CXCR2 Blockade Reduces Radical Formation in Hyperoxia-Exposed Newborn Rat Lung

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ABSTRACT: Inflammation contributes greatly to the pathogenesis of bronchopulmonary dysplasia. In previous studies, we showed that blocking neutrophil influx by treatment with SB265610, a selective CXCR2 antagonist, could partly reduce superoxide accumulation and preserve alveolar development in 60% O_2-exposed newborn rats. The purpose of this study was to further investigate the role of neutrophils in the formation of reactive oxygen and nitrogen species mediating hyperoxia-impaired lung development. We found that hydroxyl radical formation and lipid peroxidation in rat lungs were significantly increased during 60% O_2 exposure. These increases were attenuated by the administration of SB265610. In addition, SB265610 largely inhibited protein nitration induced by hyperoxia. SB265610 partly prevented the hyperoxia-enhanced bronchoalveolar lavage (BAL) protein content in 60% O_2-exposed animals. Our results demonstrate that neutrophils have a pivotal role in hydroxyl radical formation, lipid peroxidation and protein nitration. Taken together with our previous studies, the present findings show that blocking neutrophil influx protects alveolar development and improves lung function in part by preventing reactive oxygen/nitrogen species accumulation. (*Pediatr Res* **60:** 299–303, 2006)

Inflammation is a common mechanism uniting factors linked to the development of bronchopulmonary dysplasia (BPD), such as oxidative stress, mechanical injury, and defective antioxidant defenses (1). Premature newborns born to mothers with chorioamnionitis are at high risk of developing BPD (2). Likewise, premature newborns with respiratory distress syndrome (RDS) with elevated neutrophil counts in tracheal aspirates at birth are at high risk of developing BPD (3). In the baboon model of BPD, neutrophil chemokine IL-8 is elevated in tracheal aspirates a few days after delivery (4).

While there are no published studies that directly and specifically antagonize neutrophil influx in premature newborn babies, a number of experimental models show beneficial effects. Depletion of circulating neutrophils in preterm lambs lessened the severity of pulmonary leak of protein and fluid in experimental RDS (5). Blocking hyperoxia-induced pulmonary neutrophil influx in newborn rats by treatment with neutralizing antibodies to neutrophil chemokines (6) prevented impaired lung compliance (7), DNA oxidation (8), and protected alveolar development (7) in 95% O_2-exposed newborn rats.

To determine the mechanisms by which blocking neutrophil influx could protect lung development, we exposed newborn rat pups to air or $FiO_2 = 0.6$ for two weeks and treated them with SB-265610, a small molecule selective antagonist of C-X-C chemokine receptor-2 (CXCR2), a dominant neutrophil chemokine receptor. In our previous studies, SB265610 prevented neutrophil accumulation, partly reduced tissue superoxide accumulation, and accelerated alveolar development (9). To determine whether blocking neutrophil influx through this approach could reduce pulmonary hydroxyl radical accumulation, lipid peroxidation, and protein nitration, we performed similar hyperoxia exposures ± treatment with the CXCR2 antagonist SB265610 and measured hydroxyl radical reaction products, free and esterified 8-isoprostane, and 3-nitrotyrosine. We found that hyperoxia increased bronchoalveolar lavage protein, lung tissue hydroxyl radical, 8-isoprostane and 3-nitrotyrosine accumulation, and that treatment with SB265610 substantially prevented each of these adverse effects.

METHODS

Materials. SB265610 was kindly provided by Dr. Skip Sarau (Glaxo SmithKline, King of Prussia, PA). A rabbit polyclonal antibody to myeloperoxidase (MPO, for neutrophil identification) was from NeoMarker (Fremont, CA). Proliferating cell nuclear antigen (PCNA) MAb was purchased from BD Transduction Laboratories (San Diego, CA). Rabbit polyclonal antibody to

