Role of CXCR2 in cigarette smoke-induced lung inflammation


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Submitted 20 January 2005; accepted in final form 10 April 2005

Thatcher, T. H., N. A. McHugh, R. W. Egan, R. W. Chapman, J. A. Hey, C. K. Turner, M. R. Redonnet, K. E. Seweryniak, P. J. Sime, and R. P. Phipps. Role of CXCR2 in cigarette smoke-induced lung inflammation. Am J Physiol Lung Cell Mol Physiol 289: L322–L328, 2005. First published April 15, 2005; doi:10.1152/ajplung.00039.2005.—It has been hypothesized that the destruction of lung tissue observed in smokers with chronic obstructive pulmonary disease and emphysema is mediated by neutrophils recruited to the lungs by smoke exposure. This study investigated the role of the chemokine receptor CXCR2 in mediating neutrophilic inflammation in the lungs of mice acutely exposed to cigarette smoke. Exposure to dilute mainstream cigarette smoke for 1 h, twice per day for 3 days, induced acute inflammation in the lungs of C57BL/6 mice, with increased neutrophils and the neutrophil chemotactic CXC chemokines macrophage inflammatory protein (MIP)-2 and KC. Treatment with SCH-N, an orally active small molecule inhibitor of CXCR2, reduced the influx of neutrophils into the bronchoalveolar lavage (BAL) fluid. Histological changes were seen, with drug treatment reducing perivascular inflammation and the number of tissue neutrophils. β-Glucuronidase activity was significantly reduced in the BAL fluid of mice treated with SCH-N, indicating that the reduction in neutrophils was associated with a reduction in tissue damaging enzymes. Interestingly, whereas MIP-2 and KC were significantly elevated in the BAL fluid of smoke-exposed mice, they were further elevated in mice exposed to smoke and treated with drug. The increase in MIP-2 and KC with drug treatment may be due to the decrease in lung neutrophils that either are not present to bind these chemokines or fail to provide a feedback signal to other cells producing these chemokines. Overall, these results demonstrate that inhibiting CXCR2 reduces neutrophilic inflammation and associated lung tissue damage due to acute cigarette smoke exposure.

CXCR2, neutrophils, emphysema; chronic obstructive pulmonary disease; macrophage inflammatory protein-2; KC

IN 2002, AN ESTIMATED 24 MILLION Americans were afflicted with chronic obstructive pulmonary disease (COPD; emphysema and chronic bronchitis), including 2.4 million adults with moderate to severe airflow obstruction (18). COPD is the fifth-leading cause of death worldwide (26). Recent research has focused on the role of inflammation and inflammatory cell mediators in the pathogenesis of COPD (10). Cigarette smoke is a strong inflammatory stimulus that induces proinflammatory cytokines such as IL-6 and TNF-α and recruits activated macrophages and neutrophils to lung tissue (8, 23). It is believed that emphysema results from an imbalance between proteases produced by smoke-recruited inflammatory cells and the antiprotease defenses of the lung (1, 6, 32). Instillation of neutrophil elastase into the lungs of experimental animals has long been a model of emphysema (15). Recent work has confirmed that while macrophage proteases play a key role in cigarette smoke-induced inflammation (6), neutrophils are also required for tissue breakdown following cigarette smoke exposure in an animal model (8, 9).

Recruitment of neutrophils to inflammatory sites is mediated by a number of factors including adhesion molecules, multifunctional proinflammatory cytokines, and chemokines of the CXC family. The predominant neutrophil chemokine in humans is IL-8, while mice lack IL-8 but have two neutrophilic CXC chemokines, macrophage inflammatory protein (MIP)-2 and KC (13). The importance of these chemokines in promoting inflammation has been investigated in vitro and in vivo. Bronchoalveolar lavage (BAL) fluid from COPD patients contains increased neutrophils, TNF-α, and IL-8 (25, 29), whereas we and others have shown that cigarette smoke extract stimulates human lung cells (epithelial cells, fibroblasts, and macrophages) to release IL-8 (3, 19, 20, 30). In mice, MIP-2 is essential for neutrophil recruitment to the lungs following inflammatory injury (22).

