Nonpeptide CXCR2 Antagonist Prevents Neutrophil Accumulation in Hyperoxia-Exposed Newborn Rats

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ABSTRACT

Neutrophil influx in lung injury is controlled in part by chemokines acting through the receptor, CXCR2. To avoid adverse effects of steroids typically used to modify inflammation, we evaluated the effects of competitive blockade of CXCR2 in rats on neutrophil function in vitro and on neutrophil influx in vivo in hyperoxia-induced newborn lung injury, a model of bronchopulmonary dysplasia. In vitro, SB-265610 antagonizes rat cytokine-induced neutrophil chemoattractant-1 (CINC-1)-induced calcium mobilization, IC50 = 3.7 nM, and rat neutrophil chemotaxis in a concentration-dependent manner, IC50 = 70 nM. In vivo, newborn rats exposed to 95% O2 for 8 days had increased lung neutrophil content. Injection with 1 to 3 mg/kg SB-265610 on days 3 to 5 reduced hyperoxia-induced neutrophil accumulation in bronchoalveolar lavage and whole lung myeloperoxidase accumulation at the highest doses. To determine whether these effects might be due in part to increased neutrophil apoptosis, peripheral neutrophils were cultured with and without SB-265610. Apoptosis was assessed by morphologic, viability, and terminal transferase deoxynucleotidyl nick-end labeling. Treatment of neutrophils with CINC-1 reduced apoptosis compared with untreated neutrophils. SB-265610 reduced the antipapoptotic effect of CINC-1 to the levels of those untreated with CINC-1. A selective CXCR2 antagonist may be useful in diseases where neutrophil-mediated exacerbation is present.

Inflammatory cell influx precedes the development of bronchopulmonary dysplasia in premature newborns, and inflammation can exacerbate oxygen-induced lung injury (Jobe and Ikegami, 1998). Neutrophils in particular can exacerbate oxidant stress to organs in part due to respiratory burst activation. There are reports showing that neutrophil depletion strategies have been successfully used to reduce inflammation-associated lung injury (Bando et al., 1990).

Neutrophil influx is predominantly regulated by neutrophil chemokines, such as interleukin (IL)-8 and growth-related oncogene (GRO) α in humans, or cytokine-induced neutrophil chemoattractant-1 (CINC-1) and macrophage inflammatory protein-2 (MIP-2) in rats, which can all activate the CXCR2 receptor (White et al., 1998). CXCR2 receptor activation controls neutrophil sLex expression, membrane attack complex-1 expression (Rot et al., 1996), neutrophil chemotaxis (Magazin et al., 1992), and respiratory burst in rats (Prevert et al., 1995). It has been shown that neutralizing antibodies against the CXCR2 ligands CINC-1, GROα, MIP-2, and IL-8 in rats, mice, and rabbits, respectively, have reduced neutrophil-mediated inflammatory responses (Driscoll et al., 1996; Deng et al., 2000). Treatment with neutralizing antibodies to CINC-1 and MIP-2 reduced neutrophil accumulation and preserved pulmonary alveolar development in newborn rats exposed to hyperoxia (Deng et al., 2000). Targeted disruption of the CXCR2 receptor in mice reduced neutrophil adhesion (Morgan et al., 1997) and lipopolysaccharide-mediated neutrophil influx (Cacalano et al., 1994). Blockade of the receptor using a competitive antagonist has led to a reduction in neutrophil sequestration and should reduce neutrophil influx and avoid neutrophil activation in vulnerable tissues (White et al., 1998).

Activation of CXCR2 may also contribute to prolonged inflammation by maintaining neutrophil viability. Control of inflammation is regulated in part by neutrophil apoptosis (Haslett, 1999). Neutrophil death will reduce neutrophil-secreted chemokines, thereby attenuating the cycle of injury and inflammation. Neutrophil viability is regulated by chemokines and in part by the preservation of CXCR2 expres-

ABBREVIATIONS: IL, interleukin; GRO, growth-related oncogene; CXCR2, C-X-C chemokine receptor 2; TUNEL, terminal transferase deoxyuridine triphosphatidyl nucleotide nick-end labeling; HBSS, Hanks’ balanced salt solution; MPO, myeloperoxidase; DAPI, 4’,6-diamidino-2-phenylindole; BAL, bronchoalveolar lavage; PMN, polymorphonuclear neutrophil; CINC-1, cytokine-induced neutrophil chemoattractant-1; MIP-2, macrophage inflammatory protein-2; BPD, bronchopulmonary dysplasia; C5a, human complement 5a.
sion (Duncan et al., 2000b). Competitive blockade of CXCR2 may also block chemokine effects on neutrophil viability and promote apoptosis, thus leading to more rapid clearance of neutrophils at sites of inflammation.