Abbreviations: BAL, bronchoalveolar lavage; **BPD,** bronchopulmonary dysphasia; **CXCR2,** C-X-C chemokine receptor-2; **2,3-DHBA,** 2,3-dihydroxybenzoic acid; **2,5-DHBA,** 2,5-dihydroxybenzoic acid; **MPO,** myeloperoxidase; **WBC,** white blood cell; **WW/DW,** lung wet-to-dry weight ratio

nitrotyrosine was obtained from Upstate Biotechnology (Lake Placid, NY). Biotinylated goat anti-rabbit IgG, biotinylated goat anti-mouse IgG, biotinylated rabbit anti-goat IgG'streptavdin-peroxidase and 3,3'-diaminobenzidine were from Dako (Carpinteria, CA). 8-isoprostane enzyme immunoassay kits were from Cayman Chemical Co. (Ann Arbor, MI). All organic solvents were of HPLC grade. A total protein assay kit was purchased from Bio-Rad (Hercules, CA). Sep Pak C18 cartridges were from Waters (Mississauga, Canada).

Institutional review. All procedures involving animals were conducted according to criteria established by the Chinese Council for Animal Care. Approval for the study was obtained from the Animal Care Review Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Exposure system. The exposure system has been described in detail previously (10). Briefly, pathogen-free, timed-pregnant Sprague Dawley rats were obtained from the animal center of Tongji Medical College. Experiments were conducted as paired exposures, with one chamber receiving 60% O_2 and the other receiving air. On the anticipated day of delivery, each dam was placed in a $50 \times 35 \times 40$-cm plastic chamber with 12h/12h light/dark cycles, and a temperature of 25°C. Food and water were given *ad libitum*. Dams were exchanged daily between paired chambers to prevent maternal O_2 toxicity.

Intervention. Rat pups were maintained in paired chambers (air and O_2) for the 14-d exposures. Each paired litter received either 0.1% DMSO in saline (vehicle control) or SB265610 in 0.1% DMSO in saline (4 μg/g and 4 μL/g) daily s.c. on day 3–10.

Bronchoalveolar lavage (BAL) cell counting and protein concentration. After 60% O_2 exposure for 7 and 14 d, pups were anesthetized with 150 mg/kg sodium pentobarbital IP. Both lungs were slowly lavaged five times via a tracheal cannula with 0.5 mL 0.9% NaCl containing 1 mM EDTA. Lavage volumes were recorded, and cell counts measured with a hemocytometer. Differential counts of $\geq$ 200 cells were obtained using Wright-Giemsa stain. Protein concentration was measured by the method of Bradford (11).

Lung wet-to-dry weight ratio (WW/DW). The unlavaged lungs were removed after sacrifice and the wet weight was measured. The lungs were then placed in pre-weighed tubes and dried to a stable weight at 60°C over 48 h.

Sample collection. Animals were anesthetized with 150 mg/kg sodium pentobarbital IP. While the heart was beating, the pulmonary circulation was flushed with PBS containing 1 U/mL heparin to clear the lungs of blood, and perfusion fixed with 4% (wt/vol) paraformaldehyde with a constant airway pressure of 10 cm H_2O, which was maintained via a tracheal catheter.

Immunohistochemistry. Lung right middle lobes were embedded in paraffin and cut in 5-μm sections. Sections were immunostained using the strepavidin-biotin technique. For neutrophil identification, dilutions of rabbit anti-MPO and biotinylated goat anti-rabbit IgG were 1:200 and 1:250, respectively. For the detection of 3-nitrotyrosine, the dilutions of rabbit anti-nitrotyrosine and biotinylated goat anti-rabbit IgG were 1:100 and 1:200, respectively. For all antibodies, antibody specificity was verified by replacing the primary antibody with rabbit IgG. Slides were counterstained with Carazzi hematoxylin, dehydrated, cleared in xylene, and mounted.

Hydroxyl radical measurement. The pups were injected with 100 mg/kg of sodium salicylate IP in 0.9% NaCl 1 h before euthanasia. Hydroxyl radical formation was assessed by an aromatic hydroxylation assay using gas chromatography/mass spectrometry (GC/MS) with salicylate as the probe. Hydroxyl radical reactions with salicylate produce characteristic decarboxylation and hydroxylation products, 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA). As previously described (12), lungs

were homogenized in PBS with EDTA (400 μM), butylated hydroxytoluene (20 μM), and deferoxamine (20 μM) to prevent auto-peroxidation during sample preparation.

