Reversal of Vascular Macrophage Accumulation and Hypertension by a CCR2 Antagonist in Deoxycorticosterone/Salt-Treated Mice

Christopher T. Chan, Jeffrey P. Moore, Klaudia Budzyn, Elizabeth Guida, Henry Diep, Antony Vinh, Emma S. Jones, Robert E. Widdop, James A. Armitage, Samy Sakkal, Sharon D. Ricardo, Christopher G. Sobey, Grant R. Drummond

Abstract—Infiltration of macrophages into the artery wall plays detrimental roles during hypertension by promoting vascular inflammation and endothelial dysfunction, and it occurs via a chemo-attractant action of chemokines on macrophage cytokine receptors. We sought to identify the key chemokine receptors associated with macrophage infiltration into the vascular wall during deoxycorticosterone acetate (DOCA)/salt-induced hypertension in mice and to evaluate the impact of pharmacological inhibition of these receptors on blood pressure and leukocyte accumulation. Mice treated with DOCA/salt for 21 days displayed markedly elevated systolic blood pressure (158±2 versus 114±5 mm Hg in sham group; \( P < 0.0001 \)). Polymerase chain reaction screening via a gene array of 20 chemokine receptors indicated an increased expression of CCR2 in aortas of DOCA/salt-treated mice. Real-time polymerase chain reaction confirmed mRNA upregulation of CCR2 in aortas from DOCA/salt-treated animals and of the CCR2 ligands CCL2, CCL7, CCL8, and CCL12 (all >2-fold versus sham; \( P < 0.05 \)). Flow cytometry revealed 2.9-fold higher macrophage numbers (ie, CD45+CD11b+ F4/80+ cells) in the aortic wall of DOCA/salt versus sham-treated mice. Intervention with a CCR2 antagonist, INCB3344 (30 mg/kg per day, IP), 10 days after the induction of hypertension with DOCA/salt treatment, reduced the aortic expression of CCR2 mRNA and completely reversed the DOCA/salt-induced influx of macrophages. Importantly, INCB3344 substantially reduced the elevated blood pressure in DOCA/salt-treated mice. Hence, our findings highlight CCR2 as a promising therapeutic target to reduce both macrophage accumulation in the vascular wall and blood pressure in hypertension. (Hypertension. 2012;60:1207-1212.) • Online Data Supplement

Key Words: hypertension ♦ chemokine receptors ♦ CCR2 antagonist ♦ macrophages

Macrophages accumulate in the vascular wall during hypertension and likely contribute to the oxidative stress, endothelial dysfunction, and vascular inflammation that are hallmarks of the condition and that ultimately contribute to clinically relevant end points, such as atherosclerosis and arterial remodeling and stiffening.1,2 The circulating precursors of macrophages are monocytes, and depletion of these cells in mice is known to provide protection against experimentally induced hypertension.3

Chemokines are chemotactic cytokines that can be released from cells at sites of injury or infection. By binding to specific receptors expressed on the surface of leukocytes, chemokines stimulate the extravasation and accumulation of leukocytes at sites of damage. Receptors for chemokines belong to the G-protein–coupled receptor superfamily, the largest and most tractable drug targets in the human genome.4,6 Twenty chemokine receptors have been identified to date, each of which may be stimulated by one or several chemokine ligands.5

In the present study, we performed a polymerase chain reaction (PCR) screen to identify chemokine receptor genes that are upregulated in the vascular wall of mice after induction of hypertension by treatment with a combination of deoxycorticosterone acetate (DOCA) and salt. Having identified CCR2 as one such chemokine receptor, we then examined the effect of a recently described and highly selective CCR2 antagonist, INCB3344,7,9 on macrophage accumulation and blood pressure (BP) in the DOCA/salt model. Treatment of mice with INCB3344 reversed DOCA/salt-induced increases in CCR2 expression and macrophage accumulation in the vascular wall. Importantly, these effects were accompanied by a reduction in BP, highlighting CCR2 as a promising drug target in hypertension.