Through chemical modification of a previously reported competitive nonpeptide CXCR2 antagonist SB-225002, we were able to improve its pharmacokinetic properties (Fig. 1) (Sarau et al., 2001). We now report the effects of SB-265610 on in vitro rat neutrophil calcium mobilization and chemotaxis. In addition, the effect of the compound on in vivo neutrophil accumulation in hyperoxia-exposed newborn rat lung and on neutrophil apoptosis in vitro was examined.

Materials and Methods

Calcium Mobilization Studies. Studies were approved by the Institutional Animal Care and Use Committee. Neutrophils from adult male Sprague-Dawley rats were separated from venous blood in a density gradient according to the manufacturer’s directions, with the following modifications (NIM-2, Cardinal Associates, Santa Fe, NM). Whole blood anticoagulated with Ca-EDTA (3–4 ml) was added to 5 ml of the gradient at room temperature then centrifuged at 900g for 45 min. The gradient just above the red blood cell pellet was removed because this demonstrated the highest purity (neutrophils >95%). Gradient was removed by washing in 10-fold excess of cold Hanks’ balanced salt solution (HBSS), followed by centrifugation at 500g for 15 min. The cell pellets were resuspended in ice-cold deionized water three times to lyse red blood cells, followed by addition of an equal volume of 2× HBSS. Cells were pelleted and viability assessed by trypan blue exclusion and was routinely found to be >90%. Neutrophils (10^6/ml in HBSS, 10 mM HEPES) were loaded with Fura-2/AM at a final concentration of 1 μM for 1 h at 37°C. Cells were washed with HEPES-buffered RPMI 1640 medium and suspended at 10^6 cells/ml. Calcium analyses were made after treating with 0 to 1000 nM SB-265610 and then with 1 nM CINC-1, MIP-2, or human complement 5a (C5a), and then calculated as treating with 0 to 1000 nM SB-265610 and then with 1 nM CINC-1, pH 7.5, after clamping the left atrial appendage. The lungs were then lavaged with four changes of buffer. Cells from pooled lavages were centrifuged, counted, and cytospin before staining with Wright-Giemsa. A differential count was determined on at least 200 cells/lavage. Lavaged perfused lungs were flash frozen until later measurement of myeloperoxidase (MPO) activity using dianisidine, as previously described (Deng et al., 2000).

Apoptosis Measurements in Cultured Neutrophils. Neutrophils were isolated from whole blood obtained from healthy adult Sprague-Dawley rats as described above. Blood was pooled from at least four animals, and cells were resuspended in RPMI medium supplemented with 10% fetal calf serum, 100 U/ml penicillin G (Pen/Strep, Invitrogen, Bethesda MD) at a cell density of 10^6 cells/ml. Viability before and after culture was determined by trypan blue exclusion. Cells were incubated at 37°C, 5% CO2, and 95% air in medium alone; medium + 10 nM SB-265610; and medium + 100 nM CINC-1; and 0, 10, 100, and 1000 nM SB-265610. Aliquots of cultured neutrophils from each condition were obtained at 2, 4, and 6 h. Cell counts were determined with a hemacytometer, and cell morphology was determined by cytospin samples stained with Wright-Giemsa. Viability was determined by trypan blue exclusion. As positive controls for apoptosis, neutrophils were subjected to heat shock at 43°C for 1 h then returned to 37°C for culture or were cultured in the absence of CINC-1 supplementation (Callahan et al., 1999).

Terminal Transferase Deoxyuridine Triphosphatidyl Nucleotide Nick-End Labeling (TUNEL) Measurements. Aliquots of resuspended cells from at least four animals were cytospun and fixed in acetone. Fixed cells were labeled with a rhodamine-conjugated deoxyuridine triphosphate and terminal transferase for 45 min at 37°C under hydrophobic plastic coverslips (GelBond, BioWhittaker Molecular Applications, Rockland, ME) according to the manufacturer’s directions (Cell Death Detection kit, Roche Molecular Biochemicals, Indianapolis, IN). Cells were mounted in 4,6-diamidino-2-phenylindole (DAPI) containing mounting medium diluted 1:3 in DAPI-free medium (Vectashield, Vector Laboratories, Burlingame, CA). Digital images were obtained with a Nikon Diaphot inverted fluorescence microscope using an ultraviolet light source and a D990 digital camera. Images were obtained at emission wavelengths of 590 (rhodamine: TUNEL positive) and 380 nm (DAPI). An apoptosis index was obtained by counting 200 cells/condition: TUNEL positive/DAPI positive. Results for each condition are means from three separate experiments using pooled blood from at least four animals per experiment.

