Published in final edited form as:
Hypertension. 2008 August ; 52(2): 256–263. doi:10.1161/HYPERTENSIONAHA.108.112706.

Role of inflammation in the development of renal damage and dysfunction in Angiotensin II-induced hypertension

Tang-Dong Liao,
Hypertension & Vascular Research Division, Department of Internal Medicine, Henry Ford Hospital, Detroit, MI 48202, USA

Xiao-Ping Yang,
Hypertension & Vascular Research Division, Department of Internal Medicine, Henry Ford Hospital, Detroit, MI 48202, USA

Yun-He Liu,
Hypertension & Vascular Research Division, Department of Internal Medicine, Henry Ford Hospital, Detroit, MI 48202, USA

Edward G. Shesely,
Hypertension & Vascular Research Division, Department of Internal Medicine, Henry Ford Hospital, Detroit, MI 48202, USA

Maria A. Cavasin,
Hypertension & Vascular Research Division, Department of Internal Medicine, Henry Ford Hospital, Detroit, MI 48202, USA

William A. Kuziel,
PDL BioPharma, Inc., Fremont, CA 94555, USA

Patrick J. Pagano, and
Hypertension & Vascular Research Division, Department of Internal Medicine, Henry Ford Hospital, Detroit, MI 48202, USA

Oscar A. Carretero
Hypertension & Vascular Research Division, Department of Internal Medicine, Henry Ford Hospital, Detroit, MI 48202, USA

Abstract

Angiotensin II (Ang II)-induced hypertension is associated with an inflammatory response that may contribute to development of target organ damage. We tested the hypothesis that in Angiotensin II-induced hypertension, CC chemokine receptor 2 (CCR2) activation plays an important role in development of renal fibrosis, damage and dysfunction by causing: a) oxidative stress, b) macrophage infiltration, and c) cell proliferation. To test this hypothesis we used CCR2 knockout mice (CCR2−/−). The natural ligand of CCR2 is monocyte chemoattractant protein-1 (MCP-1), a chemokine important for macrophage recruitment and activation. CCR2−/− and age-matched wild-type (CCR2+/+) C57BL/6J mice were infused continuously with either Ang II (5.2 ng/10 g/min) or vehicle *via* osmotic mini-pumps 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−/− 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

Correspondence to: Oscar A. Carretero, M.D.,.Hypertension and Vascular Research Division, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit MI 48202-2689, Phone: (313) 916-2103, Fax: (313) 916-1479, Email: ocarret1@hfhs.org.

CCR2+/+ mice. We concluded that in Ang II-induced hypertension, CCR2 activation plays an important role in development of hypertensive nephropathy *via* increased oxidative stress and inflammation.

Keywords

inflammation; chemokine receptors; macrophage; reactive oxygen species; kidney diseases; albuminuria; fibrosis

Introduction

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 chemoattractant 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−/−), Ang II-induced vascular inflammation and remodeling are significantly reduced[6]. In a unilateral ureteral obstruction model of renal fibrosis and inflammation, CCR2 blockade ameliorates fibrosis and macrophage infiltration[7,8]. Although these studies suggest that MCP-1/CCR2 activation, *via* macrophage infiltration, plays a crucial role in 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: a) oxidative stress, b) macrophage infiltration, and c) cell proliferation. To test this hypothesis we used CCR2 knockout mice (CCR2−/−) with Ang II-induced hypertension.

Methods

Male 12–14 week old homozygote CCR2−/− mice with a C57BL/6J genetic background (maintained in our mutant core facility), and matched male C57BL/6J, CCR2+/+ (Jackson Lab, Bar Harbor, Maine), were used in this study. All animal procedures, care, and housing were in accordance with guidelines of the Institutional Animal Care and Use Committee (IACUC) 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 0.01N acetic acid saline solution to prevent it from attaching to the pump wall. Using sterile technique, mini pumps were placed subcutaneously in the intrascapular area to deliver Ang II at a dose of 5.2ng/10g/min. Vehicle groups were given 0.01N acetic acid saline solution. Prior to 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)[9]. SBP was measured weekly. Three sets of 10 measurements were made for each recording.

Experimental groups

CCR2−/− and age-matched CCR2+/+ mice were randomly divided into four groups (11–15 mice per group): 1) CCR2+/+ plus vehicle, 2) CCR2−/−plus vehicle, 3) CCR2+/+ plus 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 hr urine collection. Volume was recorded and albumin measured using a commercially available enzyme-linked immunosorbent assay kit (ELISA; Alpha Diagnostic International, TX). Small groups of mice (n=5 per group) were studied after 2 weeks of Ang II-infusion. Since they did not yet have a significant renal disease, they were only used to determine macrophage infiltration and cell proliferation.

Glomerular filtration rate (GFR)

GFR was measured as previously described using fluorescein isothiocyanate label inulin (FITC-inulin, Sigma) [10]. Briefly, FITC-inulin was injected as a bolus at 3 μl/g bw (body weight) and followed immediately by constant infusion of 0.15 μl/min/g bw. Following a 30 minute stabilization period, urine was collected for 30 minutes with a 100μl blood sample taken before and after urinary collection. Samples of FITC-inulin standards, plasma and diluted urine were individually transferred to a 96-well black microplate in triplicate and mixed with 10 mM HEPES buffer (N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid), *p*H 7.4). Plates were read with a micro-plate fluorescence reader (Labsystems Fluoroskan II) at excitation 485 nm, emission 538 nm. GFR was calculated using the following formula: GFR = (urine fluorescence value × urine volume/blood fluorescence value)/collection time. GFR was corrected by kidney weight (kw) with units expressed as fl/min/100 mg of kidney weight.

Immunohistochemistry staining for macrophage and proliferated cells

Paraffin-embedded sections (6 μm) were deparafinized and endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. The antigens were unmasked by microwave heat-induced epitope retrieval method in citrate acid buffer (*p*H 6.0). Nonspecific binding was blocked with 5% normal rabbit serum, then primary monoclonal antibodies; rat anti-mouse to F4/80 antigen, macrophage marker (clone: A3-1, 1:50, AbD SEROTEC)[11], or cell proliferation marker (monoclonal Ki-67 antibody, clone: TEC-3, 1:50 DAKO)[12]; were applied and incubated overnight at 4° C. Then the sections were incubated with secondary biotinylated antibody (rabbit anti-rat IgG). Immunoreactivity was detected with an ABC kit (Vectastain *Elit e* ABC peroxidase kit, Vector Lab) and visualized by 3-amino-9-ethylcarbazole solution (AEC, Zymed Lab). PBS buffer alone and nonspecific purified rat anti-mouse IgG (AbD Serotec) were used as a negative control and an isotype IgG control, respectively. Reddish-brown color was considered a positive stain. Sections were counterstained with hematoxylin. Images of 12 regions of the section were captured at 400 × magnifications using an inverted microscope (IX81, Olympus America, Center Valley, PA) with a digital camera (DP70, Olympus America, Center Valley, PA) and evaluated by a computerized image analysis system (Microsuite Biological imaging software, Olympus America, Center Valley, PA). F4/80 positive-staining cells were recognized as macrophages and those with KI-67 positive staining were counted as proliferating cells. Macrophages and Ki-67 positive cells are expressed as number of cells/mm^2.