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From the Vascular Biology and Immunopharmacology Group (C.T.C., J.P.M., K.B., E.G., H.D., C.G.S., G.R.D.), Department of Pharmacology (A.V., E.S.J., R.E.W.), Department of Anatomy and Developmental Biology (J.A.A.), and Monash Immunology and Stem Cell Laboratories (S.S., S.D.R.), Monash University, Clayton, Victoria, Australia.

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Correspondence to Christopher G. Sobey, Department of Pharmacology, Building 13E, Room E148, Wellington Road, Monash University, Clayton, Victoria 3800, Australia. E-mail Chris.sobey@monash.edu

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1207
Methods

Model of DOCA/Salt-Induced Hypertension and INCB3344 Intervention Protocol

Male C57BL/6J mice (8–10 weeks old) were obtained from Monash Animal Services (Clayton, Victoria, Australia). Normal chow and drinking water (or 0.9% saline) were provided ad libitum. The study was approved by the Monash University Animal Research Platform Animal Ethics Committee.

Hypertension was induced in mice by uninephrectomy and subsequent treatment with DOCA and salt.11,13 Mice were anesthetized via intraperitoneal injection of ketamine (100 mg/kg; Parnell Laboratories, Australia) and xylazine (10 mg/kg; Troy Laboratories), and a dorsal-lateral incision was made through the skin and muscle layers to expose the left kidney. The renal artery was then tied off with sutures and cut, distal to the ligature, to allow removal of the kidney. Before closing, the same incision site was used to implant a 21-day continuous-release DOCA pellet (2.4 mg/d; Innovative Research of America) subcutaneously in the scapular region. Finally, the drinking water was replaced with 0.9% saline. In sham-treated animals, the kidney was exposed but not removed, and a placebo pellet was implanted. These animals continued receiving normal drinking water.

In a subset of experiments, DOCA/salt-treated mice were further randomly assigned to receive the CCR2 antagonist, INCB3344 (30 mg/kg per day;3 Hayuan Chemexpress Co Ltd) or vehicle (10% DMSO/0.9% carboxymethylcellulose) via daily intraperitoneal injections commencing 10 days after induction of hypertension and continuing until the end of the 21-day treatment period. The normotensive control group for these experiments consisted of sham-treated mice that received vehicles from days 10 to 21.

Blood Pressure Measurements

After induction of hypertension (or sham treatment), systolic BP was measured via tail cuff plethysmography on days 0, 3, 7, 10, 14, 17, and 21 using the MC4000 Multichannel system (Hatteras Instruments).

Quantification of Chemokine Gene Expression in Vessel Wall

At the end of the treatment period, mice were killed and thoracic aortas (with perivascular adipose tissue intact) were harvested and snap frozen in liquid N2. RNA was extracted from aortic samples using the RNeasy Micro Kit-RNA (Qiagen), quantified using the Nanodrop 1000D spectrophotometer (Thermo Scientific), and reverse transcribed into cDNA using the RT2 First Strand Kit (Qiagen). Initially, a PCR screen of 84 chemokine-related genes indicated that 21 days of DOCA/salt-induced hypertension was associated with an increase (≥3-fold versus sham, n=3) in vascular expression of CCR2, CXCR2, and CCR5 (Table S1 in the online-only Data Supplement). Moreover, expression of several ligands for each of these receptors also appeared to be elevated in aortas from DOCA/salt-treated mice, including CCL2, CCL7, CCL8, and CCL12 (CCR2 ligands); CXCL1 and CXCL2 (CXCR2 ligands); and CCL5 (ligand for CCR5) (Table S1).

