recognized as the extracellular matrix. Twenty-five to 30 glomeruli in each section were imaged at ×400×=magnification. Data were analyzed by computerized imaging software (Microsuite Biological imaging software, Olympus America). The glomerular matrix was expressed as a percentage of the glomerular area.

Renal Cortex Collagen Determination by Hydroxyproline Assay

Collagen content of kidney cortex tissue was determined using the hydroxyproline method, as described previously.14 A piece of the kidney cortex was dried, weighed, homogenized, and then hydrolyzed with 6 N HCl for 18 hours at 110°C. Hydroxyproline content was determined using a colorimetric assay and a standard curve of 0 to 10 μg hydroxyproline. Data were expressed as micrograms of collagen per milligram of dry weight, assuming that collagen contains an average of 13.5% hydroxyproline.15

Data Analysis

Data are expressed as means ± SEMs. SBP among the groups was compared using regression coefficient and average increasing rates. For the parameters, including histological and immunohistochemical changes, glomerular matrix, renal collagen content, GFR, and 24-hour urinary albumin. Student 2-sample test was used to compare differences between groups, either between strains with the same treatment or within 1 strain between different treatments. When multiple comparisons were performed, Hochberg’s step-up procedure was used to adjust the P values. Type 1 error rate was set at α=0.05. The differences were considered statistically significant when P<0.05.

Results

SBP and Left Ventricle

SBP before Ang II infusion was similar among groups. Ang II increased SBP in both CCR2−/− and CCR2+/+ strains (P<0.001 versus vehicle within strain). The slope and average increase in SBP from basal were not statistically different between CCR2−/− and CCR2+/+ mice (Figure 1, top). Ang II also increased left ventricular (LV) weight, and LV hypertrophy did not differ between CCR2−/− and CCR2+/+ mice (Figure 1, bottom).
Ang II increased significantly in CCR2+/+ mice infused with either vehicle or Ang II. SBP was similar in vehicle groups of both strains. Ang II infusion increased SBP and LVW significantly but similarly in both strains. **P<0.001, CCR2+/+ and CCR2−/− vehicle vs Ang II; n=11 to 13 per group.

**GFR and Proteinuria**

GFR was similar in vehicle-treated groups of both strains. After 4 weeks of Ang II infusion, GFR decreased significantly in CCR2+/+ controls but remained unchanged in CCR2−/− mice (Figure 2, top). Urinary albumin excretion was significantly increased in CCR2+/+ mice with Ang II–induced hypertension, whereas it remained unchanged in CCR2−/− mice (Figure 2, bottom).

**Macrophage Infiltration**

Because inflammatory cell infiltration is an early response to Ang II, we studied macrophage infiltration as indicated by the number of F4/80-positive cells at 2 and 4 weeks after vehicle or Ang II infusion. In vehicle groups, there were few F4/80-positive cells in both strains. In the Ang II–treated CCR2+/+ mice, F4/80-positive cells increased significantly at 2 and 4 weeks. The number of F4/80-positive cells was higher at 2 weeks compared with 4 weeks (Figure 3). In CCR2−/− mice, Ang II did not increase F4/80-positive cells at 2 or 4 weeks. F4/80-positive cells were mainly located in the tubulointerstitial space and glomerulus (Figure 3).

**Nitrotyrosine and gp91phox Protein Expression**

3-Nitrotyrosine staining, a marker of oxidative stress, was almost imperceptible in vehicle-treated groups of both strains. Ang II increased 3-nitrotyrosine staining (intensity and area) in both CCR2+/+ and CCR2−/− mice at 4 weeks; however, the increase was significantly lower in CCR2−/− mice. In CCR2−/− mice with Ang II–induced hypertension, gp91phox protein expression also significantly increased in CCR2−/− mice with Ang II infusion at 4 weeks, and this response was absent in CCR2−/− mice (Figure 5). Immunoblotting exhibited 1 positive band at 58 kDa. This molecular weight for gp91phox was less than that reported for humans but similar to that in the mouse phagocyte gp91phox clone.16

**Cell Proliferation**

The number of Ki-67–positive cells was studied at 2 and 4 weeks. In CCR2−/− mice, Ki-67–positive cells were significantly increased at both 2 and 4 weeks of Ang II infusion. In CCR2−/− mice, these increases were not present. Ki-67–positive cells were found mainly in the glomerulus, tubule, and tubulointerstitial area (Figure 6).

