Background: The recruitment of leukocytes to the vascular wall is a key step in hypertension development. Chemokine receptor CXCR2 mediates inflammatory cell chemotaxis in several diseases. However, the role of CXCR2 in hypertension development and the underlying mechanisms remain unknown.

Methods: Angiotensin II (490 ng·kg\(^{-1}\)·min\(^{-1}\)) or deoxycorticosterone acetate (DOCA) salt–induced mouse hypertensive models in genetic ablation, pharmacologic inhibition of CXCR2, and adoptive bone marrow transfer mice were used to determine the role of CXCR2 in hypertension (measured by radiotelemetry and tail-cuff system), inflammation (verified by flow cytometry and quantitative real-time polymerase chain reaction [PCR] analysis), vascular remodeling (studied by haematoxylin and eosin and Masson’s trichrome staining), vascular dysfunction (assessed by aortic ring), and oxidative stress (indicated by nicotinamide adenine dinucleotide phosphate [NADPH] oxidase activity, dihydroethidium staining, and quantitative real-time PCR analysis). Moreover, the blood CXCR2\(^{+}\) cells in normotensive controls and hypertension patients were analyzed by flow cytometry.

Results: Angiotensin II significantly upregulated the expression of CXCR2 mRNA and protein and increased the number of CD45\(^{+}\) CXCR2\(^{+}\) cells in mouse aorta (n=8 per group). Selective CXCR2 knockout (CXCR2\(^{-/-}\)) or pharmacological inhibition of CXCR2 markedly reduced angiotensin II- or DOCA-salt-induced blood pressure elevation, aortic thickness and collagen deposition, accumulation of proinflammatory cells into the vascular wall, and expression of cytokines (n=8 per group). CXCR2 inhibition also ameliorated angiotensin II-induced vascular dysfunction and reduced vascular superoxide formation, NADPH activity, and expression of NADPH oxidase subunits (n=6 per group). Bone marrow reconstitution of wild-type mice with CXCR2\(^{-/-}\) bone marrow cells also significantly abolished angiotensin II-induced responses (n=6 per group). It is important to note that CXCR2 blockade reversed established hypertension induced by angiotensin II or DOCA-salt challenge (n=10 per group). Furthermore, we demonstrated that CXCR2\(^{+}\) proinflammatory cells were higher in hypertensive patients (n=30) compared with normotensive individuals (n=20).

Conclusions: Infiltration of CXCR2\(^{+}\) cells plays a pathogenic role in arterial hypertension and vascular dysfunction. Inhibition of CXCR2 pathway may represent a novel therapeutic approach to treat hypertension.
Clinical Perspective

What Is New?

- The recruitment of leukocytes to the vascular wall is an early step to the pathological process of hypertension. Chemokines and their respective receptors play a critical role in mediating inflammatory cells chemotaxis in cardiovascular diseases. Therefore, identifying specific therapeutic targets in the chemokine system is crucial for the development of new therapeutic strategies against hypertension.

- In this study, we provide experimental evidence that CXCR2+ macrophages are mediators of hypertension, vascular collagen deposition, inflammation, vascular dysfunction, and oxidative stress in angiotensin II- or DOCA-salt-induced animal models. Moreover, CXCR2+ proinflammatory cells were higher in hypertensive patients compared with normotensive individuals.

What Are the Clinical Implications?

- The findings that CXCR2 inhibition prevents and reverses hypertension and vascular dysfunction in response to multiple hypertensive stimuli increase our understanding of the mechanisms involved in CXCR2 action and the potential clinical use of CXCR2 inhibitor for the treatment of hypertension.

- Thus, selective inhibition of CXCR2 signal may be a novel therapeutic approach to prevent and cure hypertension and vascular remodeling in patients.

Hypertension is the major risk factor for stroke, coronary heart disease, and chronic kidney disease. Despite extensive investigation for more than a century, the exact mechanisms responsible for essential hypertension remain unclear. Increasing evidence has established that inflammation plays a critical role in vascular dysfunction and hypertension.1–3 Various hypertensive stimuli, including angiotensin II and reactive oxygen species, are able to stimulate migration and infiltration of proinflammatory cells, including macrophages, neutrophils, and T cells, into the vasculature, kidney, and heart. Studies have demonstrated that T-cell activation promotes hypertension development in mice.4 T-cell co-stimulation by B7 ligand is also important for the development of experimental hypertension.5 It is interesting to note that recent evidence suggests that monocytes/macrophages rather than neutrophils are essential for the development of angiotensin II-induced hypertension and vascular dysfunction.6 However, the precise mechanisms by which hypertensive stimuli recruit these cells into the vasculature during hypertension have not been fully elucidated.

Numerous factors have been shown to stimulate leukocyte migration during the inflammatory response.7 Chemokines are low molecular weight proteins of the cytokine family, which activate G-protein-coupled receptors and induce the migration of neutrophils and monocytes/macrophages to the damaged vascular wall.7,8 Four major classes of chemokines have been identified: CXC, CC, C, and CX3C. The CXC ELR+ chemokines, including CXCL1, 2, 3 (GROα, β, and γ), 5, 6, 7, and 8, act through the chemokine receptor CXCR2 and are critical for neutrophil and mononuclear cell recruitment in the mouse.9 CXCR2 is of particular interest as accumulating evidence indicates a pivotal role for this receptor in tumor development and a variety of inflammatory disorders. For example, depletion of CXCR2 impairs neutrophil recruitment and protects against ischemia-reperfusion-induced cardiac injury.10 Neutralization of CXCR2 was also shown to inhibit neutrophil accumulation in the adventitia and suppress aortic rupture after acute aortic dissection.11 In addition, pharmacologic blockade of CXCR2 was shown to prevent inflammation and autoimmune responses in experimental models of arthritis,12,13 neuroinflammation,14 and CD4+ T cells-mediated hypersensitivity reactions in the lung.15