Taqman real-time PCR was used to validate findings obtained with PCR array. Consistent with the array data, expression of CCR2 was found to be significantly elevated (≥2-fold higher) in aortas from DOCA/salt-treated versus sham-treated mice (Figure 1B), as were expression levels of CCL2 (≥2-fold; Figure 1C), CCL7 (≥4-fold; Figure 1D), CCL8 (≥3-fold; Figure 1E), and CCL12 (≥4-fold; Figure 1F) (P<0.05 for all genes, n=7 per group). Although Taqman PCR also confirmed that expression of CCR5 was 2-fold higher in aortas from DOCA/salt-treated mice (Figure S1A in the online-only Data Supplement), no significant change in expression of its cognate ligand, CCL5, was detected (P=0.11, n=6; Figure S1B). Likewise, we were unable to demonstrate a consistent increase in CXCR2 expression using Taqman PCR (P=0.15, n=6; Figure S1C). Hence, on the basis of these data, CCR2 was identified as the most promising candidate as a drug target to prevent leukocyte accumulation in the vascular wall during hypertension.

Statistical Analysis

Data are expressed as mean±SE. Systolic BPs were analyzed by 2-way ANOVA followed by Bonferroni post hoc tests. Other data sets were compared using either Student unpaired t tests or 1-way ANOVA followed by Bonferroni post hoc tests. P<0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism v5.0d (GraphPad Software Inc).

Results

DOCA/Salt-Induced Hypertension Is Associated With Increased Vascular Expression of CCR2 and Its Ligands

After uninephrectomy and DOCA/salt treatment in mice, systolic BP rose gradually, reaching a plateau that was ≈40 mm Hg higher than the baseline BP by day 7 (P<0.05, n=7 versus sham; Figure 1A) and remained elevated for the duration of the experiment (Figure 1A). By contrast, systolic BP was unchanged throughout the 21-day study period in mice that underwent sham surgery and implantation of a placebo pellet (Figure 1A).

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Analysis of Leukocyte Subsets in Vascular Wall

In a separate set of treated mice, thoracic aortas (with perivascular adipose tissue intact) were harvested and digested enzymatically by incubation in a solution containing collagenase type IX (125 U/mL), collagenase type I (sterile-filtered) (450 U/mL), and hyaluronidase IS (60 U/mL) dissolved in PBS for 30 minutes at 37°C.11,13 The digested tissue was then passed through a 70 μm sterile cell strainer (Falcon, BD Biosciences) to yield a single cell suspension.11,13 Cells were washed and resuspended in fluorescence-activated cell sorter buffer (0.5% BSA in PBS) containing a mixture of antibodies including APC-Cy7 anti-CD45, fluorescein isothiocyanate anti-Ly6C, phycoerythrin-Cy7 anti-Ly6G (all from Biolegend), Pacific Blue anti-CD11b, and APC anti-F4/80 (both from eBiosciences). After immunostaining, cell populations were analyzed by flow cytometry using a LSR-II Flow Cytometer run with DIVA software (Becton Dickinson). Data were analyzed with FlowJo software (version 8.8.1, Tree Star Inc). The numbers of total leukocytes (CD45+) and macrophages (CD45+CD11b+Ly6G-F4/80+) in the vessel wall were normalized to CountBright counting beads (Invitrogen) and expressed as total cells per thoracic aorta.
A CCR2 Antagonist, INCB3344, Prevents DOCA/Salt-Induced Changes in Vascular Expression of CCR2

In a separate series of experiments, we compared the effects of interventions with either INCB3344 or vehicle on DOCA/salt-induced changes in vascular expression of CCR2 and its ligands. Vascular expression of CCR2 was ≈2-fold higher in mice that received vehicle from days 7 to 21 of the DOCA/salt treatment regime than in sham-treated animals (P<0.05, n=6; Figure 2A). CCR2 expression was also elevated (≈1.5-fold higher) in aortas from mice that received INCB3344 from days 7 to 21 of the DOCA/salt treatment period compared with sham animals; however, this level of CCR2 expression was significantly lower than that observed in the vehicle-treated
group \( (P<0.05, n=6; \text{Figure 2A}) \). Likewise, increased expression of its receptor ligand CCL2 in DOCA/salt-treated mice was blunted in mice receiving INCB3344 \( (P<0.05, n=6; \text{Figure 2B}) \). By contrast, levels of CCL7, CCL8, and CCL12 were elevated to similar extents in DOCA/salt-treated mice receiving vehicle or INCB3344 (Figure 2C through 2E).