**Glomerular Matrix and Collagen Content**

In the vehicle groups, glomerular matrix was similar between strains. After 4 weeks of Ang II infusion, glomerular matrix increased significantly in CCR2+/+ but not in CCR2−/− mice.
Our data demonstrate that Ang II infusion caused similar increases in SBP and LV hypertrophy in CCR2^+/+ and CCR2^-/- mice. However, in CCR2^-/- mice with Ang II–induced hypertension, reactive oxygen species (ROS), macrophage infiltration, albuminuria, and renal damage were significantly decreased, and GFR was significantly higher than in CCR2^+/+ mice. We concluded that, in Ang II–induced hypertension, CCR2 activation plays an important role in the development of hypertensive nephropathy via increased oxidative stress and inflammation.

The most important ligand for the CCR2 is MCP-1, although CCR2 also binds MCP-2, MCP-3, and MCP-4. Both Ang II and mechanical strain cause MCP-1 expression in vascular cells, and MCP-1 plasma concentrations in normotensive CCR2^+/+ and CCR2^-/- mice are similar. Ang II infusion for 28 days increased plasma MCP-1 concentrations in both strains; however, in CCR2^-/- mice, the increase was significantly greater compared with CCR2^+/+ mice. Thus, it is possible that, in our study, Ang II, either directly or via elevation of blood pressure, caused MCP-1 release and CCR2 activation, resulting in ROS generation, macrophage infiltration (inflammation), cell proliferation, and subsequent development of renal fibrosis and damage.

Because both mouse strains developed similar degrees of hypertension and LV hypertrophy, but the severity of renal damage was significantly different, it could be assumed that high blood pressure itself is not responsible for renal damage. However, it could be that high blood pressure acts via mechano-transduction, causing MCP-1 release and CCR2 receptor activation, inflammation, and renal damage. Similarly, it has been reported that Ang II infusion for 28 days causes similar degrees of hypertension and LV hypertrophy in CCR2^+/+ and CCR2^-/- mice; however, vascular inflammation and remodeling are significantly more severe in CCR2^-/- mice. This study and ours suggest that, in the absence of the CCR2, Ang II–induced hypertension causes less vascular and renal damage. However, it is possible that, in a more chronic model of hypertension, renal damage occurs independent of CCR2 activation.

Ang II, in addition to vasoactive and hemodynamic effects, can also act directly as a growth factor and proinflammatory cytokine. In our study, Ang II–induced hypertension showed increased renal ROS generation in CCR2^-/- mice, and this effect was significantly decreased in the absence of the CCR2 receptor. We assessed ROS generation by immunostaining of 3-nitrotyrosine, a peroxynitrite marker. Ang II administration increased both intensity and area of positive 3-nitrotyrosine immunostaining in kidneys of CCR2^+/+ mice; however, vascular inflammation and remodeling were significantly more severe in CCR2^-/- mice. This study and ours suggest that, in the absence of the CCR2, Ang II–induced hypertension causes less vascular and renal damage. However, it is possible that, in a more chronic model of hypertension, renal damage occurs independent of CCR2 activation.
found in macrophages and various renal cells. We found that gp91phox expression was significantly increased in CCR2+/+ mice, but this increase was not present in CCR2-/- mice. The present study supports the hypothesis that, in Ang II–induced hypertension, CCR2 receptor activation participates in the development of oxidative stress and inflammation and the development of renal fibrosis and disease.25 Inflammation itself also increases oxidative stress.26 Thus, Ang II could cause oxidative stress directly by stimulating reduced nicotinamide-adenine dinucleotide phosphate oxidase in renal tissue, as well as by increasing macrophage infiltration in the kidney.27–29 Oxidative stress has also been implicated in the pathogenesis of Ang II–induced hypertension.30–33 However, in our study blood pressure was similar in CCR2+/+ and CCR2-/- mice, despite the observation that only the former had a significant increase in oxidative stress. Thus, our study does not support the hypothesis that oxidative stress participates in chronic elevation of blood pressure during Ang II infusion. Similar dissociation between oxidative stress and development of hypertension, especially at chronic stages, has been reported by Zhou et al34 and by Touyz et al.35