This study aimed to define the role of CXCR2 in the pathogenesis of hypertension and vascular dysfunction induced by hypertensive stimuli. Hypertension was studied in CXCR2 knockout (CXCR2−/−) mice, wild-type (WT) mice treated with CXCR2 inhibitor, and chimeric mice engineered to lack CXCR2 only in hematopoietic cells. Our results demonstrate that angiotensin II significantly increases the expression of CXCR2 on macrophages. Knockout or inhibition of CXCR2 markedly reduces macrophage infiltration into the vascular wall, limits activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, and attenuates arterial hypertension and vascular dysfunction. Reconstitution of CXCR2−/− mice with WT bone marrow cells reestablished hypertension, vascular remodeling, and oxidative stress in response to angiotensin II, whereas reconstitution of WT mice with CXCR2−/− bone marrow cells prevented hypertension, suggesting that hematopoietic cell CXCR2 expression plays a critical role in hypertension development.

METHODS

Detailed methods are described in the online-only Data Supplement.

Animals and Experimental Protocols

WT mice (C57BL/6J, male) and CXCR2 knockout mice (CXCR2−/−, B6.129S2(C)-Cxcr2tm1Mwm/J) were purchased from Jackson Laboratory (Sacramento, CA). Mice at 8 to 12 weeks of age were used in an angiotensin II-induced hypertension model by subcutaneous infusion of angiotensin II (Sigma-Aldrich, St. Louis, MO) at a dose of 490 ng/kg/min or saline using osmotic mini-pumps (Alzet MODEL 1007D; DURECT, Cupertino, CA) for 14/28 days as described previously.16 Deoxycorticosterone acetate (DOCA)-salt-induced hypertension was also created as previously described.17 The selective CXCR2 antagonist...
SB265610 (Axon, Groningen, The Netherlands) was administered intraperitoneally (2 mg/g/d) beginning 1 day before angiotensin II/DOCA infusion and continued during angiotensin II/DOCA-salt treatment. To determine whether SB265610 could reverse established hypertension, SB265610 treatment was initiated 2 weeks after either angiotensin II infusion (for 28 days) or DOCA-salt challenge (for 21 days). All investigations were approved by the Animal Care and Use Committee of Dalian Medical University and conformed to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Blood Pressure Recordings
Blood pressure was measured by the tail-cuff system (SofronBP-98A; Softron, Tokyo, Japan) and the telemetric blood pressure system (TA-PA11C10, Data Science International, Tilburg, The Netherlands).

Histopathology
Aorta sections were fixed in formalin, embedded in paraffin, sectioned (5 µm), and stained with haematoxylin and eosin and Masson’s trichrome reagent as described previously. Cryosections were stained with the dihydroethidine (1 µM in PBS) for 30 minutes at 37°C. Green auto-fluorescence and red dihydrothididine fluorescence were detected using Nikon Labophot 2 microscope (Nikon, Tokyo, Japan).

Quantitative Real-Time PCR Analysis
Total mRNA was extracted by the Trizol Reagent method (Invitrogen, New York, NY), and the first strand of cDNA was synthesized with moloney murine leukemia virus reverse transcriptase (Promega, Southampton, UK). Quantitative real-time polymerase chain reaction (qPCR) was performed with an iCycler IQ system (Bio-Rad, CA) as described previously. The primer sequences synthesized with moloney murine leukemia virus reverse transcriptase (Promega, New York, NY), and the first strand of cDNA was synthesized with moloney murine leukemia virus reverse transcriptase (Promega, Southampton, UK). Quantitative real-time polymerase chain reaction (qPCR) was performed with an iCycler IQ system (Bio-Rad, CA) as described previously. The primer sequences are described in online-only Data Supplement Table S1.

Flow Cytometry
Inflammatory cells in aortic vessels and blood were analyzed by flow cytometry as previously described. Single-cell suspensions were treated with Fc block, washed, and stained with CD45 V500, CD3 PE-CF594, CD11b APC-Cy7, Ly6G PE-Cy7, CD45 V500, CD3 PE-CF594, CD11b APC-Cy7, Ly6G PE-Cy7, CD13 APC, CD15 FITC, CD64 V450, and CXCR2 PE (BD) as described previously. Cryosections were stained with the dihydroethidine (1 µM in PBS) for 30 minutes at 37°C. Green auto-fluorescence and red dihydrothididine fluorescence were detected using Nikon Labophot 2 microscope (Nikon, Tokyo, Japan).

Constitution of Bone Marrow Transplantation Chimeric Mice
We used bone marrow transplantation to produce chimeric mice to study the contribution of bone marrow-derived CXCR2-positive cells to hypertension as previously described.

Vascular Relaxation Studies
The thoracic aorta was cut into 4-mm segments and gently mounted on force transducers (Power Laboratory, AD Instruments, Bella Vista, Australia) in organ chambers. After stimulation by norepinephrine, vascular responses to increasing concentrations of acetylcholine and sodium nitroprusside (SNP) were recorded.