**INCB3344 Reduces Systolic BP and Macrophage Accumulation in the Aortic Wall of DOCA/Salt-Treated Mice**

Flow cytometric analysis revealed a marked increase (compared with sham animals) in total leukocyte \( (\text{CD45}^+ \text{ cells}) \) numbers in DOCA/salt-treated mouse aortas that had received vehicle for the final 14 days of the treatment period \( (P<0.05; n=7–10; \text{Figure 3A}) \). Although there appeared to be fewer leukocytes in the aortic wall of DOCA/salt-treated mice maintained on INCB3344 versus those on vehicle, there was no significant difference between the 2 treatment groups \( (P>0.05, n=7–8; \text{Figure 3A}) \). By contrast, whereas macrophage \( (\text{CD45}^+\text{CD11b}^+\text{F4/80}^+) \) numbers were significantly elevated (compared with sham animals) \( \approx 3\)-fold in DOCA/salt-treated mice that received vehicle \( (P<0.05, n=5–8; \text{Figure 3B}) \), this was abrogated in mice receiving INCB3344 \( (P>0.05, n=6–8; \text{Figure 3B}) \). Finally, INCB3344 (but not vehicle) reversed DOCA/salt-induced elevations in systolic BP in mice by \( \approx 50\% \) over the 14-day intervention period \( (P<0.05 \text{ versus DOCA/salt+vehicle, } n=9–11; \text{Figure 3C}) \).

**Discussion**

The main findings from this study are that DOCA/salt-induced hypertension in mice is associated with marked increases in expression of the chemokine receptor, CCR2, and its ligands CCL2, CCL7, CCL8, and CCL12 in the vascular wall, and intervention with a selective CCR2 antagonist, INCB3344, significantly reduces both leukocyte and macrophage accumulation in the aortic wall and systolic BP.

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**Figure 3.** Effect of intervention with a CCR2 antagonist, INCB3344 (30 mg/kg per day), on deoxycorticosterone acetate (DOCA)/salt-induced increases in accumulation of leukocytes (A) and macrophages (B) in aortas of mice, and on systolic blood pressure (BP) (C). In A and B, left panels are representative flow cytometry event plots, whereas right panels show the group data (mean±SEM) from \( n \geq 5 \) experiments. In C values are expressed as mean±SEM of \( n \geq 10 \) experiments. Dashed line indicates time point at which interventions were administered to mice. \( *P<0.05 \text{ vs sham}, #P<0.05 \text{ vs vehicle/DOCA/salt for Bonferroni after 1-way ANOVA (in A and B) and 2-way repeated-measures ANOVA (C)}. \) SSC indicates side scatter.
antagonist, INCB3344, prevents macrophage accumulation in the vessel wall and markedly reverses DOCA/salt-induced increases in BP.

The proinflammatory leukocyte chemokine receptor CCR2 is most highly expressed on monocytes and their activated cell type, macrophages. Previous studies have demonstrated that CCR2 is essential for macrophage accumulation in the vascular wall and for the subsequent development of vascular hypertrophy and fibrosis. For example, essential hypertension in humans and angiotensin II–induced hypertension in rats were both shown to be associated with increases in expression of CCR2 on circulating monocytes and in the vascular wall. Furthermore, in CCR2-deficient mice and in chimeric mice (bone marrow transplanted) with leukocyte-specific CCR2 deficiency, angiotensin II–induced macrophage accumulation, aortic wall thickening, and collagen deposition were blunted as compared with wild-type mice. In the present study, we showed that DOCA/salt-induced hypertension in mice is also associated with augmented aortic expression of CCR2—presumably as a result of the accumulation of CCR2-expressing leukocytes in the vessel wall. Moreover, a CCR2 antagonist, INCB3344, reduced vascular CCR2 expression and completely reversed macrophage accumulation in the vessel wall of mice with established hypertension. Importantly, CCR2 antagonism also caused a marked reduction in the DOCA/salt-induced increase in systolic BP. Although further studies are needed to assess whether the effects of INCB3344 in this model extend to reductions in other disease parameters, such as vascular and cardiac fibrosis, our findings nonetheless highlight CCR2 as a promising target for new therapies that tackle both the BP and inflammatory components of hypertension.