Immunohistochemistry staining and semi quantitative analysis for nitrotyrosine

Transverse kidney sections were frozen, cut into 6 μm slices, and used for immunostaining of 3-nitrotyrosine, a marker for oxidative stress. A monoclonal antibody to 3-nitrotyrosine was used as primary antibody (clone: 1A6, 1:100, Millipore, Billerica, MA). The detection method was similar to that used for macrophage immunohistochemistry, except slices were fixed with cool acetone for frozen sections. For each slice, 24 images from the renal cortex were taken at ×400 magnification. Area and intensity of positive staining were scored separately by 3

independent observers unaware of treatment allocation. Area of positive staining was scored from 0 to 3 as follows: 0, no visible area; 1, small area; 2, medium area; and 3, large area. Intensity of positive staining was also scored from 0 to 3: 0, no visible staining; 1, faint staining; 2, moderate staining; and 3, strong staining.

Western blot of NADPH oxidase gp91^{phox} in the kidney

Eight fg protein from kidney cortex extracts were subjected to 10% SDS-PAGE under reducing conditions. Proteins were transferred to a nitrocellulose membrane. A monoclonal antibody against NADPH oxidase gp91^{phox} was used as primary antibody (supplied by Mark T Quinn, Ph.D., Montana State University). Signals were revealed with chemiluminescence (ECL kit, Chemicon) and visualized by autoradiography. Membranes were then stripped (Pierce) and reprobed with β-actin (polyclonal antibody, sc-1616, 1:1000, Santa Cruz) to verify equal loading. Bands' optical density was quantified with a bioscanner and expressed as ratio of gp91^{phox} to β-actin. The positive band of gp91^{phox} was at molecular weight of 58 kDa and β-actin was at 43 kDa.

Glomerular matrix

The glomerular matrix was evaluated by periodic acid-Schiff staining (PAS, Sigma) as described previously[13]. Dark purple color in the glomerulus was recognized as extracellular matrix. Twenty-five to thirty glomeruli in each section were imaged at 400× magnification. Data were analyzed by computerized imaging software (Microsuite Biological imaging software, Olympus America, Center Valley, PA). The glomerular matrix was expressed as percentage of 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 hr 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 fg collagen per mg dry weight, assuming that collagen contains an average of 13.5% hydroxyproline[15].

Data Analysis

Data are expressed as mean ± SEM. 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-hr urinary albumin, student's two-sample *t*-test was used to compare differences between groups, either between strains with the same treatment or within one strain between different treatments. When multiple comparisons were performed, Hochberg's step-up procedure was used to adjust the *p* values. Type one error rate was set at a=0.05. The differences were considered statistically significant when $p < 0.05$.

Results

SBP and left ventricle weight

SBP before Ang II infusion was similar among groups. Ang II increased SBP in both CCR2+/+ and CCR2−/− strains ($p < 0.001$ *vs* vehicle within strain). The slope and average increase in SBP from basal were not statistically different between CCR2+/+ and CCR2−/− mice (Fig. 1, upper panel). Ang II also increased left ventricular (LV) weight, and LV hypertrophy did not differ between CCR2+/+ and CCR2−/− mice (Fig. 1, lower panel).

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 (Fig. 2, upper panel). Urinary albumin excretion was significantly increased in CCR2+/+ mice with Ang II-induced hypertension while it remained unchanged in CCR2−/− mice (Fig. 2, lower panel).

Macrophage infiltration

Since 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 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 to 4 weeks (Fig. 3). In CCR2−/− mice, Ang II did not increase F4/80-positive cells neither at 2 or 4 weeks. F4/80-positive cells were mainly located in the tubulointerstitial space and glomerulus (Fig. 3).

Nitrotyrosine and gp91^{phox} proteins 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 (Fig. 4). gp91^{phox} protein expression also significantly increased in CCR2 +/+ mice with Ang II infusion at 4 weeks, and this response was absent in CCR2−/− mice (Fig. 5). Immunoblotting exhibited one positive band at 58 kDa. This molecular weight for gp91^{phox} was less than that reported for humans but similar to that in the mouse phagocyte gp91^{phox} 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 −/−, these increases were not present. Ki-67positive cells were found mainly in the glomerulus, tubule and tubulointerstial area (Fig. 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−/− (Fig. 7). In CCR2+/+, Ang II also increased renal collagen content measured using the hydroxyproline assay. Renal collagen content in CCR2−/− mice was unchanged after Ang II infusion (Fig. 8).

Discussion

Our data demonstrate that Ang II infusion caused similar increases in systolic blood pressure and left ventricular 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 glomerular filtration rate was significantly higher than in CCR2+/+ mice. We concluded that in Ang II-induced hypertension, CCR2 activation plays an important role in development of hypertensive nephropathy *via* increased oxidative stress and inflammation.

The most important ligand for the CCR2 is MCP-1 though CCR2 also binds MCP-2, MCP-3 and MCP-4[17]. Both Ang II and mechanical strain cause MCP-1 expression in vascular

cells[18,19]. 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 to CCR2+/+ mice[6]. 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.

Since both mouse strains developed similar degrees of hypertension and left ventricular 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* mechanotransduction, 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 left ventricular hypertrophy in CCR2 +/+ and CCR2−/− mice, however vascular inflammation and remodeling are significantly more severe in CCR2+/+ mice[6]. 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[20–24]. 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-trotyrosine immunostaining in kidneys of CCR2+/+ but not CCR2−/− mice. Nitrotyrosine staining was mainly in the glomerular area and some in the tubulointerstitial area and vessel walls. We also assessed the potential for ROS generation by measuring gp91phox, the main membrane component of the NADPH oxidase complex found in macrophages and various renal cells. We found that gp91phox expression was significantly increased in CCR2 +/+, but this increase was not present in CCR2−/−. The present study supports the hypothesis that in Ang II-induced hypertension, CCR2 receptor activation participates in development of oxidative stress and inflammation and development of renal fibrosis and disease[25]. Inflammation itself also increases oxidative stress[26]. Thus, Ang II could cause oxidative stress directly by stimulating NADPH 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 Raij et al[34] 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 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 IL-1, IL-2 and TNF-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 tubulointerstitial area in CCR2+/+ with Ang II-induced hypertension. It could be that mesangial cells and fibroblast proliferation participate in 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, following Ang II administration, mice lacking CCR2 exhibit less: a) ROS generation, b) macrophage infiltration, c) cell proliferation, d) glomerular matrix, and e) collagen deposition in the kidney compared to CCR2+/+. Lower ROS generation and macrophage infiltration could lead to reduced injury to the glomerular filtration membrane and tubules, while 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 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 development of renal nephrosclerosis and end stage renal disease. Hypertensive renal disease varies markedly between individuals with similar blood pressure; for example, it has been shown to be more common in African Americans than Caucasians. Also Dahl salt-sensitive rats have more severe renal damage than spontaneously hypertensive rats with similar blood pressure[46–48]. 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 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.