NADPH Oxidase Activity
Aortas were cut into pieces and homogenized by ultrasonic processor in PBS buffer and protein concentration was determined by BCA Protein Assay Kit (Life Technologies, Waltham, MA). The aorta lysate was mixed with 0.1 mM NADPH, 5 µM lucigenin, PBS buffer, and water in 96-well plates and incubated in 37°C for 30 min. NADPH oxidase activity of the aorta was measured by photon emission from the chromogenic substrate lucigenin by lumimeter at 450-nm wavelength. The data were converted to relative light units/min/mg of protein.

Study Patients and Flow Cytometry Analysis
This investigation was performed with approval by the Institutional Ethics Committee of First Affiliated Hospital of Dalian Medical University. Thirty new-onset essential hypertensive patients (systolic blood pressure ≥140 mmHg and diastolic blood pressure ≥90 mmHg) and 20 normotensive individuals were included in the study. The baseline characteristics of normotensive control subjects and hypertensive patients are shown in Table 1. Blood samples were obtained from all participants in the recumbent position with a 21-gauge needle by antecubital venipuncture. Coagulation was blocked by K3EDTA. Blood was erythrocyte-depleted with RBC lysis buffer (BD) and incubated with CD45 PerCP-cy5.5, CD13 APC, CD15 FITC, CD64 V450, and CXCR2 PE (BD) as previously described. On the basis of a live gate, events were acquired on a FACS Canto II™ flow cytometer (BD) and analyzed.

Table 1. Baseline Characteristics of Normotensive Control Subjects and Hypertensive Patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normotensive Controls (n=20)</th>
<th>Hypertensive Patients (n=30)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>37 (10)</td>
<td>44 (13)*</td>
<td>0.039</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>10 (50)</td>
<td>15 (50)</td>
<td>0.172</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>110±8</td>
<td>163±28***</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>68±7</td>
<td>100±17***</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>71±7</td>
<td>74±11</td>
<td>0.124</td>
</tr>
<tr>
<td>Cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total, mmol/L</td>
<td>4.2±0.6</td>
<td>4.9±1**</td>
<td>0.007</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>2.6±0.4</td>
<td>3.0±0.7*</td>
<td>0.016</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.4±0.2</td>
<td>1.2±0.2**</td>
<td>0.002</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.1±0.3</td>
<td>1.5±0.8*</td>
<td>0.019</td>
</tr>
<tr>
<td>Creatinemia, umol/L</td>
<td>57±12</td>
<td>61±13</td>
<td>0.381</td>
</tr>
<tr>
<td>Fasting blood glucose, mmol/L</td>
<td>4.4±0.8</td>
<td>4.5±1.0</td>
<td>0.248</td>
</tr>
<tr>
<td>White blood cell count (10^9/L)</td>
<td>6.0±1.3</td>
<td>7.0±2.8</td>
<td>0.294</td>
</tr>
</tbody>
</table>

Hypertensive patients versus normotensive control. HDL indicates high-density lipoprotein; and LDL, low-density lipoprotein. All the parameters are mean (SD) except Male, n (%).

*P<0.05, **P<0.01, ***P<0.001.
Statistical Analysis

SPSS 16.0 was used to perform the statistical calculations. All data are expressed as mean±SE. First, the normality test (Shapiro-Wilk) was performed to determine whether the data were normally distributed with SPSS 16.0. If data were normally distributed (or in Gaussian distribution), the Student’s t test was used to test for the difference between 2 groups. If the data were not normally distributed, then the Mann-Whitney test was used to test for the difference between two groups. For acetylcholine or SNP-induced vasodilatation tests in aortic rings, repeated-measures analysis of variance was used. If the analysis of variance demonstrated a significant effect, then post hoc comparisons were made pairwise with the Fisher least significant difference test. Multivariable logistic regression models were used to evaluate the association of human hypertension and blood CXCR2+ cell numbers (including CD45+CXCR2+ cells, CD45+CD13+CXCR2+ monocytes, CD45+CD13+CD15+CXCR2+ neutrophils, and CD45+CD13+CD64+CXCR2+ macrophages) while adjusting for sex, age, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides. P<0.05 was considered statistically significant.

RESULTS

Angiotensin II Induces CXCR2-Positive Myelomonocytes Infiltration in the Vascular Wall During Hypertension

Chemokines are known to play a critical role in the infiltration of myelomonocytes.6 We first used a microarray assay to evaluate the chemokine gene expression profiles in the aortas of mice exposed to angiotensin II. We found that chemokine CXCL1 was the most highly expressed (13.9-fold vs saline-treated) in the aorta 1 day after angiotensin II treatment (Figure IA in the online-only Data Supplement). CXCL1 expression was further confirmed by qPCR analysis (Figure IB in the online-only Data Supplement). Because CXCL1 signals through the G protein-coupled receptor CXCR2, we analyzed the aortic infiltration of CXCR2+ cells by flow cytometry. Compared with saline infusion, angiotensin II infusion caused a significant increase of CD45+CXCR2+ cells in mice aortas (Figure 1A). Real-time qPCR showed that angiotensin II infusion significantly increased CXCR2 mRNA content in the aorta as well (Figure 1B). Immunohistochemical staining further demonstrated that angiotensin II infusion increased CXCR2 protein expression in aorta (Figure 1C).