The concept of a therapy that reverses BP while simultaneously reducing the accumulation of macrophages in the vascular wall is highly attractive, because these cells are likely to represent important links between hypertension and pathophysiological changes, such as vascular stiffening and atherosclerosis, which may ultimately give rise to clinically relevant end points, such as renal damage, myocardial ischemia, myocardial infarction, and stroke. Macrophages are highly plastic cells that assume different states of activation depending on the cytokine milieu to which they are exposed. Thus, in the presence of T-helper 2 cytokines, such as interleukins 4 and 13, macrophages become polarized toward an alternative or M2 phenotype. M2 macrophages could conceivably play a role in vascular hypertrophy and stiffening via their ability to release trophic factors, such as insulin-like growth factor-1, profibrotic factors, such as transforming growth factor-α, and extracellular matrix components, such as fibronectin. By contrast, in the presence of T-helper 1 cytokines, such as interferon-α, macrophages become classically activated toward an M1 phenotype. M1 macrophages are likely to be proatherosclerotic, releasing inflammatory cytokines, such as tumor necrosis factor-α and interleukins-1α, -6, and -12, as well as vasoactive and pro-oxidant factors, such as prostanooids, nitric oxide, and reactive oxygen species. In addition to directly promoting inflammation, macrophages have a propensity to engulf modified lipid particles and subsequently differentiate into foam cells, which are the primary constituent of atherosclerotic plaques. To date, no studies have examined the activation state(s) of macrophages that accumulate in the vessel wall of hypertensive humans or animals. Whatever role such studies identify for macrophages, the findings of the present study suggest that they are likely to be obviated by targeting CCR2.

Regarding the translatability of the present findings to the clinic, INCB3344 possesses several pharmacokinetic and pharmacodynamic properties that indicate it may be a promising lead compound for the development of novel drugs to treat hypertension. First, INCB3344 is equally effective at antagonizing the human CCR2 receptor as it is at antagonizing the rodent receptor, with binding affinities in the low nanomolar range. Second, INCB3344 is highly selective (>100-fold) for CCR2 more than even the most closely related chemokine receptor subtypes, such as CCR1 and CCR5, suggestive of limited off-target effects. Finally, INCB3344 was shown to have high oral bioavailability in mice (>45%).

It has been shown that the CCR2 ligand, CCL2 (formerly known as monocyte chemo-attractant protein-1), is upregulated in the vascular wall during experimental hypertension. In the present study, we confirmed this finding and then showed for the first time that several additional CCR2 ligands are also upregulated in the vascular wall during DOCA/salt-induced hypertension, including CCL7 (MCP-3), CCL8 (MCP-2), and CCL12 (MCP-5). Hence, the attractant forces for CCR2-expressing leukocytes into the vascular wall during hypertension would appear to be high. Upregulation of multiple CCR2 ligands during hypertension is indicative of a high degree of redundancy in the system and suggests that targeting the common receptor for all these ligands is likely to be a more effective strategy for disrupting leukocyte entry than targeting any one of the individual chemokines (eg, with neutralizing antibodies).

Perspectives

Hypertension is now recognized as an immune disorder in which leukocytes, including macrophages and T cells, become activated and accumulate in the vascular wall where they promote inflammation, fibrosis, oxidative stress, and endothelial dysfunction. Such pathophysiological changes are not only likely to increase vascular resistance and thereby exacerbate the primary symptom of hypertension, namely elevated BP, but they may also be precursors to vascular stiffening and atherosclerosis, which are responsible for clinical events such as renal and heart failure, myocardial ischemia, and stroke. Chemokine receptors play a major role in regulating the trafficking of leukocytes from the circulation into tissues and as such represent attractive targets for therapies aimed at modulating immune responses. In this study, we identified CCR2 as an important mediator of leukocyte trafficking into the vascular wall during hypertension. Moreover, we demonstrated that administration of a small molecule antagonist of this receptor, INCB3344, to mice with established hypertension had a profound effect in reversing BP and reducing macrophage accumulation, highlighting CCR2 as a promising target for the next generation of antihypertensive drugs.