Total lung 8-isoprostane (free and esterified) measurement. To prevent auto-oxidation, lung tissue was immediately flash frozen in liquid N_2 at harvest and stored at -80°C until analysis. As previous described (13), upon thawing, the tissue sample was homogenized in PBS with 0.005% (vol/vol) butylated hydroxytoluene, and 100 ng isoprostane was added to quantify recovery during purification. Proteins were precipitated with ethanol and removed by centrifugation. The supernatant was incubated with an equal volume of 15% (wt/vol) potassium hydroxide at 40°C for 1h for alkaline hydrolysis before solid-phase extraction using Sep Pak C18 cartridges. After purification, samples were analyzed in duplicate for 8-isoprostane content using a commercially available ELISA kit.

Statistical analysis. All data except survival rate are presented as mean $\pm$ SEM of five litters. Differences for survival rates between the groups were compared using Fisher's exact test. Differences for other data were evaluated with the SPSS 12.0 package (SPSS, Chicago, IL) using one-way analysis of variance (ANOVA), followed by assessment of differences using Student-Newman-Keuls test. Statistical significance was defined as a *p* value < 0.05.

RESULTS

Effects of the CXCR2 antagonist SB265610 on survival rate and lung injuries after 14 d exposure to air or 60% O_2 are shown in Table 1. Survival rate and body weight were recorded at the end of the 14-d exposure. There were no significant differences in either survival rate or body weight between groups. However, the survival rate decreased in 60% O_2-exposed animals with or without SB265610 treatment, compared with air-exposed animals. Lung injury was assessed by lung WW/DW ratio and protein concentration in BAL fluid. Compared with air-exposed animals with or without SB265610 injection, WW/DW ratio was significantly increased after 14 d exposure to 60% O_2, and there was no significant difference between the two hyperoxia-exposed groups. The content of protein in BAL fluid was significantly increased after both 7- and 14-d exposure to 60% O_2, which was partially prevented by SB265610 treatment.

Total white blood cell (WBC) counts and neutrophils in BAL fluid were significantly elevated in pups after both 7- and 14-d exposures to 60% O_2, as compared with air-exposed animals. In the SB265610-treated group, the increases in total WBC were significantly reduced, and elevated neutrophil count was completely attenuated (Fig. 1). MPO immunohistochemistry showed an increased abundance of neutrophils on exposure to 60% O_2 for 7 and 14 d, which was reduced by treatment with SB265610(data not shown), parallel to the

Table 1. *The effect of SB265610 on survival rate, lung wet/dry weight ratio and protein concentration in BAL after a 14-day exposure*

Group	Survival rate % (survival/total)	Body weight (g)	Lung wet/dry weight ratio	Protein concentration in BAL (g/ml)	
				7d	14d
Air					
Vehicle	100 (51/51)	25.24 ± 0.78	5.16 ± 0.14	10.11 ± 0.19	23.80 ± 1.67
SB265610	100 (53/53)	26.69 ± 0.58	5.09 ± 0.06	12.30 ± 0.56	20.54 ± 1.14
60% O_2					
Vehicle	90.9 (50/55)	25.09 ± 0.25	6.72 ± 0.29*	123.41 ± 5.87*	168.94 ± 9.41*
SB265610	90.7 (49/54)	25.74 ± 0.26	6.58 ± 0.32*	65.18 ± 2.32*†	74.52 ± 3.68*†

Results except survival rate are presented as means $\pm$ SEM. Numbers in brackets under survival rate are the sample sizes of survived and total pups in each group.

* *p* < 0.01, by one-way ANOVA for animals exposed to 60% O_2 compared with those in air in the same treatment group.