CXCR2 Knockout Abolished Angiotensin II-Induced Hypertension, Vascular Hypertrophy, Aortic Macrophage Infiltration, and Inflammatory Gene Expression

We next determined the effect of CXCR2 on blood pressure in CXCR2 knockout (CXCR2-/-) mice using both the noninvasive tail-cuff method (Figure II in the online-only Data Supplement) and invasive radio-telemetry (Figure 2A and 2B). To our surprise, angiotensin II-induced hypertension was almost totally abolished in CXCR2-/- mice compared with WT animals (Figure 2A and 2B). Radiotelemetry further confirmed that systolic pressure averaged 161±3.5 mm Hg in WT mice and 109±3.4 mm Hg in CXCR2-/- mice during the last week of angiotensin II infusion (Figure 2A and 2B). Likewise,
Figure 2. Depletion of CXCR2 prevents angiotensin II-induced hypertension, vascular inflammation, and fibrosis. 

A, Representative original systolic blood pressure recordings in WT and CXCR2-/- mice 3 days before angiotensin II treatment (baseline) and during the last 3 days of angiotensin II infusion (Ang II) by telemetric blood pressure measurements. B, Average systolic (top) and diastolic (bottom) blood pressure of WT and CXCR2-/- mice before and after angiotensin II treatment obtained by telemetry. *P<0.01 versus WT + Ang II; n=8 per group. C, H&E staining of thoracic aorta (top) and the wall thickness of each group were analyzed (bottom). Scale bar, 50 μm. D, Masson staining of thoracic aorta (top) and the percentage of fibrotic (Continued)
diastolic pressure was reduced from 129±1.4 mmHg to 95±0.7 mmHg in CXCR2-/- mice (Figure 2B). Angiotensin II also causes aortic wall thickening and collagen deposition in WT animals, and this finding was significantly blunted in CXCR2-/- mice (Figure 2C and 2D). Moreover, angiotensin II-induced infiltration of CD45+ myelomonocytes, especially F4/80+ macrophages in the aorta, was markedly attenuated in CXCR2-/- mice. It is interesting to note that CD3+ T cells were also markedly decreased in CXCR2-/- mice compared with WT mice after angiotensin II infusion. Depletion of CXCR2 did not change the number of macrophages and T cells after saline infusion (Figure 2E). In addition, angiotensin II-induced expression of several proinflammatory genes and fibrotic markers (IL-1α, IL-6, TNF-α, CD68, VCAM, COX-2, α-SMA, collagen I, and collagen III) in aortic tissue was significantly reduced in CXCR2-/- mice (Figure 2F and 2G).

**Pharmacological Inhibition of CXCR2 Profoundly Suppressed Angiotensin II-Induced Hypertension, Vascular Remodeling, and Inflammation**

To further assess the role of CXCR2 in hypertension, we performed additional experiments in WT mice treated with CXCR2 inhibitor SB265610. Consistent with the results obtained from CXCR2-/- mice, treatment with SB265610 fully attenuated angiotensin II-induced hypertension (Figure 3A). Furthermore, angiotensin II induced aortic wall thickness, collagen deposition, and accumulation of CD45+ myeloid-derived inflammatory cells, including macrophages and T cells, and expression of proinflammatory cytokines and fibrotic markers (IL-1β, IL-6, TNF-α, CD68, VCAM, COX-2, α-SMA, collagen I, and collagen III) in aortic tissue was also significantly attenuated in SB265610-treated mice (Figure 3B through 3F). These results suggest that CXCR2 contributes to angiotensin II-induced hypertension and vascular injury.

**Inhibition of CXCR2-Attenuated Angiotensin II-Induced Vascular Dysfunction and Oxidative Stress**

To examine how CXCR2 inhibition ameliorated vascular dysfunction, we measured ex vivo vascular function in WT, CXCR2-/-, or SB265610-treated mice with angiotensin II. Intact aortas were isolated from WT and CXCR2-/- or SB265610-treated mice 14 days after saline or angiotensin II infusion, and concentration-relaxation curves in response to acetylcholine or SNP were examined. Consistent with previous observations,6,18–20 chronic angiotensin II infusion significantly impaired endothelium-dependent vasodilatation to acetylcholine compared with saline-treated control, whereas these effects were markedly preserved in CXCR2-/- and SB265610-treated mice. Angiotensin II infusion only mildly affected endothelium-independent vasodilatation to SNP in WT mice, and there was no statistically significant difference between angiotensin II-treated mice and saline control. Acetylcholine and SNP-induced vasodilatation was not different between WT and CXCR2-/- mice under control conditions (treated with saline) (Figure 4A and 4B). These results indicate that blocking CXCR2 function prevented angiotensin II-induced endothelial dysfunction.

Increased vascular tone is thought to be mediated by an increase in vascular superoxide production.21,22 We found that angiotensin II significantly increased aortic superoxide (as indicated by dihydroethidine staining), and this effect was abrogated in CXCR2-/- or SB265610-treated mice (Figure 4C and 4D). Moreover, the increase of superoxide formation was accompanied by increased aortic NADPH oxidase activity in both groups after angiotensin II infusion (Figure 4E). qPCR analysis demonstrated that aortic NADPH oxidase NOX1, NOX2, NOX4 catalytic subunits, and p22phox mRNA levels were significantly elevated by angiotensin II treatment in WT mice, but this increase was markedly attenuated in aortas from CXCR2-/- mice or in WT mice treated with SB265610 (Figure 4F and 4G). Thus, CXCR2 drives angiotensin II-induced increases in aortic NADPH oxidase activity and superoxide formation, which likely contribute to vascular dysfunction.