In our study, Ang II–induced hypertension increased renal macrophage infiltration in CCR2+/+ but not CCR2-/- mice, indicating that macrophage infiltration is mediated by CCR2 receptor activation. Similar to other studies, we found that macrophage infiltration was higher at 2 than at 4 weeks after Ang II infusion began.36,37 Macrophages were found mainly

**Figure 4.** A, Representative immunohistochemical staining for nitrotyrosine (a peroxynitrite marker) in mice infused for 4 weeks with either vehicle (Veh) or Ang II. Reddish-brown color was considered a positive stain. Positive stain can be found in the glomerulus and tubulointerstitial area. Scale bar=50 μm. B, Semiquantitative analysis of both intensity (top) and area (bottom) of nitrotyrosine-positive staining. CCR2+/+ mice with Ang II–induced hypertension have a significant increase in both intensity and area of staining for nitrotyrosine (p<0.001; vehicle vs Ang II). In CCR2-/- mice with Ang II–induced hypertension, these effects were significantly blunted (p<0.001, CCR2+/+ vs CCR2-/- mice), but still the increases were significant vs vehicle group (p<0.001; vehicle vs Ang II).

**Figure 5.** Renal tissue Western blot analysis of gp91phox proteins (Nox2) in mice treated with either vehicle or Ang II. The top panel shows representative Western blot for gp91phox (58 kDa) and β-actin. The bottom panel shows a quantification of the ratio of gp91phox to β-actin. gp91phox expression increased significantly in CCR2+/+ mice with Ang II–induced hypertension (p<0.005, vehicle vs Ang II). gp91phox did not increase in CCR2-/- mice with Ang II–induced hypertension (p<0.006, CCR2+/+ Ang II vs CCR2-/- Ang II).
in the tubulointerstitial space and, to a lesser extent, in the glomerulus, suggesting that infiltrated macrophages in the kidney may affect both tubular and glomerular cells. Macrophages, by their destructive potential and ability to secrete regulators of neighboring cells, contribute to renal and vascular damage in Ang II–induced hypertension.38,39

We also observed that, in Ang II–induced hypertension, there is an increase in renal cell proliferation. Mesangial cells and interstitial fibroblasts are the major cells involved in renal fibrosis. They proliferate in response to macrophage-derived cytokines such as interleukin -1, interleukin-2, and tumor necrosis factor-α.40,41 Also, Ang II, directly and/or via ROS generation, could increase renal cell proliferation.42,43 It has been shown that the number of mesangial cells and interstitial fibroblasts correlates with renal fibrosis and dysfunction.44