Acknowledgements

Sources of Funding: This work was supported by National Institutes of Health grants HL-28982(O.A.C.).

References

1. Ruiz-Ortega M, Esteban V, Ruperez M, Sanchez-Lopez E, Rodriguez-Vita J, Carvajal G, Egido J. Renal and vascular hypertension-induced inflammation: role of angiotensin II. Curr Opin Nephrol Hypertens 2006;15:159–166. [PubMed: 16481883]
2. Li JJ, Fang CH, Hui RT. Is hypertension an inflammatory disease? Med Hypotheses 2005;64:236–240. [PubMed: 15607546]
3. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. Am J Physiol Cell Physiol 2007;292:C82–C97. [PubMed: 16870827]

4. Boring L, Gosling J, Chensue SW, Kunkel SL, Farese RV Jr, Broxmeyer HE, Charo IF. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. J Clin Invest 1997;100:2552–2561. [PubMed: 9366570]
5. Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in CCR2$^{-/-}$ mice reveals a role for chemokines in the initiation of atherosclerosis [letter]. Nature 1998;394:894–897. [PubMed: 9732872]
6. Ishibashi M, Hiasa K, Zhao Q, Inoue S, Ohtani K, Kitamoto S, Tsuchihashi M, Sugaya T, Charo IF, Kura S, Tsuzuki T, Ishibashi T, Takeshita A, Egashira K. Critical role of monocyte chemoattractant protein-1 receptor CCR2 on monocytes in hypertension-induced vascular inflammation and remodeling. Circ Res 2004;94:1203–1210. [PubMed: 15059935]
7. Kitagawa K, Wada T, Furuichi K, Hashimoto H, Ishiwata Y, Asano M, Takeya M, Kuziel WA, Matsushima K, Mukaida N, Yokoyama H. Blockade of CCR2 ameliorates progressive fibrosis in kidney. Am J Pathol 2004;165:237–246. [PubMed: 15215179]
8. Wada T, Furuichi K, Sakai N, Iwata Y, Kitagawa K, Ishida Y, Kondo T, Hashimoto H, Ishiwata Y, Mukaida N, Tomosugi N, Matsushima K, Egashira K, Yokoyama H. Gene therapy via blockade of monocyte chemoattractant protein-1 for renal fibrosis. J Am Soc Nephrol 2004;15:940–948. [PubMed: 15034096]
9. Krege JH, Hodgin JB, Hagaman JR, Smithies O. A noninvasive computerized tail-cuff system for measuring blood pressure in mice. Hypertension 1995;25:1111–1115. [PubMed: 7737724]
10. Lorenz JN, Gruenstein E. A simple, nonradioactive method for evaluating single-nephron filtration rate using FITC-inulin. Am J Physiol 1999;276:F172–F177. [PubMed: 9887093]
11. Hudson Keenihan SN, Robertson SA. Diversity in phenotype and steroid hormone dependence in dendritic cells and macrophages in the mouse uterus. Biol Reprod 2004;70:1562–1572. [PubMed: 14766730]
12. Peng H, Carretero OA, Vuljaj N, Liao T-D, Motivala A, Peterson EL, Rhaleb N-E. Angiotensin-converting enzyme inhibitors. A new mechanism of action. Circulation 2005;112:2436–2445. [PubMed: 16216963]
13. Raij L, Azar S, Keane W. Mesangial immune injury, hypertension, and progressive glomerular damage in Dahl rats. Kidney Int 1984;26:137–143. [PubMed: 6239058]
14. Woessner JF Jr. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 1961;93:440–447. [PubMed: 13786180]
15. Chiariello M, Ambrosio G, Cappelli-Bigazzi M, Perrone-Filardi P, Brigante F, Sifola C. A biochemical method for the quantitation of myocardial scarring after experimental coronary artery occlusion. J Mol Cell Cardiol 1986;18:283–290. [PubMed: 3083110]
16. Bjorgvinsdottir H, Zhen L, Dinauer MC. Cloning of murine gp91phox cDNA and functional expression in a human X-linked chronic granulomatous disease cell line. Blood 1996;87:2005–2010. [PubMed: 8634451]
17. Eis V, Vielhauer V, Anders H-J. Targeting the chemokine network in renal inflammation. Arch Immunol Ther Exp 2004;52:164–172.
18. Capers Q, Alexander RW, Lou P, De LH, Wilcox JN, Ishizaka N, Howard AB, Taylor WR. Monocyte chemoattractant protein-1 expression in aortic tissues of hypertensive rats. Hypertension 1997;30:1397–1402. [PubMed: 9403559]
19. Wang DL, Wung BS, Shyy YJ, Lin CF, Chao YJ, Usami S, Chien S. Mechanical strain induces monocyte chemotactic protein-1 gene expression in endothelial cells. Effects of mechanical strain on monocyte adhesion to endothelial cells. Circ Res 1995;77:294–302. [PubMed: 7614716]
20. Dzau VJ. Tissue angiotensin and pathobiology of vascular disease. A unifying hypothesis. Hypertension 2001;37:1047–1052. [PubMed: 11304501]
21. Ray PE, Aguilera G, Kopp JB, Horikoshi S, Klotman PE. Angiotensin II receptor-mediated proliferation of cultured human fetal mesangial cells. Kidney Int 1991;40:764–771. [PubMed: 1745028]
22. Itoh H, Mukoyama M, Pratt RE, Gibbons GH, Dzau VJ. Multiple autocrine growth factors modulate vascular smooth muscle cell growth response to angiotensin II. J Clin Invest 1993;91:2268–2274. [PubMed: 8486785]