**CXCR2 Knockout Prevents DOCA-Salt-Induced Hypertension**

To determine whether the reduced hypertensive response in CXCR2-/- or SB265610-treated mice was specific to angiotensin II, we also examined a mouse model of DOCA-salt-induced hypertension. Similar to the angiotensin II study, DOCA-salt-induced hypertension was markedly attenuated in CXCR2-/- mice compared with WT mice (Figure 5A). Likewise, DOCA-salt-induced increases in aortic wall thickness and fibrosis,
Figure 3. The CXCR2 inhibitor SB265610 alleviates hypertension, vascular inflammation, and fibrosis after angiotensin II infusion. A, Systolic blood pressure was measured by the noninvasive tail-cuff method in vehicle or SB265610-treated mice before (C) and after angiotensin II treatment (T, 490ng/kg/min). B, H&E staining of thoracic aorta (left) (Continued)
superoxide production (red dihydroethidium-derived fluorescence), and mRNA expression of proinflammatory cytokines (IL-1β, IL-6, TNF-α, CD68, VCAM, and iNOS) and NADPH oxidase subunits (NOX1, NOX2, NOX4, and p22phox) were markedly reduced in CXCR2−/− mice (Figure 5B through 5F).

Selective Deletion of CXCR2 on Bone Marrow–Derived Cells Attenuates Angiotensin II–Induced Hypertension, Inflammation, Vascular Dysfunction, and Oxidative Stress

Because CXCR2 is expressed on macrophages and neutrophils and on nonmyeloid cells such as fibroblasts, smooth muscle cells, and endothelial cells 23–28 we specifically examined the role of myeloid cell CXCR2 expression in the development of hypertension and vascular dysfunction. We generated chimeric mice by transplanting bone marrow (BM)–derived cells from WT or CXCR2−/− mice into lethally irradiated WT and CXCR2−/− mice, respectively. Four weeks later, these mice were infused with either angiotensin II or vehicle for 2 weeks. WT mice transplanted with CXCR2−/− BM showed significantly reduced systolic pressure, aortic thickening, collagen deposition, and F4/80+ macrophages infiltration into the adventitia, and reduced mRNA expression of proinflammatory cytokines and fibrotic markers (IL-6, IL-1β, TNF-α, CD68, VCAM, COX-2, α-SMA, collagen I, and collagen III) compared with WT mice transplanted with WT BM. In contrast, transplantation of WT BM into CXCR2−/− mice completely restored these pathologic features of hypertension. CXCR2−/− mice transplanted with CXCR2−/− BM responded the same as WT mice transplanted with CXCR2−/− BM (Figure 6A through 6D, 6H, Figure IIIA and IV in the online-only Data Supplement).

The effect of angiotensin II infusion on vascular dysfunction was also examined in BM-transplanted mice. Angiotensin II-induced impairment of endothelium-dependent vasodilatation was much greater in WT mice transplanted with WT BM than in WT mice transplanted with CXCR2−/− BM, but there was no significant difference of endothelium-independent vasodilatation between each group (Figure 6E). Likewise, angiotensin II-induced aortic superoxide production (as indicated by dihydroethidium staining), NADPH oxidase activity, and expression of NOX2, NOX4, and p22phox were markedly attenuated in WT mice transplanted with CXCR2−/− BM cells. CXCR2−/− mice transplanted with CXCR2−/− BM responded the same as WT mice transplanted with CXCR2−/− BM (Figure 6F and 6G, Figure IIIB in the online-only Data Supplement). Overall, these findings demonstrate that inhibition of myeloid cell CXCR2 activity prevents angiotensin II–induced hypertension, superoxide production, vascular remodeling, and dysfunction.

CXCR2 Inhibitor Reverses Established Hypertension

We next tested whether inhibition of CXCR2 can reverse preestablished hypertension in mice induced by angiotensin II or DOCA-salt. Consistent with previous and present data, 5 angiotensin II infusion or DOCA-salt treatment for 2 weeks elevated blood pressure to 173±5.9 mm Hg or 131±4.5 mm Hg, respectively, compared with saline control (97±6.9 mm Hg). Mice were then divided into two groups: one received intraperitoneal injection of inhibitor SB265610 (2 mg/kg/d per mouse) for an additional 2 weeks, and another group received the vehicle only. Treatment of mice with SB265610 significantly lowered blood pressure in mice induced by either angiotensin II (Figure 7A) or DOCA-salt (Figure 7B) within 1 week of treatment. These results indicate that blockade of CXCR2 lowers hypertension and reduces preestablished hypertension.

CXCR2+ Cells Are Increased in Human Hypertension

To determine whether CXCR2+ macrophages are important in human hypertension, we analyzed blood CXCR2+ cells in patients (30 with hypertension and 20 normotensive individuals, Table 1) with flow cytometry. We found that hypertensive patients were older and had higher total cholesterol, LDL cholesterol, and triglyceride and lower HDL cholesterol concentrations in serum compared with normotensive control (Table 1). Flow cytometry showed that CXCR2 was mainly expressed in CD45+CD13+CD64+ macrophages in hypertensive individuals (Figure 8). We then evaluated the association of blood CXCR2+ cell numbers and hypertension with
Figure 4. Inhibition or genetic ablation of CXCR2 improves vascular function and reduces oxidative stress in response to Angiotensin II.

A and B, Concentration-response curves of endothelium-dependent (acetylcholine, left) and nonendothelium-dependent (SNP, right) relaxation. C and D, DHE staining of the aortic segments (left) and the DHE fluorescence intensity of each group were analyzed (right). Red fluorescence reflects the superoxide formation, whereas green fluorescence shows the laminae. Scale bar, 50 μm.