The CXC chemokines signal through a family of CXC receptors on inflammatory cells. In humans, IL-8 binds two receptors, CXCR1 and CXCR2, both of which are expressed on neutrophils (24). Mice lack a CXCR1 homolog; MIP-2 and KC signal exclusively through CXCR2 (4). The CXC chemokines and their receptors are attractive targets for therapeutic intervention in inflammatory lung diseases such as COPD. It has previously been reported that blockade of the MIP-2/CXCR2 interaction inhibits neutrophil recruitment in several mouse models of inflammation (14, 17, 21, 37). However, the role of the CXC chemokines and CXCR2 in cigarette smoke-mediated lung inflammation has not previously been investigated.

In this report we investigated the role of CXCR2 in cigarette smoke-induced inflammation using a CXCR2 inhibitor. SCH-N inhibits the binding of CXC chemokines to the human CXCR1 and CXCR2 receptors as well as the murine CXCR2 receptor and has an IC₅₀ of 3 nM in a mouse neutrophil chemotaxis assay. SCH-N inhibits neutrophilic inflammation in mice exposed acutely to cigarette smoke. Treatment with SCH-N reduced neutrophils in BAL fluid and tissue neutrophils by 50%. β-Glucuronidase activity was significantly reduced, indicating that inhibition of neutrophilic influx ameliorated the tissue damage associated with acute cigarette smoke exposure. Thus CXCR2 plays a key role in the acute inflammatory response to cigarette smoke.
MATERIALS AND METHODS

Cigarette smoke exposure. Adult female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the Inhalation Core Facility at the University of Rochester. Mice were placed in individual compartments of a wire cage, which was placed inside a closed plastic box connected to the smoke source. Research cigarettes (IR3F, University of Kentucky) were smoked according to the Federal Trade Commission protocol (1 puff/min of 2-s duration and 35-ml volume) in a Baumgartner-Jaeger CSM2072i cigarette smoking machine (CH Technologies, Westwood, NJ). mainstream cigarette smoke was diluted with filtered air and directed into the exposure chamber. The smoke exposure (total particulate matter per cubic meter of air, TPM) was monitored in real time with a MicroDust Pro aerosol monitor (Casella CEL, Bedford, UK) and verified daily by gravimetric sampling. The smoke concentration was set at a nominal value of 600 mg/m$^3$ TPM by adjusting the flow rate of the dilution air. The average actual exposure for these experiments was 647 ± 58 mg/m$^3$. Mice received two 1-h exposures, 4 h apart, for three consecutive days, and were killed on the fourth day. Control mice were exposed to filtered air in an identical chamber according to the same schedule. All animal procedures were performed under the supervision of the University Committee on Animal Research (University of Rochester).

Drug treatment. A 5 mg/ml suspension of SCH-N (provided by the Schering Plough Research Institute, Kenilworth, NJ) in vehicle (0.4% hydroxypropyl-methylcellulose) was prepared immediately before each administration. Mice received a 50-μl SCH-N suspension (or vehicle alone) po 1 h before each smoke exposure. To ensure accurate dosing, drug or vehicle was administered via Hamilton Gastight syringes fitted with Hamilton Repeating Dispensers (Hamilton, Reno, NV).

BAL. Mice were anesthetized with 2,2,2-tribromoethanol (Avertin, 250 mg/kg ip) and killed by exsanguination. The heart and lungs were removed en bloc, and the lungs were lavaged twice with 0.5 ml of PBS. The lavage fluid was centrifuged, and the cell-free supernatants were frozen for later analysis. The BAL cell pellet was resuspended in PBS. The lavage fluid was centrifuged, and the cell-free supernatants were removed en bloc, and the lungs were lavaged twice with 0.5 ml of glycine buffer (pH 4.5) as described (33).

Murine IL-6, TNF-α, MIP-2, and KC were measured using commercial ELISA (R&D Systems, Minneapolis, MN). PGE$_2$ was measured by enzyme immunoassay using commercially available reagents (Cayman Chemical, Ann Arbor, MI) as described (16). The limit of detection was 7 pg/ml for using commercially available reagents (Cayman Chemical, Ann Arbor, MI) and verified daily by gravimetric sampling. The smoke concentration was set at a nominal value of 600 mg/m$^3$ TPM by adjusting the flow rate of the dilution air. The average actual exposure for these experiments was 647 ± 58 mg/m$^3$. Mice received two 1-h exposures, 4 h apart, for three consecutive days, and were killed on the fourth day. Control mice were exposed to filtered air in an identical chamber according to the same schedule. All animal procedures were performed under the supervision of the University Committee on Animal Research (University of Rochester).