23. Ruperez M, Ruiz-Ortega M, Esteban V, Lorenzo O, Mezzano S, Plaza JJ, Egido J. Angiotensin II increases connective tissue growth factor in the kidney. Am J Pathol 2003;163:1937–1947. [PubMed: 14578193]
24. Ruiz-Ortega M, Ruperez M, Lorenzo O, Esteban V, Blanco J, Mezzano S, Egido J. Angiotensin II regulates the synthesis of proinflammatory cytokines and chemokines in the kidney. Kidney Int 2002;62 (Suppl 82):S12–S22.
25. Ruiz-Ortega M, Ruperez M, Esteban V, Rodriguez-Vita J, Sanchez-Lopez E, Carvajal G, Egido J. Angiotensin II: a key factor in the inflammatory and fibrotic response in kidney diseases. Nephrol Dial Transplant 2006;21:16–20. [PubMed: 16280370]
26. Forman HJ, Torres M. Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. Am J Respir Crit Care Med 2002;166:S4–S8. [PubMed: 12471082]
27. Pagano PJ, Clark JK, Cifuentes-Pagano ME, Clark SM, Callis GM, Quinn MT. Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. Proc Natl Acad Sci USA 1997;94:14483–14488. [PubMed: 9405639]
28. Pagano PJ, Chanock SJ, Siwik DA, Colucci WS, Clark JK. Angiotensin II induces $p67^{phox}$ mRNA expression and NADPH oxidase superoxide generation in rabbit aortic adventitial fibroblasts. Hypertension 1998;32:331–337. [PubMed: 9719063]
29. Keidar S, Kaplan M, Hoffman A, Aviram M. Angiotensin II stimulates macrophage-mediated oxidation of low density lipoproteins. Atherosclerosis 1995;115:201–215. [PubMed: 7661879]
30. Kopkan L, Castillo A, Navar LG, Majid DS. Enhanced superoxide generation modulates renal function in ANG II-induced hypertensive rats. Am J Physiol Renal Physiol 2006;290:F80–F86. [PubMed: 16106039]
31. Kanellis J, Nakagawa T, Herrera-Acosta J, Schreiner GF, Rodríguez-Iturbe B, Johnson RJ. A single pathway for the development of essential hypertension. Cardiol Rev 2003;11:180–196. [PubMed: 12852795]
32. Kawada N, Imai E, Karber A, Welch WJ, Wilcox CS. A mouse model of angiotensin II slow pressor response: role of oxidative stress. J Am Soc Nephrol 2002;13:2860–2868. [PubMed: 12444204]
33. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase. Role in cardiovascular biology and disease. Circ Res 2000;86:494–501. [PubMed: 10720409]
34. Zhou M-S, Schulman IH, Pagano PJ, Jaimes EA, Raij L. Reduced NAD(P)H oxidase in low renin hypertension. Link among angiotensin II, atherogenesis, and blood pressure. Hypertension 2006;47:81–86. [PubMed: 16344366]
35. Touyz RM, Mercure C, He Y, Javeshghani D, Yao G, Callera GE, Yogi A, Lochard N, Reudelhuber TL. Angiotensin II-dependent chronic hypertension and cardiac hypertrophy are unaffected by gp91phox-containing NADPH oxidase. Hypertension 2005;45:530–537. [PubMed: 15753233]
36. Bush E, Maeda N, Kuziel WA, Dawson TC, Wilcox JN, DeLeon H, Taylor WR. CC chemokine receptor 2 is required for macrophage infiltration and vascular hypertrophy in angiotensin II-induced hypertension. Hypertension 2000;36:360–363. [PubMed: 10988265]
37. Rodriguez-Iturbe B, Quiroz Y, Nava M, Bonet L, Chavez M, Herrera-Acosta J, Johnson RJ, Pons HA. Reduction of renal immune cell infiltration results in blood pressure control in genetically hypertensive rats. Am J Physiol Renal Physiol 2002;282:F191–F201. [PubMed: 11788432]
38. Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. J Clin Invest 1996;98:2572–2579. [PubMed: 8958220]
39. Hwang D, Fischer NH, Jang BC, Tak H, Kim JK, Lee W. Inhibition of the expression of inducible cyclooxygenase and proinflammatory cytokines by sesquiterpene lactones in macrophages correlates with the inhibition of MAP kinases. Biochem Biophys Res Commun 1996;226:810–818. [PubMed: 8831694]
40. Wilson HM, Walbaum D, Rees AJ. Macrophages and the kidney. Curr Opin Nephrol Hypertens 2004;13:285–290. [PubMed: 15073486]
41. Siragy HM, Awad A, Abadir P, Webb R. The angiotensin II type 1 receptor mediates renal interstitial content of tumor necrosis factor-alpha in diabetic rats. Endocrinology 2003;144:2229–2233. [PubMed: 12746279]

42. Kobayashi M, Sugiyama H, Wang DH, Toda N, Maeshima Y, Yamasaki Y, Masuoka N, Yamada M, Kira S, Makino H. Catalase deficiency renders remnant kidneys more susceptible to oxidant tissue injury and renal fibrosis in mice. Kidney Int 2005;68:1018–1031. [PubMed: 16105032]
43. Zhuo J, Maric C, Harris PJ, Alcorn D, Mendelsohn FA. Localization and functional properties of angiotensin II AT1 receptors in the kidney: focus on renomedullary interstitial cells. Hypertens Res 1997;20:233–250. [PubMed: 9453258]
44. Eardley KS, Cockwell P. Macrophages and progressive tubulointerstitial disease. Kidney Int 2005;68:437–455. [PubMed: 16014021]
45. Ninichuk V, Khandoga AG, Segerer S, Loetscher P, Schlapbach A, Revesz L, Feifel R, Khandoga A, Krombach F, Nelson PJ, Schlondorff D, Anders HJ. The role of interstitial macrophages in nephropathy of type 2 diabetic db/db mice. Am J Pathol 2007;170:1267–1276. [PubMed: 17392166]
46. Bataineh A, Raij L. Angiotensin II, nitric oxide, and end-organ damage in hypertension. Kidney Int Suppl 1998;68:S14–S19. [PubMed: 9839277]
47. Toto RB. Hypertensive nephrosclerosis in African Americans. Kidney Int 2003;64:2331–2341. [PubMed: 14633170]
48. Fogo AB. Hypertensive risk factors in kidney disease in African Americans. Kidney Int 2003;63 (Suppl 83):S17–S21.
49. Brown DM, Provoost AP, Daly MJ, Lander ES, Jacob HJ. Renal disease susceptibility and hypertension are under independent genetic control in the fawn-hooded rat. Nat Genet 1996;12:44–51. [PubMed: 8528250]
50. Freedman BI, Yu H, Spray BJ, Rich SS, Rothschild CB, Bowden DW. Genetic linkage analysis of growth factor loci and end-stage renal disease in African Americans. Kidney Int 1997;51:819–825. [PubMed: 9067916]