E, Aortic NADPH oxidase activity was measured. F and G, qPCR analysis of NOX1, NOX2, NOX4, and p22phox mRNA expression. *P<0.05, **P<0.01 versus WT/vehicle + saline, #P<0.05, ##P<0.01 versus WT + Ang II; n=6 per group. A indicates adventitia; Ach, acetylcholine; Ang II, angiotensin II; DHE, dihydroethidine; E, endothelium; M, media; NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidase; SNP, sodium nitroprusside; and WT, wild-type.
multivariable logistic regression models. After adjusting for sex, age, total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides, a statistically significant association was found between the numbers of CXCR2+ cells, including CD45+ cells (odds ratio [OR] 1.422), monocytes (OR 1.499), neutrophils (OR 1.481), macrophages (OR 1.145), and hypertension (Table 2).

**DISCUSSION**

In this study, we demonstrated that inhibition of chemokine receptor CXCR2 activity by genetic deletion or pharmacologic CXCR2 inhibitor attenuated hypertension development in mice. CXCR2 disruption reduced angiotensin II or DOCA-salt-induced aortic infiltration of macrophages, proinflam-
Figure 6. CXCR2 deficiency in bone marrow-derived cells prevents angiotensin II–induced hypertension, vascular inflammation, fibrosis, dysfunction, and oxidative stress. A, Representative telemetric trace recording of systolic blood pressure of each group during the last 3 days of angiotensin II infusion. B, Average systolic blood pressure of each group before and after angiotensin II treatment obtained by telemetry. C and D, H&E and Masson staining of thoracic aorta (left), wall thickness, and percentage of fibrotic area of each group were analyzed (right). Scale bar, 50 μm. E, Concentration-response curves of endothelium acetylcholine-dependent (Continued)
matory cytokine expression, and vascular superoxide production and prevented development of vascular endothelial dysfunction. In addition, adoptive transfer of BM-derived cells from WT mice into CXCR2−/− mice reestablished angiotensin II-induced hypertension, vascular dysfunction, inflammation, fibrosis, and oxidative stress, indicating a specific involvement of leukocyte CXCR2 expression in the development of hypertension. It is important to note that CXCR2 blockade also reversed established angiotensin II or DOCA-salt-induced hypertension.

In the present study, our data revealed that ablation or inhibition of CXCR2 activity reduced vascular endothelial dysfunction, attenuated vascular oxidative stress, decreased the extent of vascular medial hypertrophy and collagen deposition (Figure 2 through 5), and, most important, nearly abolished the increase in systolic blood pressure induced by angiotensin II or DOCA-salt. In addition, the number of vascular macrophages and T lymphocytes, and the expression of proinflammatory cytokines, were markedly reduced by knockout of CXCR2 or inhibitor SB265610 (Figure 2E and 3D), demonstrating a critical role for CXCR2 in vascular infiltration of inflammatory cells, oxidative stress, and hypertension.

Inflammatory cells, including monocytes/macrophages, neutrophils, and T lymphocytes, exert differential effects in experimental models of hypertension, vascular inflammation, and dysfunction. For example, infiltration of neutrophils is important to initially activate or fully stimulate monocytes/macrophages in the vessel wall but has no effect on arterial hypertension induced by angiotensin II. T cells but not B cells have been reported to promote vascular dysfunction and hypertension in mice after angiotensin II infusion or DOCA-salt stress. It is important to note that monocytes/macrophages but not neutrophils were shown to be responsible for angiotensin II-induced vascular dysfunction and hypertension. In this study, our flow cytometry measurements showed that CXCR2 was highly expressed in macrophages and neutrophils but...
almost undetectable in T lymphocytes (Figure IC in the online-only Data Supplement). Furthermore, depletion or inhibition of CXCR2 effectively reduced blood pressure elevation and infiltration of F4/80+ macrophages in aorta in response to angiotensin II (Figure 2 and 3). Although adoptive transfer of CXCR2−/− BM into WT mice markedly attenuated hypertension, vascular dysfunction, and oxidative stress, transfer of WT BM into CXCR2−/− mice reestablished these effects in response to angiotensin II infusion (Figure 6). These findings demonstrate that CXCR2+ macrophages mediate angiotensin II- or DOCA-salt-induced arterial hypertension and vascular dysfunction.

Early studies demonstrated that angiotensin II-induced hypertension and vascular dysfunction are associated with increased vascular superoxide production.21 NADPH oxidase isoforms NOX1-5 are the major source of superoxide in vessels.32,33 NOX1 is expressed mainly in vascular smooth muscle cells; NOX2 in endothelial cells, cardiomyocytes, and some VSMCs; NOX4 in endothelial cells, VSMCs, and other cells; and NOX5 in human endothelial cells.34 The various NOX isoforms are believed to play important roles in vascular remodeling and dysfunction. Here we found that angiotensin II-induced production of superoxide, NADPH oxidase activity, and the mRNA levels of NADPH oxidase subunits NOX1, NOX2, NOX4, and p22phox were markedly abrogated in CXCR2−/− or SB265610-treated mice (Figure 4E through 4G). Proinflammatory cells are believed to be the main source of superoxide in the vascular wall.35 As shown in our study, inhibition or ablation of CXCR2 markedly reduced the number of CD45+ cells, particularly macrophages, which infiltrated the vascular wall after angiotensin II infusion. Together these data suggest that inhibition or ablation of CXCR2 at-