† *p* < 0.01, by one-way ANOVA for SB265610-treated animals compared with control animals exposed to the same gas.
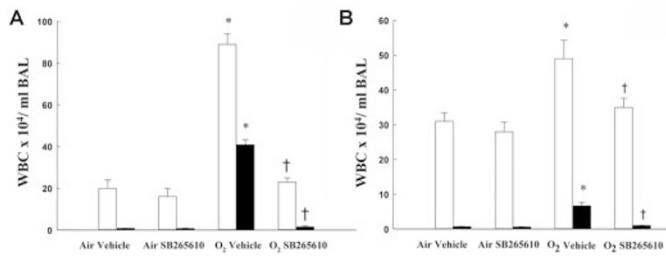
Figure 1. Total WBC (□) and neutrophil (■) counts in BAL of neonatal rats exposed to air or 60% O_2 + SB265610 or vehicle (*A*, day 7; *B*, day 14). Bars represent mean ± SEM, $n = 5$. * $p < 0.01$, by one-way analysis of variance, for animals exposed to 60% O_2 compared with those in air in the same treatment group. † $p < 0.01$, by one-way analysis of variance, for SB265610-treated animals compared with control animals exposed to the same gas.

effects on BAL neutrophils. These results were consistent with our previous findings with MPO immunostaining and activity measurements, showing that SB265610 treatment via s.c. as well as IP injection can effectively block O_2-dependant pulmonary neutrophil influx in neonatal rats.

Hydroxyl radical formation in rat lungs, as measured by the salicylate hydroxylation products 2,3-DHBA and 2,5-DHBA, was significantly increased after both 7 and 14 d of exposure to 60% O_2, and this was significantly prevented by the administration of SB265610 (Fig. 2).

As shown in Fig. 3, lung 8-isoprostane content was markedly increased in 60% O_2-exposed rats, as compared with air- and vehicle-exposed animals. SB265610 treatment partly reduced the increase in 8-isoprostane content in the lungs of neonatal rats after exposure to 60% O_2 for 7 and 14 d.

As detected by immunohistochemistry, faint diffuse nitrotyrosine immunoreactivity was present in lungs of air-exposed pups with (Fig. 4*B*) or without SB265610 treatment (Fig. 4*A*). However, lungs of rats exposed to 60% O_2 showed widespread and more intense immunostaining for nitrotyrosine (Fig. 4*C*), which was largely prevented by SB265610 treatment (Fig. 4*D*).

DISCUSSION

Mounting evidence links maladaptive inflammation to abnormal postnatal lung development. Earlier studies showed
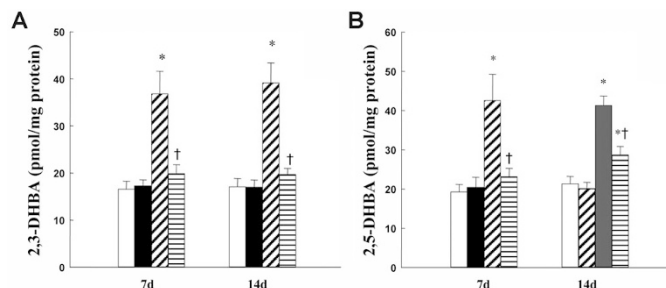

Figure 2. Hydroxyl radical detection in lungs of neonatal rats exposed to air or 60% O_2 + SB265610 or vehicle on days 7 and 14 (A. 2,3-DHBA; B. 2,5-DHBA). Bars represent mean ± SEM, $n = 5$. * $p < 0.01$, by one-way analysis of variance, for animals exposed to 60% O_2 compared with those in air in the same treatment group. † $p < 0.01$, by one-way analysis of variance, for SB265610-treated animals compared with control animals exposed to the same gas. Air (□), Air + SB265610 (■), O_2 (▨), O_2 + SB265610 (▤)
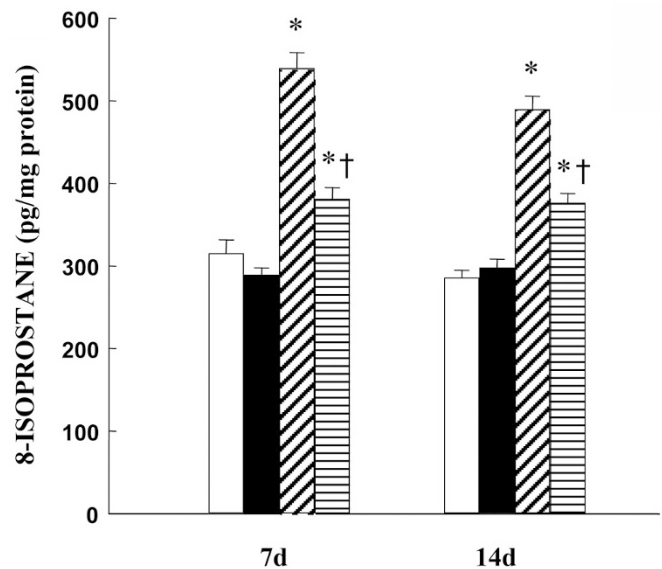