Analysis of BAL fluid. Murine IL-6, TNF-α, MIP-2, and KC were measured in BAL samples by commercial ELISA (R&D Systems, Minneapolis, MN). PGE$_2$ was measured by enzyme immunoassay using commercially available reagents (Cayman Chemical, Ann Arbor, MI) as described (16). The limit of detection was 7 pg/ml for IL-6, MIP-2, and KC; 15 pg/ml for TNF-α; and 30 pg/ml for PGE$_2$. To assay β-glucuronidase activity, the liberation of p-nitrophenol from 4-nitrophenol glucuronide (Sigma, St. Louis, MO) was measured at 405 nm in glycine buffer (pH 4.5) as described (33).

Histological analysis and immunohistochemistry. Mouse lungs (which had not been lavaged) were fixed by inflation with 10% neutral formalin at 405 nm in glycine buffer (pH 4.5) as described (33).

Statistics. All experiments were performed in random and blinded, 10 high-power fields (500 × 500 μm) per section were counted, and the average number of neutrophils per mm$^3$ of tissue was determined.

RESULTS

Acute cigarette smoke-induced inflammation is significantly reduced by inhibiting CXCR2. C57BL/6 mice were exposed to cigarette smoke for 1 h, twice a day for 3 days, and killed on the fourth day. This exposure protocol elicited a strong inflammatory response characterized by significant increases in the number of neutrophils, lymphocytes, and eosinophils recovered by BAL, with neutrophils comprising up to 50% of total BAL cells (Fig. 1). This acute cigarette smoke exposure protocol did not result in significant increases in BAL macrophages. SCH-N is a novel CXCR2 antagonist that is a potent inhibitor of mouse neutrophil chemotaxis in vitro (IC$_{50}$ of 3 nM; N. A. McHugh and R. W. Egan, unpublished data). Pretreatment with SCH-N before each smoke exposure significantly reduced the neutrophil influx into the lungs, with a 50%
decrease in the number of neutrophils in BAL (Fig. 1A). SCH-N did not affect the number of lymphocytes or eosinophils in BAL (Fig. 1, D and E). Levels of β-glucuronidase, one of several hydrolytic enzymes found in phagocytic cells and thought to play a role in tissue breakdown in inflammatory lung disease (27), were elevated 96% in the BAL of cigarette smoke-exposed mice. β-Glucuronidase activity was elevated 71% in SCH-N treated, smoke-exposed mice compared with nonsmoke-exposed controls, representing a 26% reduction in smoke exposure-specific activity (Fig. 1F).

Inhibition of CXCR2 reduces airway inflammation and infiltration of lung tissue by neutrophils in cigarette smoke-exposed mice. Lungs from mice exposed to air or cigarette smoke and treated with SCH-N were fixed, and sections were stained with hematoxylin and eosin or with an antibody to mouse neutrophils. Exposure to cigarette smoke induced a significant perivascular inflammation with prominent neutrophils and mononuclear cells (Fig. 2C). Adherent neutrophils were frequently seen in the lumen of pulmonary blood vessels (Fig. 2C), and neutrophils and macrophages were prominent in alveolar capillaries (Fig. 2E). Treatment with SCH-N reduced the size and frequency of perivascular infiltrates, as well as the number of adherent neutrophils inside blood vessels (Fig. 2D). Although macrophages were still prominent in alveolar capillaries, neutrophils were largely absent (Fig. 2E). These observations were confirmed by immunohistochemical staining using an antibody that recognizes a surface protein on mouse neutrophils. Neutrophils are prominent in perivascular infil-