Figure 8. Blood CXCR2+ cells are increased in hypertensive patients.
A–D, Flow cytometric analysis of circulating CD45+CXCR2+ inflammatory cells (A), CD45+CD13+CXCR2+ monocytes (B), CD45+CD13+CD15+CXCR2+ neutrophils (C), and CD45+CD13+CD64+CXCR2+ macrophages (D) in both hypertensive patients (n=30) and normotensive individuals (n=20). ***P<0.001 hypertensive patients versus normotensive control.
tenuates hypertension by reducing inflammatory cell infiltration and subsequent vascular oxidative stress. CXCR2 has been implicated in a number of immune-mediated inflammatory diseases.\textsuperscript{36} Although several CXCR2-specific inhibitors have been developed as anti-inflammatory agents, there are currently no clinically approved CXCR2 inhibitors. Recently, agents that inhibit CXCR2 activation, such as SB265610, reparixin, and CX797, have been developed and examined in various animal disease models, including autoimmune diseases, ischemia-reperfusion injury, diabetes, lung injury, and vascular injury.\textsuperscript{11,15,37–39} It is interesting to note that comparison of these inhibitors shows a distinct mechanism of action on CXCR2. For example, CX797 inhibits IL-8-mediated β-arrestin-2 recruitment and cell migration,\textsuperscript{36} whereas SB265610 inhibits CXCR2 function by binding to an allosteric site.\textsuperscript{40} Recently, reparixin was used to effectively decrease systolic blood pressure in spontaneously hypertensive rats.\textsuperscript{41} Our results also demonstrated that SB265610 markedly inhibited vascular inflammation and hypertension induced by angiotensin II. It is important to note that SB265610 treatment reversed established hypertension in our model (Figure 7), suggesting that CXCR2 may be a new therapeutic target for treatment of arterial hypertension.

\textbf{CONCLUSIONS} 

Our study demonstrated for the first time that hypertension induced by various stimuli, including angiotensin II, mineralocorticoids, and high salt, requires CXCR2 activity, which is essential for vascular infiltration of macrophages, dysfunction, and oxidative stress (Figure V in the online-only Data Supplement). These findings may be clinically relevant because CXCR2-positive cells were also markedly elevated in hypertensive patients. Therefore, selective blockade of proinflammatory CXCR2\textsuperscript{+} cells, especially macrophages, might represent a novel therapeutic target for arterial hypertension treatment.

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\textbf{DISCLOSURES} 

None.

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\textbf{FOOTNOTES} 

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\textbf{REFERENCES} 

CXCR2 Mediates Experimental Hypertension


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Genetic and Pharmacologic Inhibition of the Chemokine Receptor CXCR2 Prevents Experimental Hypertension and Vascular Dysfunction
Lei Wang, Xue-Chen Zhao, Wei Cui, Yong-Qiang Ma, Hua-Liang Ren, Xin Zhou, John Fassett, Yan-Zong Yang, Yingjie Chen, Yun-Long Xia, Jie Du and Hui-Hua Li

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**SUPPLEMENTAL MATERIAL**

**Table SI Primers used for quantitative real-time PCR**

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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>5'-GTTGGTCTCTCGGAGGCTTCA-3'</td>
<td>5'-GTTGGTCTCTCGGAGGCTTCA-3'</td>
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</table>

CXCR2, chemokine (C-X-C motif) receptor 2; VCAM, vascular cell adhesion molecule; COX2, cytochrome c oxidase subunit II; IL-1β, Interleukin-1β; IL-6, Interleukin-6; NOX1, NADPH oxidase 1; NOX2, NADPH oxidase 2; NOX4, NADPH oxidase 4; TNF-α, Tumor necrosis factor; P22phox, cytochrome b-245, alpha polypeptide; α-SMA, α-smooth muscle actin; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.
1. Supplemental Methods

1.1 Animals and Treatment

Mice were anaesthetized with ketamine (200 mg/kg) and xylazine (10 mg/kg) by intra-peritoneal injection (i.p.). Anaesthesia was monitored by pinching the toe. Post-operative anaesthesia (buprenorphine, 0.05 mg/kg/12h, i.p.) was administered for 48 h. On the last day of angiotensin II/DOCA infusion, all mice were anaesthetized by an overdose of pentobarbital (100 mg/kg, intra-peritoneal injection, Sigma-Aldrich). The aortas were removed and prepared for further histological and molecular analysis.

1.2 Telemetric Blood Pressure Recording

Mice were anesthetized with isoflurane. The left common carotid artery was isolated carefully, the catheter which was connected to the transducer was introduced into the carotid and advanced until the tip was just inside the thoracic aorta. One of the ECG transmitters was positioned along the right flank, the other was positioned close to the hindlimb. The transducer was implanted abdominal subcutaneously. Sutured the skin after all the above device was positioned appropriate. All the above operation procedure was completed under sterile conditions. Then mice were allowed to recover for 2 weeks, and blood pressure before and during angiotensin II treatment were recorded continuously in freely moving animals using receiver platforms (DSI), data was taken using the Data Quest system (DSI).

1.3 Histopathology

For the immunohistochemistry staining of CXCR2, F4/80 and rabbit Ig G in the aorta, the sections were deparafinated, antigen retrieved by citrate buffer, blocking antigen by 3% BSA, incubated CXCR2, F4/80-antibody (1:200, Abcam Cambridge, MA) in 4°C overnight, finally detected CXCR2, F4/80 in the aorta by HRP-DAB detection method. For DHE staining, the isolated aorta was cut into 3 mm rings and incubated in Krebs-Hepes-solution for 15 min at 37°C, then it was embedded in OCT and frozen in liquid nitrogen, sectioned (8 µm).