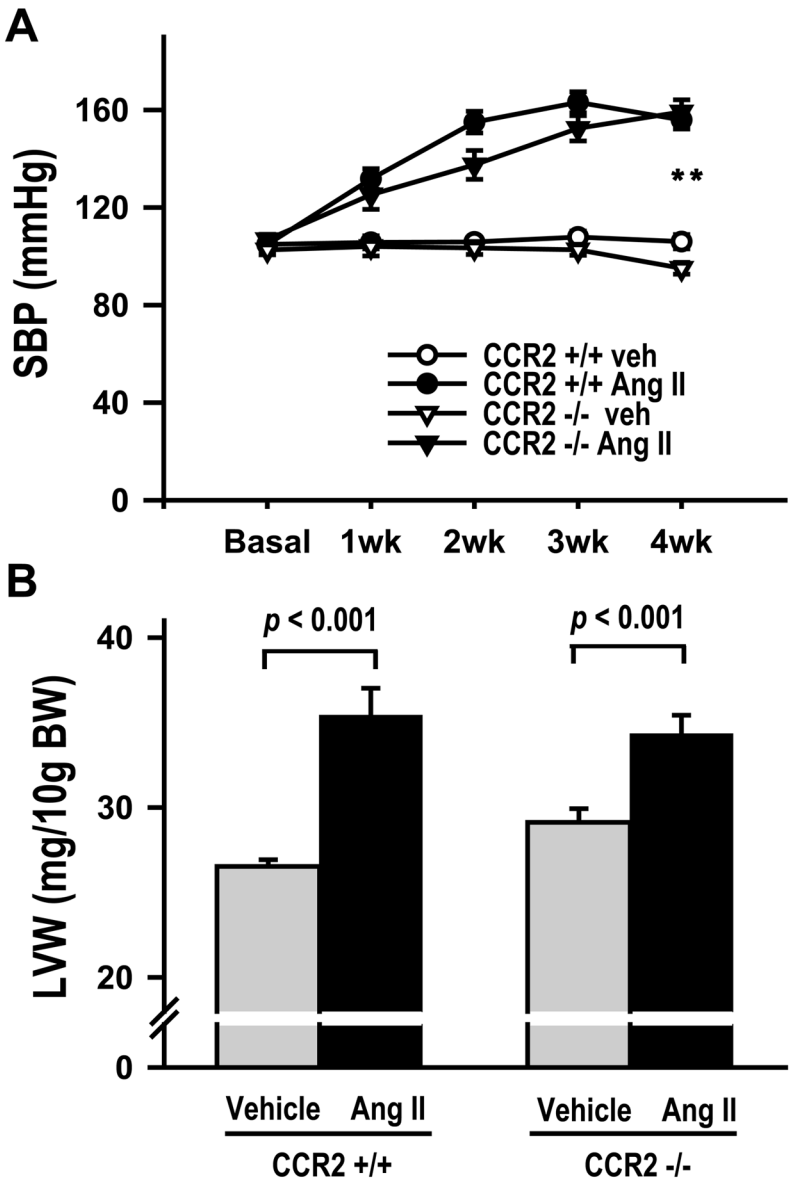

Figure 1.

(A) Systolic blood pressure (SBP), and (B) Left ventricular weight (LVW) corrected for body weight (BW) in CCR2+/+ and 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.

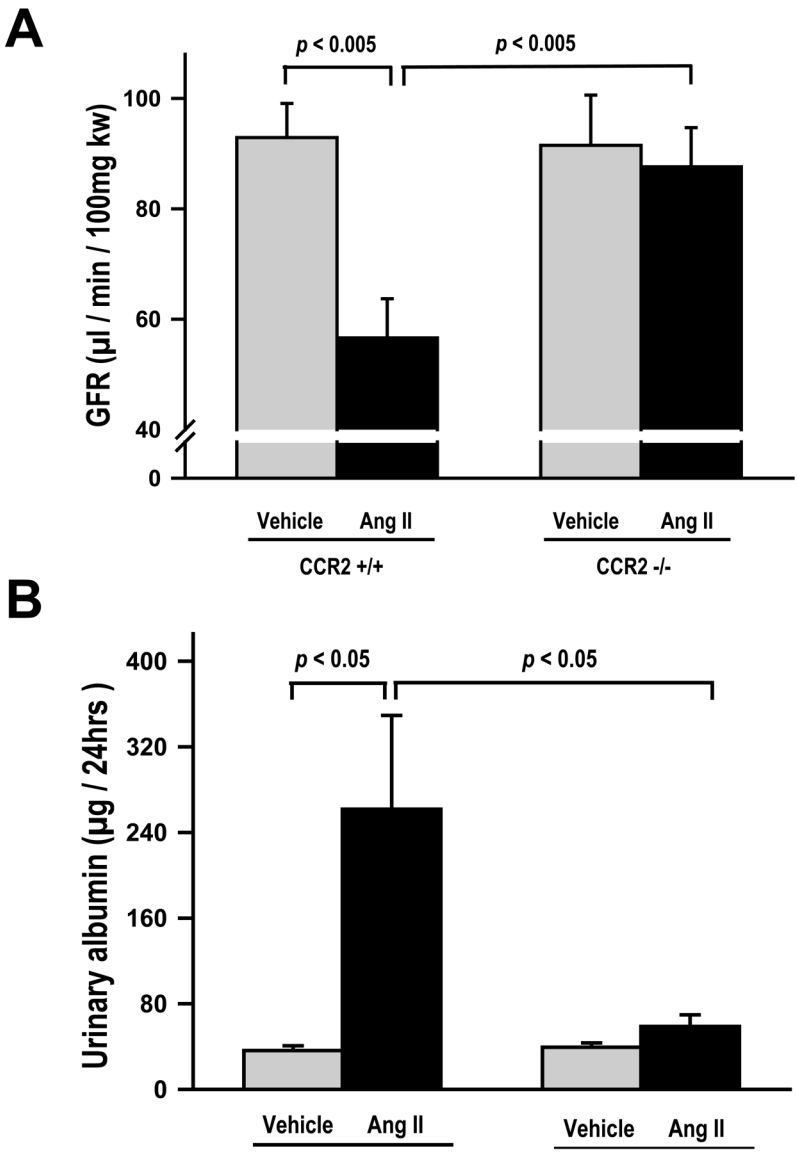

Figure 2.

(A) Glomerular filtration rate (GFR), and (B) urinary albumin in CCR2+/+ and CCR2−/− mice infused with either vehicle or Ang II. GFR was similar in vehicle groups of both strains. In CCR2+/+ with Ang II-induced hypertension, GFR was significantly lower compared to vehicle ($p<0.005$, vehicle *vs* Ang II) and also compared to CCR2−/− with Ang II-induced hypertension ($p < 0.005$, CCR2+/+ *vs* CCR2−/−). In CCR2−/− with Ang II-induced hypertension, GFR did not change (NS; vehicle vs Ang II), (n = 11 to 13 per group). Urinary albumin excretion increased significantly in CCR2+/+ with Ang II-induced hypertension ($p < 0.05$, vehicle *vs* Ang II), while in CCR2−/−, it did not change. (n = 8 to 12 per group).