We found proliferating cells in the glomerular and tubuloin-

Figure 6. A, Representative immunohistochemical staining for Ki-67–positive cells (an indicator for cell proliferation) in mice infused for 4 weeks with vehicle (Veh) or Ang II. Reddish-brown color in the nucleioli was considered positive. Positive cells were found in the tubulointerstitial area (arrows) and glomerulus (arrowheads). Scale bar=50 μm. B, Quantitative analysis of Ki-67–positive cells in mice treated with vehicle or Ang II. In CCR2+/− mice with Ang II–induced hypertension, the number of cells proliferating in the kidney increased significantly at both 2 (top) and 4 (bottom) weeks (P<0.002, vehicle vs Ang II). However, the increase at 2 weeks was greater than at 4 weeks. In CCR2−/− mice with Ang II–induced hypertension, the number of proliferating cells did not change at either 2 or 4 weeks. Ki-67–positive cells in Ang II–induced hypertension were significantly higher in CCR2+/− vs CCR2−/− mice (P<0.002; 2 weeks, n=5 per group; 4 weeks, n=8 to 10 per group).

Figure 7. A, Representative periodic acid-Schiff staining for glomerular matrix. Dark purple color in the glomerulus is extracellular matrix. Scale bar=25 μm. B, Quantitative analysis of glomerular matrix area in mice treated with vehicle (Veh) or Ang II. In CCR2+/− mice with Ang II–induced hypertension, glomerular matrix area increased significantly (P<0.001, vehicle vs Ang II), whereas in CCR2−/− mice, it did not change. Glomerular matrix in Ang II–induced hypertension was significantly higher in CCR2+/− vs CCR2−/− mice; P<0.001 (n=7 per group).
terstitial area in CCR2 \(^{-/-}\) mice with Ang II–induced hypertension. It could be that mesangial cells and fibroblast proliferation participate in the development of renal fibrosis.\(^{21,42}\)

Renal fibrosis was evaluated by measuring renal cortex collagen and glomerular matrix. Ang II administration increased renal cortex fibrosis and glomerular matrix significantly in CCR2 \(^{+/+}\) but not in CCR2 \(^{-/-}\) mice. These results agree with other studies stating that CCR2 blockade and/or decreasing macrophage infiltration ameliorated progressive renal fibrosis in unilateral ureteral obstruction and type 2 diabetic db/db mice.\(^{7,8,45}\) Increased renal fibrosis in Ang II–induced hypertension may be related to augmented oxidative stress and macrophage infiltration and subsequent mesangial cell and fibroblast proliferation. In contrast, mice lacking CCR2 have less cell proliferation and better renal function. Decreased collagen synthesis in the cortex and extracellular matrix deposition in the glomerulus may help to maintain GFR.

In summary, after Ang II administration, mice lacking CCR2 exhibit less ROS generation, macrophage infiltration, cell proliferation, glomerular matrix, and collagen deposition in the kidney compared with CCR2 \(^{+/+}\) mice. Lower ROS generation and macrophage infiltration could lead to reduced injury to the glomerular filtration membrane and tubules, whereas lower extracellular matrix deposition and cell proliferation in the glomerulus are beneficial for preserving renal function. These results suggest that CCR2 plays an important role in the development of renal injury and dysfunction, which are associated with ROS generation, macrophage infiltration, cell proliferation, and extracellular matrix deposition.

**Perspectives**

Chronic hypertension is a major risk factor in the development of renal nephrosclerosis and end-stage renal disease. Hypertensive renal disease varies markedly between individuals with similar blood pressure; eg, it has been shown to be more common in black subjects than in white subjects. Also, Dahl salt-sensitive rats have more severe renal damage than spontaneously hypertensive rats with similar blood pressure.\(^{56–48}\) These studies suggest that, in addition to high blood pressure, other factors, including genetic characteristics and inflammation, participate in the pathogenesis of renal disease in hypertension.\(^{49,50}\) Our study provides experimental evidence that a lack of CCR2 ameliorates renal inflammation injury and dysfunction induced by Ang II. These findings may lead to novel therapies directed at blockade of either MCP-1 or CCR2.

**Sources of Funding**

This work was supported by National Institutes of Health grants HL-28892 (to O.A.C.).

**Disclosures**

None.

**References**


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Hypertension, published online June 9, 2008;

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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/early/2008/06/09/HYPERTENSIONAHA.108.112706.citation

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