1.4 Flow Cytometry

Aortic vessels were cleaned of fatty tissue, minced, and digested with aorta dissociation enzyme stock solution (125 U/ml collagenase type XI, 60 U/ml hyaluronidase type 1, 60 U/ml DNase I, and 450 U/ml collagenase type I, in 2.5 ml of PBS) to obtain the single cell suspensions. Blood was erythrocyte-depleted by lysing buffer (BD, New Jersey, USA).
1.5 Constitution of Bone Marrow Transplantation Chimeric Mice

Briefly, the bone marrow cells were extracted from C57BL/6J WT mice and CXCR2−/− mice by flushing femurs and tibiae with RPMI-1640 medium. Recipient mice were irradiated (8.5 Gy) by cobalt and then received 5X10⁶ bone marrow cells from C57BL/6J WT mice or CXCR2−/− mice. Then mice were kept in clean, individually ventilated cages. The chimeric mice were given acidified, antibiotic water and sterilized food. After 4 weeks, the chimeric mice were confirmed by phenotyping CXCR2 expression on bone marrow. Then the chimeric mice were then used to do experiments.

1.6 Vascular Relaxation Studies

Intact aortas were gently isolated from mice and stripped of adventitial fat. 4mm segments of the thoracic aorta were mounted on force transducers gently (Power lab, AD Instruments, Germany) in organ chambers filled with Krebs-Henseleit solution (37°C, pH 7.2-7.4, containing 120 mM NaCl, 5.5 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂-6H₂O, 1.2 mM Na₂HPO₄, 20 mM NaHCO₃, 0.03 mM EDTA-Na₂, 10 mM Glucose bubbled with Carbogen gas containing 95% O₂ and 5% CO₂). Vessels were then equilibrated for 1 hour before the stimulation of vaso-activity by 60% of the maximal KCl solution (37°C, pH 7.38-7.40, containing 5.248 g NaCl, 4.473 g KCl, 0.244 g MgCl₂-6H₂O, 0.277 g CaCl₂, 2 g Glucose and 1.1915 g Hepes in 1000 ml H₂O). To test the relaxation ability of the aorta, we stimulated the aorta to contract with 1*10⁻⁶ M Noradrenalin Elisa (NE), and then applied a concentration gradient (1*10⁻⁹ M, 1*10⁻⁸ M, 1*10⁻⁷ M, 1*10⁻⁶ M, 1*10⁻⁵ M) acetylcholine (ACh) and sodium nitroprusside (SNP) to relax the aorta. Percent relaxation was calculated as described before¹.

1.7 Study Patients

Prior to blood collection, we carefully examined the medical history of control subjects and hypertensive patients. Any subjects with clear infectious diseases or immune diseases are excluded from our study. Namely, we would exclude any patients with potential diseases such as septicemia, anti-neutrophil cytoplasmic autoantibody-associated vasculitides, arthritis, inflammatory bowel disease, plasmodium vivax malaria, Helicobacter pylori-induced peptic ulceration and allergic²⁶. Both control subjects and hypertensive patients had no apparent history using any drugs within 6 months prior to our study.

References


Supplemental Figure I. Angiotensin II Infusion Significantly Increases the Expression of CXCL1 in The Aorta. A, Chemokine mRNA profiles in saline or angiotensin II-treated aortas on day 1 (n=3 per group). B, Validation of CXCL1, CXCL13, CXCL2, CXCL10 and CXCL9 mRNA expression levels by qPCR analysis. ***P<0.001 vs. saline, n=6 per group. C, Flow cytometry analysis of the percentage of CXCR2⁺ cells in CD45⁺CD13⁺ monocytes, CD45⁺CD13⁺CD15⁺ neutrophils, CD45⁺CD13⁺CD64⁺ macrophages and CD45⁺CD3⁺ T cells in human blood.
**Supplemental Figure II.** Ablation of CXCR2 Inhibits Angiotensin II-Induced Hypertension. **A,** Systolic blood pressure was measured via the noninvasive tail-cuff method in WT and CXCR2-deficient (CXCR2−/−) mice before (C) and after Ang II treatment (T) period. ###P<0.001 WT + Ang II vs. CXCR2−/− + Ang II, n=8 per group.

**Supplemental Figure III.** CXCR2 Deficiency in Bone Marrow-Derived Cells Reduces Angiotensin II-Induced Vascular Inflammation and Oxidative Stress. **A,** mRNA levels of IL-1β, IL-6, TNF-α, CD68, VCAM-1 and COX-2 in the aorta were measured by qPCR analysis. **B,** qPCR analysis of NOX1, NOX2, NOX4 and p22phox mRNA expression in the aorta. #P<0.05 vs. WT BM to WT; n=6 per group.
**Supplemental Figure IV.** CXCR2 Deficiency in Bone Marrow-Derived Cells Reduces Angiotensin II-Induced F4/80+ macrophages infiltration into the adventitia. **A,** Immunohistochemistry staining of the F4/80-positive cells in aortic sections. Arrows indicate the F4/80-positive cells. Scale bar, 50 μm.

**Supplemental Figure V.** Central illustration of the study. Hypertensive stimuli (Angiotensin II etc.) induce chemokine release (such as CXCL1) from the vessel wall, stimulating migration and infiltration of bone marrow derived CXCR2+ macrophages. The infiltration of bone marrow derived-CXCR2+ macrophages drives vascular remodeling and dysfunction by causing vascular oxidative stress and inflammation, finally leading to hypertension.