Neutrophil Cell Morphology. Cells were cytospun and air dried before staining with Wright-Giemsa. Cellular appearance consistent with apoptosis, such as pyknotic nuclei, chromatin condensation, and vacuolization, was determined by examination at 1000× magnification of Wright-Giemsa-stained neutrophils by microscopists masked to the treatment condition, as previously described (Singhal et al., 1999). An apoptotic index was obtained by counting the mean number of apoptotic neutrophils and dividing by the total number of neutrophils. At least 200 cells per condition per animal were used. Interobserver agreement was tested by regression analysis. Between groups, differences were tested by analysis of variance, and post hoc analyses were performed by Tukey-Kramer using JMP software (SAS, Cary, NC) (Carvounis, 2000). Significance was accepted at p < 0.05. Interobserver agreement was accepted at a correlation coefficient >0.8.
Results

To determine the potency of SB-265610 and the cross species specificity of the antagonist, isolated rat neutrophils loaded with Fura-2 were stimulated with rat CINC-1, with or without varying concentrations (0.1–333 nM) of SB-265610. Calcium mobilization induced by CINC-1 was inhibited in a concentration-dependent fashion with an IC50 = 3.4 nM (Fig. 2). SB-265610 inhibited Ca2+ mobilization induced by C5a, but only at IC50 = 6.8 μM, confirming selectivity of the antagonist for CXCR2. Similar results to CINC-1 were obtained in the presence of MIP-2.

Rat neutrophils chemotax to CINC and C5a in a concentration-dependent manner with equal maximum efficiency. Previous experiments determined the optimal concentrations to be 100 and 10 nM for CINC-1 and C5a, respectively. Using these two optimal concentrations of CINC and C5a, we determined the ability of SB-265610 to inhibit both CINC-1- and C5a-induced chemotaxis. As can be seen from Fig. 3, SB-265610 concentration dependently inhibited CINC-1-induced chemotaxis with an IC50 between 50 and 100 nM. The same compound failed to inhibit C5a-induced chemotaxis at concentrations up to 330 nM, thus demonstrating selectivity for rat CXCR2. Therefore, SB-265610 is a functional antagonist for CINC-1 at the rat neutrophil CXCR2.

SB-265610 was evaluated in vivo in a rat model of hyperoxia, where neonatal rats are exposed to high concentrations (95%) of O2 for up to 8 days. We and others have reported that hyperoxia leads to pulmonary neutrophil accumulation in newborns (Deng et al., 2000; Lorant et al., 2000). At day 8, neutrophils represent 60% of the total cells in the bronchoalveolar lavage (BAL) for those animals exposed to 95% O2 compared with 5% for air-exposed animals. Other cells present in the BAL of hyperoxia-exposed animals at day 8 included macrophages (32%), lymphocytes (1%), epithelial cells (5%), and mast cells (<0.5%). The neutrophil predominance at this stage of lung injury is consistent with previous reports in rats and other species, as well as in human newborns with severe respiratory distress syndrome (Lorant et al., 2000). Some hyperoxia-exposed animals were treated i.p. with SB-265610 at 1, 2, or 3 mg/kg on days 3 to 5 and lung neutrophil numbers assessed on day 8. As can be seen from Fig. 4, SB-265610 significantly decreased infiltrating PMN

![Fig. 2](image1.png)

**Fig. 2.** Concentration-dependent effect of SB-265610 on calcium mobilization in neutrophils treated with 0.1 nM CINC-1 (●) or 0.1 nM C5a (□), percent maximal response versus SB-265610 concentration. Results are mean ± S.E. of three experiments.

![Fig. 3](image2.png)

**Fig. 3.** Concentration-dependent effect of SB-265610 on neutrophil chemotaxis treated with 100 nM CINC-1 (●) or 10 nM C5a (□), percent maximal response versus SB-265610 concentration. Results are mean ± S.E. of three experiments.