Figure 3. Total 8-isoprostane concentrations in lungs from neonatal rats exposed to air or 60% O_2 + SB265610 or vehicle for 7 and 14 d. Bars represent mean ± SEM, $n = 5$. * $p < 0.01$, by one-way analysis of variance, for animals exposed to 60% O_2 compared with those in air in the same treatment group. † $p < 0.05$, by one-way analysis of variance, for SB265610-treated animals compared with control animals exposed to the same gas. Air (□), Air + SB265610 (■), O_2 (▨), O_2 + SB265610 (▤)
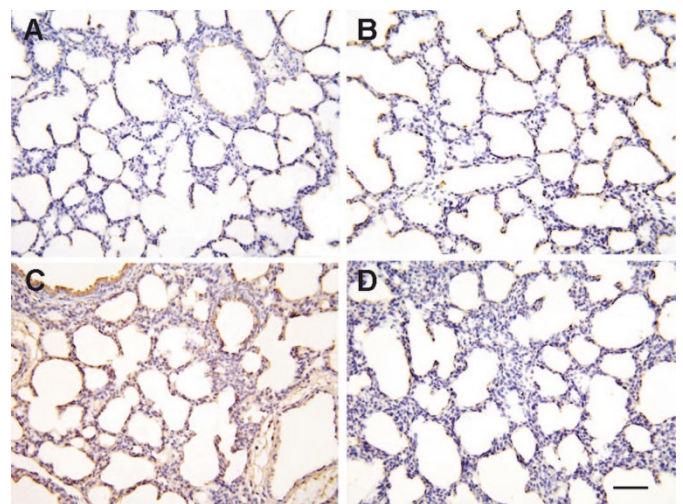

Figure 4. Detection of nitrated protein by nitrotyrosine immunostaining (brown). Neonatal rat pups were exposed to air or 60% O_2 for 7 d plus SB265610 or vehicle. Faint positive staining was evident in air-exposed newborn rats that had received vehicle (*A*) or SB265610 (*B*). Positive staining for nitrotyrosine was more intense and present diffusely in the lungs of 60% O_2-exposed newborn rats that had received vehicle (*C*). Newborn rats treated with 60% O_2 and SB265610 had a similar intensity of staining to air control animals (*D*). Bar length = 50 μm.

that blocking neutrophil influx could partly protect alveolar development (7,9). Because our earlier studies showed that DNA oxidation and superoxide accumulation were reduced following neutrophil blockade, we wanted to determine whether other molecular targets were also protected by this strategy.

We found that blocking neutrophil influx largely prevented pulmonary hydroxyl radical accumulation. There is also evidence (14) that hydroxyl radicals are the most important free

radicals responsible for the growth-inhibiting effects of hyperoxia on mouse fetal lung morphogenesis. The best recognized mechanism of hydroxyl radial formation (15) is by the Fenton reaction, in which divalent redox ions, such as iron, serve as catalysts. Romas and colleagues (16) reported a myeloperoxidase-dependent mechanism of hydroxyl radical generation which does not require a supplemental iron catalyst, that is, myeloperoxidase-generated hypohalous acid (HOCl or HOBr) reacting with intracellular superoxide to form the hydroxyl radical.