Fig. 2. SCH-N reduces neutrophil accumulation in lung tissue of cigarette smoke-exposed mice. Mice were exposed to filtered air or cigarette smoke and treated with SCH-N or vehicle. Lungs were inflated and fixed with formalin, and sections were stained with hematoxylin and eosin. Air-exposed mice have normal alveoli and blood vessels (A, B). Smoke-exposed mice exhibit extensive perivascular inflammation with extravasating monocytes and neutrophils (arrows, C) and monocytes and neutrophils in the alveolar capillaries (E). Smoke-exposed mice treated with SCH-N have less perivascular inflammation (D) and greatly reduced numbers of extravasating (D) and capillary neutrophils (F). m, Monocyte; N, neutrophil. Bar 20 μm.
brates of smoke-exposed mice treated with vehicle (Fig. 3C) but not SCH-N (Fig. 3D). SCH-N also reduced the appearance of neutrophils trapped in the alveolar capillary bed (Fig. 3E and F). Neutrophils were counted in immunostained sections by a blinded researcher, and the number of neutrophils per cubic millimeter of tissue was determined (Fig. 4). Cigarette smoke exposure induced a sevenfold increase in the number of tissue neutrophils, which was reduced by 45% by treatment with SCH-N.

Inhibition of CXCR2 does not otherwise alter the inflammatory milieu. Neutrophil migration from the peripheral blood into tissues requires both a proinflammatory milieu in the tissue, with expression of endothelial adhesion molecules, and ligation of the CXCR2 receptor on neutrophils, which induces leukocyte rolling and extravasation (11, 35, 39). We analyzed the levels of the proinflammatory cytokines IL-6 and TNF-α as well as PGE2, an inflammatory mediator produced by lung fibroblasts that is implicated in neutrophil activation and recruitment, in mice exposed to cigarette smoke with and without SCH-N treatment. Exposure to cigarette smoke resulted in a 10-fold increase in IL-6 and a two- to threefold increase in PGE2 in BAL fluid (Fig. 5). No differences were observed in mice treated with SCH-N, suggesting that SCH-N does not interfere with neutrophil migration by altering the general inflammatory microenvironment. It should be noted that TNF-α levels were not elevated by cigarette smoke exposure (Fig. 5B); however, these mice were harvested 24 h after the final smoke exposure, whereas TNF-α levels peak 2–4 h after smoke exposure (data not shown).

Inhibition of CXCR2 leads to elevated levels of CXC chemokines. IL-8, the human neutrophilic CXC chemokine, can be induced in vivo and in vitro by cigarette smoke (3, 31). To determine whether MIP-2 and KC, the mouse CXC chemokines that bind CXCR2, are induced in vivo by this acute

Fig. 3. Neutrophil immunohistochemistry in smoke-exposed and drug-treated mice. Lung sections were prepared as described and immunostained with an antibody specific for a neutrophil surface marker (Serotec, Oxford, UK) and counterstained with hematoxylin. Neutrophils are stained red. Very few neutrophils are present in air-exposed mice (A, B). The perivascular infiltrates in smoke-exposed mice contain many neutrophils (C), as do alveolar capillaries (E). Smoke-exposed mice treated with drug have reduced perivascular and capillary neutrophils (D, F). Bar 20 μm.
cigarette smoking protocol, these chemokines were measured in BAL fluid from air- and smoke-exposed mice. Cigarette smoke exposure induces a twofold increase in MIP-2 and a 30-fold increase in KC, which is consistent with cigarette smoke being a strong neutrophilic inflammatory stimulus (Fig. 6). Interestingly, treatment with SCH-N further increases MIP-2 and KC levels two- to threefold over smoke alone.