![Fig. 4](image3.png)

**Fig. 4.** Dose-dependent effects of 1 to 3 mg/kg SB-265610 compared with vehicle given on days 3 to 5 on neutrophil accumulation in bronchoalveolar lavage obtained at day 8 from 95% O2-exposed newborn rat lung. Mean ± S.E., *p < 0.05 versus vehicle.
numbers in 95% O₂ exposed animals by >95% at 3 mg/kg compared with 95% O₂ control (p > 0.05). In addition, we also monitored lung MPO accumulation, which represents adhered/infiltrated PMNs. MPO content was significantly decreased in the lung at the highest dose of SB-265610 (Fig. 5).

To determine whether the SB-265610-induced decrease in lung neutrophil accumulation could be due to increased neutrophil apoptosis, we evaluated the compound’s effect on apoptosis in freshly isolated rat peripheral blood neutrophils. Cells were cultured in the presence of CINC (100 nM) with or without SB-265610 (10–1000 nM) and in the presence of 10 nM SB-265610 without CINC. Apoptosis was induced by heat shock of neutrophils before culture. The apoptotic index was determined at 2, 4, and 6 h, using a combination of TUNEL and DAPI staining and morphologic assessments. There was good agreement assessing apoptosis between masked observers evaluating neutrophil morphology (correlation coefficient = 0.94). Treatment of neutrophils with CINC (100 nM) significantly reduced neutrophil apoptosis (29 ± 2% by morphology 11 ± 1% TUNEL positive) at 6 h compared with the untreated controls (Fig. 6). Pretreatment of CINC-exposed neutrophils with an optimal concentration (100 nM) of SB-265610 completely reversed the ant apoptotic activity of CINC and allowed the cells to proceed to apoptosis with a high proportion of TUNEL (62 ± 10% TUNEL positive, 44 ± 4% by morphology) positive cells, which was comparable with the positive-control, heat-shocked neutrophils, Fig. 6. Treatment with 10 nM SB-265610 alone significantly increased apoptosis at 4 and 6 h compared with CINC treatment. Viability was similar at 4 and 6 h among SB-265610-treated cells at concentrations >100 nM and in cells treated with 10 nM SB-265610 without CINC.

![Fig. 5. Dose-dependent effects of 1 to 3 mg/kg SB-265610 compared with vehicle given on days 3 to 5 on myeloperoxidase activity accumulation in perfused, lavaged, 95% O₂-exposed newborn rat lung at day 8. Mean ± S.E., * p < 0.05 versus vehicle.](image)

![Fig. 6. Concentration-dependent effects of SB-265610 on neutrophil apoptosis. Neutrophils were cultured in medium alone (○) (no CINC, no SB-265610), heat-shocked (□), treated with 10 nM CINC alone (●), 10 nM SB-265610 alone (△) or 10 nM CINC + 10 (○) or 100 (■) nM SB-265610. Mean ± S.E., n = 4/treatment. All SB-265610-treated groups were significantly different (*p < 0.05) from CINC only treatment. A, effects on neutrophil apoptosis as determined by morphology; B, effects on neutrophil apoptosis as determined by TUNEL; C, effects on neutrophil viability determined by trypan blue.](image)
Discussion

In these studies, we report that a nonpeptide competitive inhibitor of the CXCR2 receptor, SB-265610, can block calcium mobilization and chemotaxis in vitro in response to the dominant neutrophil chemokines in rats, CINC-1 and MIP-2, at concentrations achievable in vivo. We have further shown that treatment in vivo can prevent neutrophil accumulation in bronchoalveolar lavage and lung tissue in response to hyperoxia in newborn rats. The prevention of pulmonary neutrophil accumulation in response to hyperoxia may also be due, in part, to accelerated neutrophil apoptosis, which we demonstrated in vitro in response to CXCR2 receptor blockade.

Neutrophil chemokines are induced in a variety of inflammatory states and have been implicated in acute lung injury, infection (Strieter et al., 1999), and bronchopulmonary dysplasia (BPD) (Kotecha, 1996). Targeted blockade of neutrophil functions offers the benefit of reducing adverse effects of inflammation, while minimizing exposure to unwanted and unpredictable side effects of nonspecific anti-inflammatory treatments such as glucocorticoids and cyclooxygenase inhibitors. This would be of particular benefit in premature newborns at risk to develop BPD, who are commonly treated with postnatal glucocorticoids to prevent or treat BPD, but who are vulnerable to adverse effects of glucocorticoids on growth and development (Stark et al., 2001).

The primary importance of the CXCR2 pathway for neutrophil chemoattraction in lung inflammation in particular follows from the observations that blockade of CXCR2 ligands using neutralizing antibodies is sufficient to prevent inflammation, despite the presence of other chemokines, such as C5a or leukotriene B4. Blockade of CINC-1, for example, was superior to blocking with leukotriene B4 receptor antibodies or anticomplement in lipopolysaccharide-induced lung inflammation (Yamasawa et al., 1999).