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Disclosures
None.

References

Novelty and Significance

What Is Relevant?
• High blood pressure in mice is associated with an influx of white blood cells into the walls of arteries.

What Is New?
• In this study, we identified the chemical signals (chemokines) responsible for attracting white blood cells into the walls of arteries during hypertension.

Summary
• We further showed that treatment of mice with a compound (INCB3344) that specifically blocks these signals reduced white blood cell accumulation in arteries and dramatically reduced blood pressure.
• Our findings suggest that preventing the accumulation of white blood cells in arteries with drugs that block chemokines could be a new strategy to treat hypertension in patients.
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REVERSAL OF VASCULAR MACROPHAGE ACCUMULATION AND HYPERTENSION BY A CCR2 ANTAGONIST IN DEOXYCORTICOSTERONE / SALT-TREATED MICE.

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Vascular Biology & Immunopharmacology Group, Department of Pharmacology, Monash University, Clayton, Australia; Department of Pharmacology, Monash University, Clayton, Australia; Monash Immunology and Stem Cell Laboratories, Clayton, Australia
Table S1. Data (mean values from 3 experiments) from PCR gene array showing changes in expression of chemokine-related genes in aortas from DOCA/salt- versus sham-treated mice. Bold values show chemokine receptors for which there was >3-fold change in expression. Gene abbreviations (Full gene name): APLNR (Apelin receptor); BDNF (Brain derived neurotrophic factor); BMP 6, 10, 15 (Bone morphogenetic protein 6, 10 15); CCBP2 (Chemokine binding protein 2); CCL1-20 (Chemokine (C-C motif) ligand 1-20); CCR1L1 (Chemokine (C-C motif) receptor 1-like 1); CCR1-10 (Chemokine (C-C motif) receptor 1-10); CCRL1-2 (Chemokine (C-C motif) receptor-like 1-2); CMKLR1 (Chemokine-like receptor 1); CMTM2A-6 (CKLF-like MARVEL transmembrane domain containing 2a-6); CSF1 (Colony stimulating factor 1 (macrophage)); CSF2 (Colony stimulating factor 2 (granulocyte-macrophage)); CX3CL1 (Chemokine (C-X3-C motif) ligand 1); CX3CR1 (Chemokine (C-X3-C) receptor 1); CXCL2-15 (Chemokine (C-X-C motif) ligand 2-15); CXCR2-7 (Chemokine (C-X-C motif) receptor 2-7); GDF5 (Growth differentiation factor 5); GPR81 (G protein-coupled receptor 81); HIF1A (Hypoxia inducible factor 1, alpha subunit); IL 1α-18 (Interleukins 1α-18); INHA (Inhibin alpha); INHBB (Inhibin beta-B); LIF (Leukemia inhibitory factor); LTB4R2: (Leukotriene B4 receptor 2); MMP2 (Matrix metallopeptidase 2); MYD88 (Myeloid differentiation primary response gene 88); NFKB1 (Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105); PF4 (Platelet factor 4); PPBP (Pro-platelet basic protein); RGS3 (Regulator of G-protein signaling 3); SLIT2 (Slit homolog 2 (Drosophila)); TLR4 (Toll-like receptor 4); TNF (Tumor necrosis factor); TNFRSF (Tumor necrosis factor receptor superfamily, member 1a); TNFSF14 (Tumor necrosis factor (ligand) superfamily, member 14); TREM1 (Triggering receptor expressed on myeloid cells 1); TYMP (Thymidine phosphorylase); XCL1 (Chemokine (C motif) ligand 1)
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Figure S1. Effect of combined uninephrectomy and DOCA/salt treatment in mice on aortic mRNA expression of CCR5 (A), CCL5 (B) and CXCR2 (C). Values are expressed as mean ± S.E.M. of n ≥ 7 experiments. *P<0.05 versus sham for Student’s unpaired T test.