**DISCUSSION**

This study investigated the role of CXCR2 receptor on neutrophils in acute cigarette smoke-induced inflammation. We have developed a cigarette smoke exposure protocol that results in acute lung inflammation characterized by massive increases in BAL neutrophils (up to 50% of total BAL cells), tissue neutrophils, and production of inflammatory cytokines, prostaglandins, and neutrophil chemokines. SCH-N, a CXCR2 antagonist, inhibited smoke-induced lung neutrophilia by 50%, with reductions in BAL neutrophils and β-glucuronidase, an enzyme associated with tissue breakdown. Perivascular inflammatory infiltrates were smaller, less frequent and contained fewer neutrophils, and fewer neutrophils were observed adherent to blood vessel endothelium. SCH-N did not alter the number of inflammatory lymphocytes or eosinophils recruited by smoke and did not alter the general proinflammatory milieu, as indicated by measurement of IL-6 and PGE2 in the BAL. It should be noted that while TNF-α has been shown by some to be a key initiator of inflammation following cigarette smoke exposure (7), we did not observe changes in TNF-α with either smoke exposure or drug treatment (Fig. 5B). Because we determined that neutrophilic inflammation of the lungs peaks 24 h after the final smoke exposure in this model, the mice were killed at this time point for all analyses. However, TNF-α levels peak 2–4 h after smoke exposure (data not shown), so it is not surprising that we did not observe changes in TNF-α in these mice. However, since IL-6 and PGE2 levels are elevated by smoke exposure and unaffected by SCH-N, we expect that TNF-α would follow the same pattern if mice were killed at an earlier time point.

It was interesting that SCH-N, a CXCR2 antagonist, increased the levels of the CXC chemokines MIP-2 and KC two- to threefold over smoke alone. SCH-N should be acting against CXCR2 on circulating neutrophils, whereas MIP-2 and KC are probably produced by lung resident cells exposed to smoke. One possible explanation is that MIP-2 and KC are regulated in part by a negative feedback mechanism and that the arrival of neutrophils at the site of inflammation sends a signal that downregulates further chemokine production. Because fewer
neutrophils enter the lung with SCH-N treatment, this down-regulatory signal is not sent. It is also possible that an autocrine negative feedback loop exists in which cells that produce MIP-2 and KC modulate chemokine production via their own CXCR2 receptors. It has recently been reported that mouse alveolar type 2 cells express both MIP-2 and CXCR2 (38), so it is possible that blockade of CXCR2 blocks an autocrine regulatory mechanism resulting in increased production of MIP-2.

SCH-N is a potent CXCR2 antagonist, inhibiting mouse neutrophil chemotaxis in vitro with an IC_{50} of 3 nM (data not shown), while inhibiting the neutrophil inflammatory response to cigarette smoke by 50%. It should be noted that in mice, neutrophil migration can also be induced by the CC chemokine MIP-1α acting via the CCR1 receptor (34, 36). Cigarette smoke induces MIP-1α in rat alveolar macrophages in vitro (5), so it is likely that the partial inhibition seen in this study is due to redundancy in neutrophil chemotaxis in mice (28, 40).

Neutrophilic inflammation is a key factor in chronic bronchitis and emphysema. Disease progression is associated with a switch from a T cell-mediated inflammation in healthy smokers and patients with mild COPD to neutrophilic inflammation in severely ill patients (10), who also have higher levels of the human neutrophil CXC chemokine IL-8 (25, 29). Neutrophils have also been shown to be a key effector cell of matrix breakdown in an animal model (8, 9). Due to the fact that neutrophil recruitment in humans requires activation of the CXCR1 or CXCR2 receptors by CXC chemokines, these interactions have been explored as potential therapeutic targets (2, 12). In mouse models of inflammation, neutrophil recruitment can be blocked by treatment with antibodies to MIP-2 and KC, CXCR2 antibodies (mice lack CXCR1), and CXCR2 antagonist peptides derived from CXCR2 ligands (14, 17, 21). This study demonstrates that CXCR2 plays a key role in cigarette smoke-induced inflammation and that inhibiting chemokine binding to CXCR2 can significantly reduce neutrophil infiltration and tissue damage resulting from acute exposure to cigarette smoke. Further experiments will be needed to determine whether long-term blockade of CXCR2 can reduce the severity of emphysema-like changes in mouse models of chronic cigarette smoke exposure.

ACKNOWLEDGMENTS

SCH-N was provided by the Chemical Research Department of Schering-Plough Research Institute. R. W. Egan is currently at Inflammation Discovery Research, Millennium Pharmaceuticals, 55 Landsdowne St., Cambridge, MA.

GRANTS

This research was supported in part by National Heart, Lung, and Blood Institute Grants K08HL-04492 and HL-075432, National Institute of Environmental Health Sciences Center Grant P30ES-01247, and Environmental Protection Agency Grant R-827354.

DISCLOSURES

This research was funded in part by the Schering-Plough Research Institute.

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