We chose to target the CXCR2 receptor pathway in part because of the predominance of CXCR2 ligands such as IL-8 (Kotecha, 1996) and GROα (Inwald et al., 1998) in tracheal aspirates of newborns who develop BPD. Although there are elevations of other pro-inflammatory cytokines in tracheal aspirates in BPD and other lung injury states, IL-8 has been consistently reported to be elevated in clinical studies (Kotecha et al., 1995) and in animal models of BPD (Coalson, 1999). Neutralization of CXCR2 ligands was sufficient to prevent neutrophil accumulation in several studies of acute and subacute inflammatory lung injury (see Strieter et al., 1999 for review). This suggests that pro-inflammatory cytokines in these injury states induce neutrophil influx through effects on CXCR2 chemokine expression, in addition to direct effects of inflammatory mediators on chemokine expression (Lin et al., 1994; Xu et al., 1995). CXCR2 blockade may be sufficient to block neutrophil influx during injury states despite elevations of other pro-inflammatory cytokines.

We and others have previously shown that hyperoxia exposure in newborns can induce pulmonary accumulation of CXC neutrophil chemokines (D’Angio et al., 1999; Deng et al., 2000), and we have shown that neutralizing antibody treatment can attenuate pulmonary neutrophil influx during hyperoxia exposure (Deng et al., 2000). This approach has led to functional and developmental improvements in developing newborn rat lung (Auten et al., 2000).

In these studies we sought to test whether a nonpeptide competitive antagonist to CXCR2 would be sufficient to block neutrophil accumulation during hyperoxia in newborn rats. In theory, blockade of CXCR2 should offer the added advantage of blocking all potential ligands that might activate the receptor and bypass the effect of neutralizing a single chemokine. We found that the degree of blockade of neutrophil influx in BAL and myeloperoxidase accumulation in lung tissue was similar to that which we observed in our earlier studies using neutralizing antibodies to CINC-1 and MIP-2 (Deng et al., 2000). It may be that in our subacute inflammatory model of hyperoxia exposure in newborn rats, blocking neutrophil influx for a brief period, two days, is sufficient to attenuate the cycle of injury, inflammation, and exacerbation. This may be due to elimination of paracrine secretion of IL-8, which may serve to perpetuate neutrophil influx (Fujishima et al., 1993). The relative contributions of each CXCR2 ligand and other neutrophil chemokines probably vary among injury states and may depend on the specific cellular locations and duration of injury.

In addition to blocking neutrophil influx, CXCR2 blockade may also influence total PMN accumulation by accelerating neutrophil apoptosis. The resolution of inflammation depends on the well regulated supply and elimination of activated neutrophils, mediated in part by apoptosis (Renshaw et al., 2000). This may be of particular importance in the lung, since removal of apoptotic neutrophils by macrophages may prevent release of neutrophil proteases, which may further protect the lung and other tissues from injury (Haslett, 1999). Duncan and colleagues have shown that CXCR2 signaling is necessary to prevent neutrophil apoptosis (Duncan et al., 2000a,b). We have shown that CXCR2 competitive inhibition with SB-265610 can increase rat neutrophil apoptosis despite the presence of 100 nM CINC-1, a CXCR2 ligand, in a concentration-dependent manner. This is consistent with their findings in human neutrophils that IL-8 prevented apoptosis in a similar concentration-dependent manner.

Autocrine effects of neutrophil-produced CINC-1 may also prevent neutrophil apoptosis (Duncan et al., 2000a). Addition of 10 nM SB-265610 without added CINC-1 produced high levels of neutrophil apoptosis at 4 h (by morphology) that were intermediate between heat-shock control (maximum) and the highest SB-265610 concentration (Fig. 6). Direct cytotoxicity might have contributed to the observed effects of SB-265610. However, the higher level of apoptosis produced by the lower concentration of antagonist when unopposed by ligand (added CINC-1) suggests that the differences in neutrophil apoptosis depended on ligand-receptor interaction rather than predominantly on direct cytotoxicity, although some cytotoxic effect cannot be completely excluded.

Blockade of neutrophil influx using a competitive CXCR2 antagonist may offer more selective and effective defense against neutrophil-mediated damage in lung injury and in other conditions as well, such as rheumatoid arthritis and inflammatory bowel disease, which have recently been treated with partial success using anticytokine treatment (Moreland, 1998; Rutgeerts, 1998).

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References


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