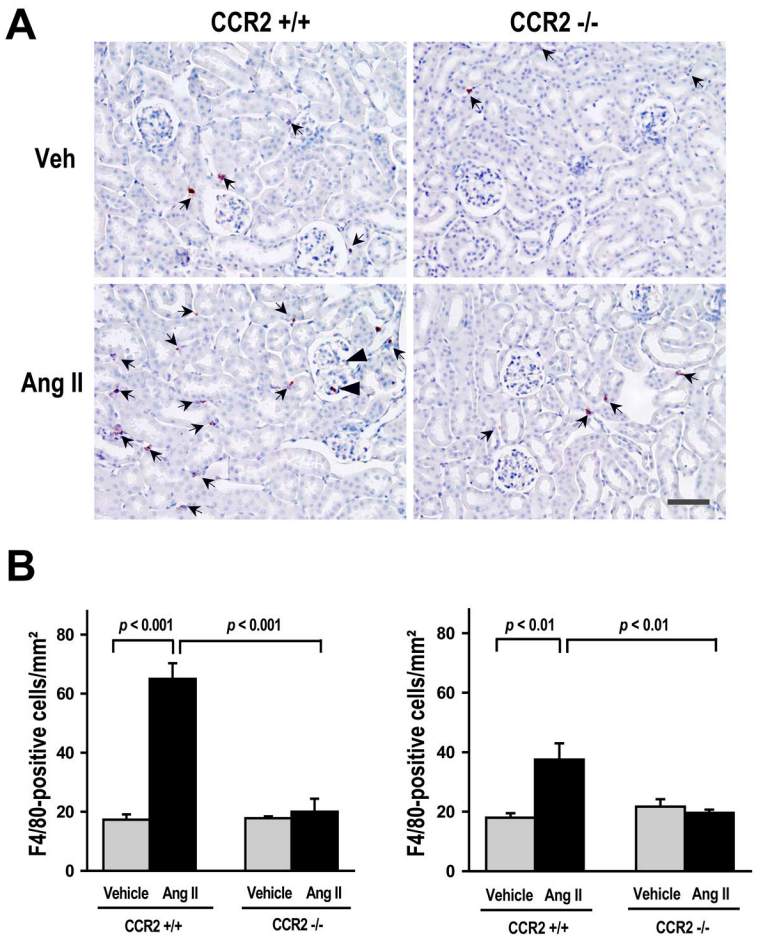

Figure 3.

(A) Representative immunohistochemical staining for F4/80-positive cells (macrophages) in mice infused for 2 weeks with either vehicle or Ang II. Reddish-brown color in the cytoplasm indicates positive staining. Positive staining for macrophages was found mainly in the tubulointerstitial space (arrows) and to a lesser extent in the glomerulus (arrow-heads). Scale Bar = 50 μm.

(B) Quantitative analysis of immunohistochemical staining for F4/80-positive cells (macrophages) at 2 (left) and 4 weeks (right). In CCR2+/+ with Ang II-induced hypertension the number of positive cells increased significantly at both 2 and 4 weeks ($p < 0.01$, vehicle *vs* Ang II). However, macrophage infiltration was higher at 2 weeks. In CCR2−/− with Ang II induced hypertension, macrophage infiltration did not increase at either 2 or 4 weeks. (2 weeks, n=5 per group; and 4 weeks, n=8 per group).

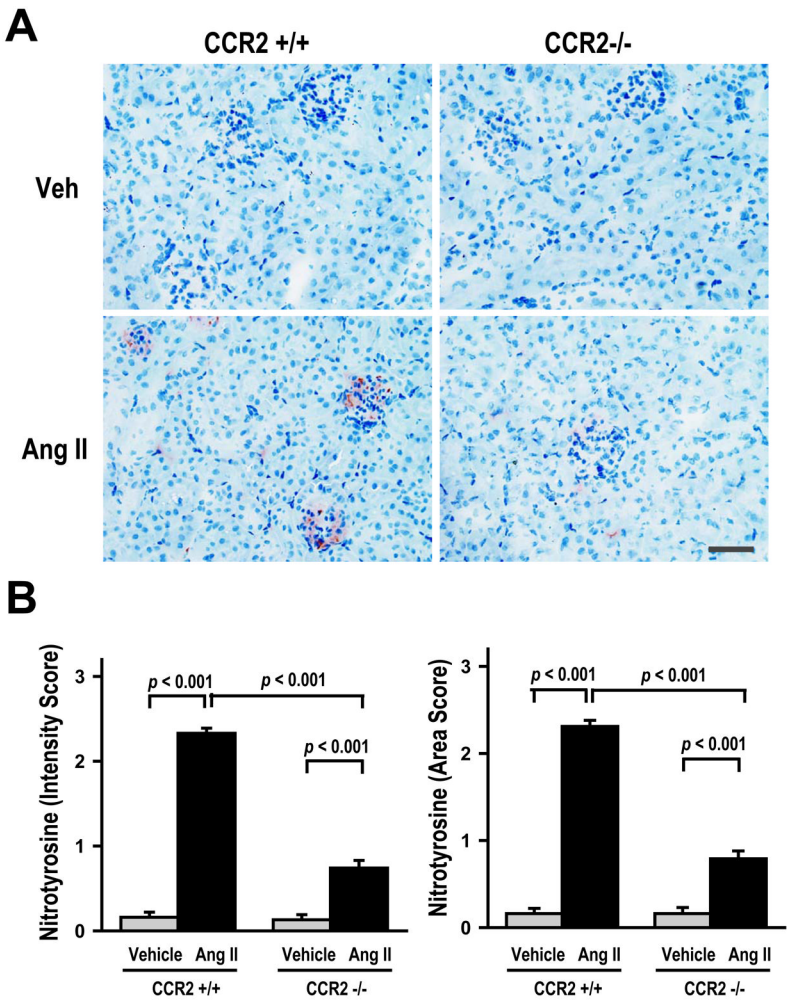

Figure 4.

(A) Representative Immunohistochemical staining for nitrotyrosine (a peroxynitrite marker) in mice infused for 4 weeks with either vehicle or Ang II. Reddish-brown color was considered positive stain. Positive stain can be found in glomerulus and tubulointerstitial area. Scale Bar = 50 μm.

(B) Semi-quantitative analysis of both intensity (left) and area (right) 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−/− with Ang II-induced hypertension these effects were significantly blunted ($p < 0.001$, CCR2+/+ *vs* CCR2−/−) but still the increases were significant compared to vehicle group ($p<0.001$; vehicle vs Ang II). Nitrotyrosine intensity and area in Ang II-induced hypertension was significantly higher in CCR2+/+ compared to CCR2−/− ($p< 0.001$; CCR2+/+ *vs* CCR2−/−). (n = 7 per group).