Hyperoxia may lead to free radical-mediated lipid peroxidation, and then result in the destruction of cell membrane and tissue injury (17). In this study, we used 8-isoprostane to monitor lipid peroxidation levels. 8-isoprostane has been widely used to quantify oxidative stress in various conditions (18). Moreover, it also exerts a number of biologic activities in the lung, including smooth muscle contraction and proliferation and fibroblast chemotaxis and proliferation (19). A recent study (20) reported that plasma 8-isoprostane concentrations in infants destined to develop BPD were significantly higher at 3 and 7 d of life than in those not developing BPD, suggesting that plasma 8-isoprostane could serve as a marker for assessing the risk for BPD development in pre-term infants. Our results suggest that activated neutrophils play a role in the process of lipid peroxidation in the lungs of newborn rats during exposure to hyperoxia. This conclusion is supported by evidence (21) that myeloperoxidase is secreted by activated neutrophils and may serve as an alternative enzymatic participant in the initiation of lipid peroxidation *in vivo*.

Our study suggested that neutrophils may also play a role in the formation of nitrotyrosine, a marker of reactive nitrogen species generation. A recent report noted that activated neutrophil heme-protein peroxidase activity, in particular neutrophil-derived myeloperoxidase (22), significantly contributes to nitrotyrosine formation *in vivo*. These results were consistent with those findings in hyperoxia-exposed adult rats, Baldus and colleagues (23) reported that myeloperoxidase immunoreactivity strongly colocalized with nitrotyrosine formation in hyperoxia-exposed adult rats, both of the signals being distributed in subendothelial and epithelial regions. Nitrotyrosine is produced by the reaction of reactive nitrogen species with proteins containing tyrosine residues. Several studies have focused on identifying target nitrated proteins, and Mn-SOD (24), tyrosine phosphatases (25), cytochrome P450 (26), ribonucleotide reductase (27) and extracellular matrix protein fibronectin (28) have been found to be nitrated. It is believed that some nitrated proteins are inactivated and dysfunctional. However, as tyrosine groups are also the target of phosphorylase, protein nitration may be an important mechanism for posttranslational protein modification (25).

Our data on protein content in BAL and WW/DW indicated that blocking neutrophil influx partly relieved hyperoxia-mediated lung edema. A recent study reported that CXCR2 knock-out adult mice had almost no signs of hyperoxia-induced lung injuries (29). Our model used a significantly lower FiO_2, since it was aimed at producing impaired lung development that mimics BPD, rather than acute lung injury. Our expected and observed degree of protein leak was less than typically observed in hyperoxia-induced injury in adult rodents (6,30).

There is a growing body of evidence that free radicals are required for normal cellular metabolism, proliferation, and differentiation (12,31–33). Antagonism of CXCR2 using SB265610 does not appear to affect basal ROS accumulation, since there were no differences between vehicle and SB265610 treated air-exposed groups, and since our previous report showed no adverse effects on lung development (9).

Expression of CXCR2 has been demonstrated in many other cell types, including non-hematopoietic cells such as endothelial cells. Although these cells could also be affected by the inhibitor, contributing to the observed protection, we found no effects, except in the hyperoxia-exposed group. It could be that antagonizing CXCR2 might affect eosinophils, monocytes (34), or mast cell targeting (35), but we did not examine this directly. CXCR2 function in pulmonary vascular endothelial cells and in bronchiolar epithelium is only now being explored, and may be required for full expression of pulmonary inflammation (36). Effects of SB265610 in these other cells types could have contributed to the observed lung protection, but would have done so predominantly via their indirect effects on neutrophil accumulation.

The present study demonstrates that neutrophils are the significant intermediate sources of hydroxyl radical that accumulates in hyperoxia-exposed newborn rat lung, and that blocking neutrophil influx can substantially reduce important downstream products of reactions with hydroxyl radicals, such as 8-isoprostane. Together with our previous studies, our present findings show that blocking neutrophil influx protects alveolar development and improves lung function, in part by preventing hydroxyl radical accumulation and lipid peroxidation.

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