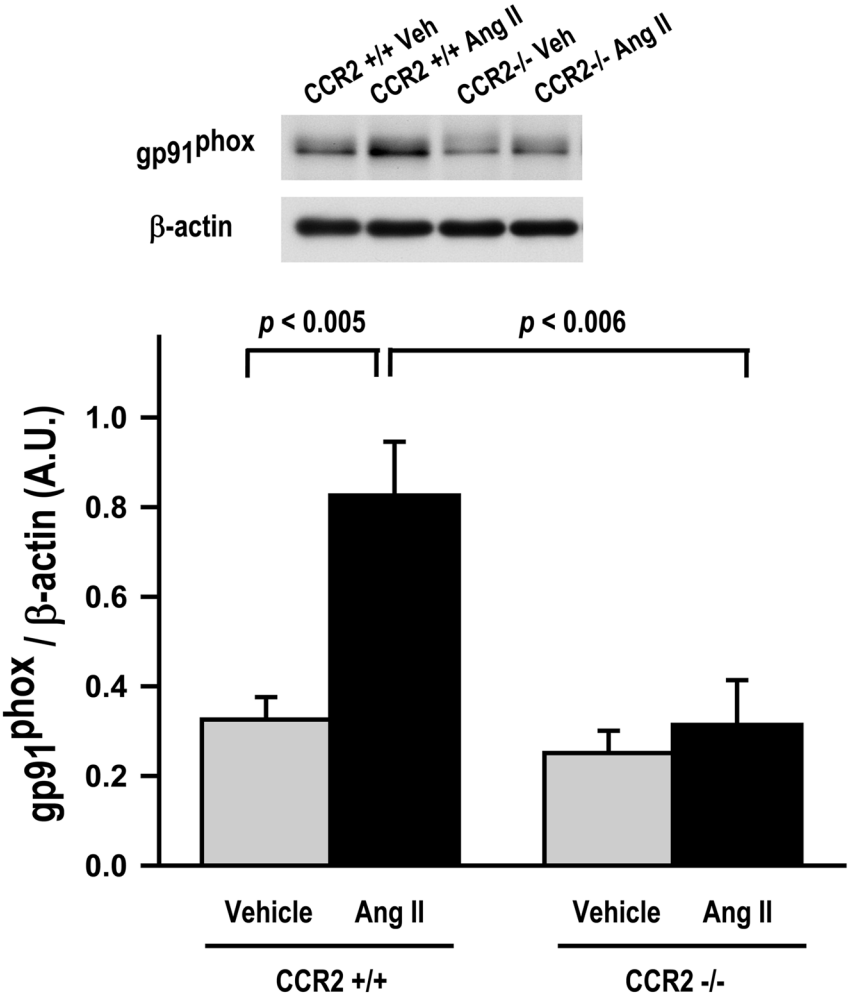

Figure 5.

Renal tissue Western blot analysis of gp91^{phox} proteins (Nox2) in mice treated with either vehicle or Ang II. The upper panel shows representative Western blot for gp91^{phox} (58 kDa) and β-actin. The lower panel shows quantification of ratio of gp91^{phox} to β-actin. gp91^{phox} expression increased significantly in CCR2+/+ with Ang II-induced hypertension ($p < 0.005$, vehicle *vs* Ang II). gp91^{phox} did not increase in CCR2−/− with Ang II-induced hypertension ($p < 0.006$, CCR2+/+ Ang II *vs* CCR2−/− Ang II). gp91^{phox} expression in Ang II-induced hypertension was significantly higher in CCR2+/+ compared to CCR2−/− ($p< 0.01$; CCR2+/+ *vs* CCR2−/−). (n=6 per group).

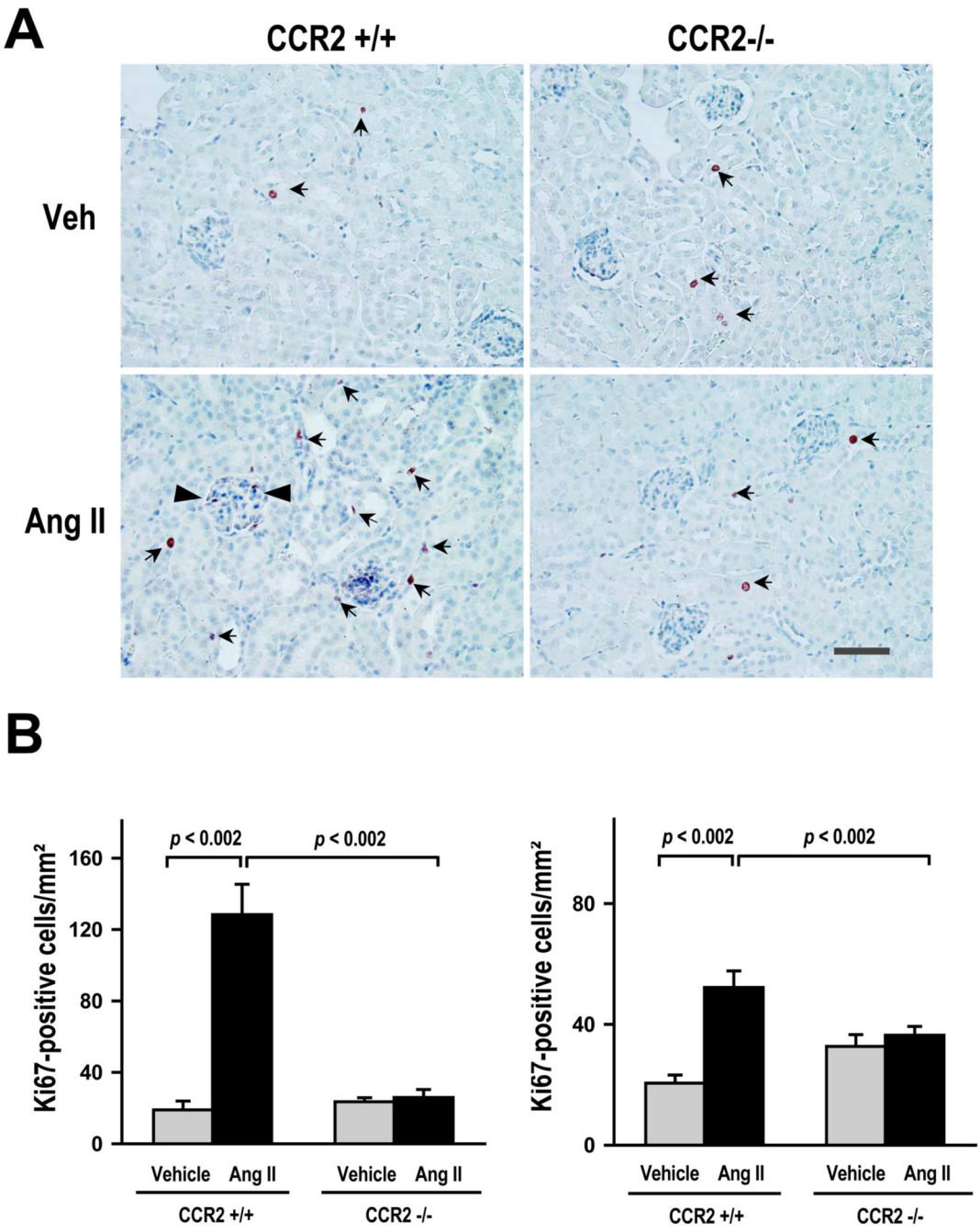

Figure 6.

(A) Representative immunohistochemical staining for Ki-67-positive cells (an indicator for cell proliferation) in mice infused for 4 weeks with vehicle or Ang II. Reddish-brown color in the nucleoli was considered positive. Positive cells were found in tubulointerstitial area (arrows) and glomerulus (arrow-heads). Scale Bar = 50 μm.

(B) Quantitative analysis of Ki-67-positive cells in mice treated with vehicle or Ang II. In CCR2+/+ with Ang II-induced hypertension, the number of cells proliferating in the kidney increased significantly at both 2 and 4 weeks ($p < 0.002$, vehicle *vs* Ang II). However, the increase at 2 weeks was higher than at 4 weeks. In CCR2−/− 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+/+ compared to CCR2−/− ($p < 0.002$; CCR2+/+ *vs* CCR2−/−). (2 weeks, n=5 per group; and 4 weeks n=8–10 per group).

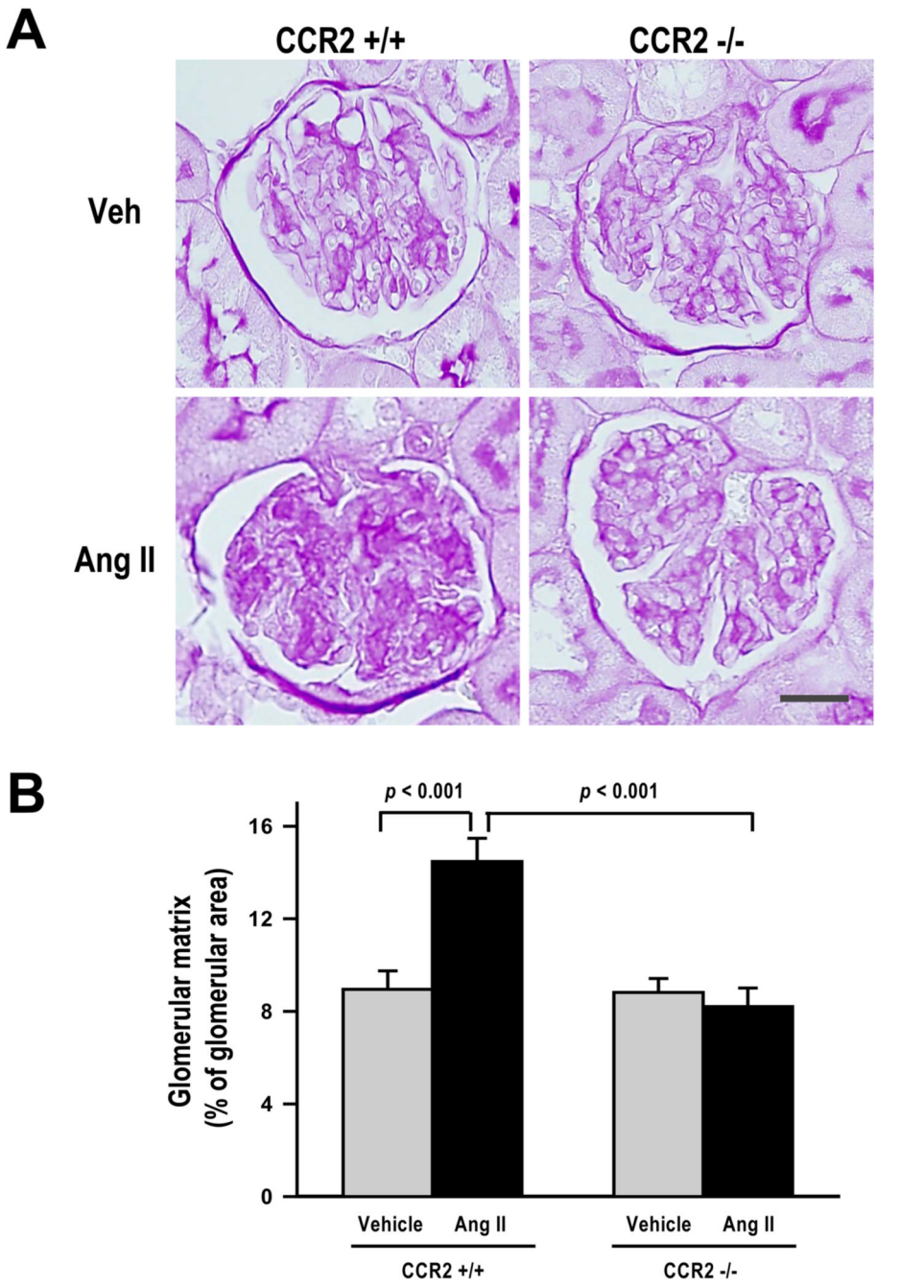

Figure 7.

(A) Representative periodic acid-Schiff (PAS) 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 or Ang II. In CCR2+/+ with Ang II-induced hypertension, glomerular matrix area increased significantly ($p < 0.001$, vehicle *vs* Ang II), while in CCR2−/−, it did not change. Glomerular matrix in Ang II-induced hypertension was significantly higher in CCR2+/+ compared to CCR2−/−; p< 0.001; CCR2+/+ *vs* CCR2−/−. (n=7 per group).

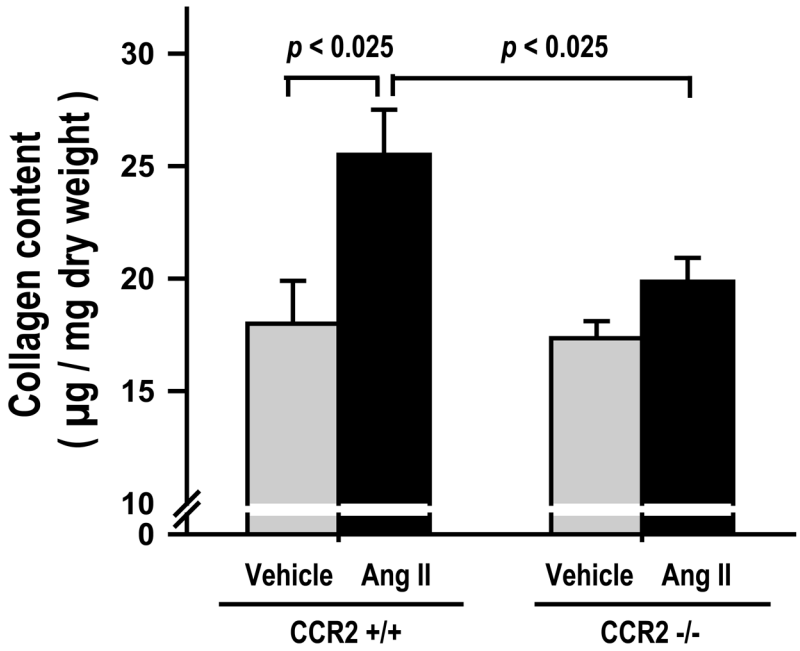

Figure 8.

Collagen content, measured by hydroxyproline assay, in kidneys from mice treated with vehicle or Ang II. In CCR2+/+ with Ang II-induced hypertension, collagen content increased significantly ($p < 0.025$, vehicle *vs* Ang II), while in CCR2 −/−, this increase was not significant. Collagen content in Ang II-induced hypertension was significantly higher in CCR2+/+ compared to CCR2−/−; $p < 0.025$, CCR2+/+ *vs* CCR2−/−. (n = 6 per group).