Suppression of inflammatory cell trafficking and alveolar simplification by the heme oxygenase-1 product carbon monoxide

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An estimate of 30% of premature infants with a birth weight between 500 and 1500 g will develop respiratory distress syndrome and its consequence, bronchopulmonary dysplasia (BPD). Pathological features of BPD include interstitial fibrosis and abnormal cellular proliferation, as well as arrested alveolar development. Infants with BPD are at risk for moribundity and mortality during the first years of life, including increased medication use, hospital readmissions (14), and lung function abnormalities that persist into adulthood (13, 24).

Recent studies implicate lung inflammation as a contributor to BPD development. Leukocyte infiltrates, in particular neutrophils and cells of the monocyte/macrophage lineage, as well as inflammatory mediators including IL-1β, IL-6, tumor necrosis factor-α, and CXC and CC-chemokines, have been detected in the lungs of patients with BPD (3, 23, 25, 30, 33). Treatment of hyperoxia-exposed neonatal lungs with antibodies or antagonists against neutrophil (CXCL1, CXCL2) and macrophage (CCL2) chemokines prevents lung inflammation and alveolar simplification (2, 9, 50, 55).

Oxidative stress has also been implicated as a cause of pulmonary injury leading to the development of BPD. Prolonged exposure to sublethal hyperoxia in animal models recapitulates some of the processes observed in BPD (18, 40, 41), such as arrested alveolar development, a paucity of α-actin-positive myofibroblasts at the alveolar septal tips, and a corresponding increase in the number of interstitial myofibroblasts (4, 21, 22, 48). Importantly, rodents exhibit a saccular stage of lung development at birth that is only completed after 4 wk of postnatal alveolarization (53). By comparison, sacules appear in the human lung by 23 wk of gestation (11, 19). In newborn animals, hyperoxia has been shown to be a strong inducer of various proinflammatory cytokines in airway cells and pulmonary tissue (51). Reactive oxygen species may be produced by resident lung epithelial cells under hyperoxic conditions or from neutrophils and macrophages invading the lungs as part of the inflammatory process (42).

Organisms faced with hyperoxic stress have evolved sophisticated mechanisms to ensure survival. Emerging evidence highlights a crucial role for stress-inducible heme oxygenase (HO)-1 and its byproduct carbon monoxide (CO) and biliverdin, in cytoprotection against hyperoxic and ischemic forms of tissue and cellular injury (12, 15, 31, 32, 34, 35, 38, 49). Although the precise mechanisms whereby CO modulates the inflammatory milieu remain under study, there is some evidence that it does so by tilting the milieu toward the oxidative and proinflammatory forms of tissue and cellular injury (10, 34, 49). The recent study has been shown that HO-1 expression is induced during lung maturation and following exposure to hyperoxia, consistent with adaptation to extrauterine conditions of increased oxidative stress and inflammation (10, 44). It has recently been shown that HO-1 and CO mitigate hyperoxia-induced pulmonary inflammation in neonatal mice (15). However, in this study, arrested alveolar development was minimally affected by CO administration, and the mechanisms by which CO reduced pulmonary inflammation are not explored.

We hypothesized that inhalation of low-dose episodic CO mitigates the effect of hyperoxia on alveolar development by an anti-inflammatory mechanism. We report here that inhalation of CO suppresses inflammatory cell trafficking, proinflam-
matory cytokine expression, and alveolar simplification in hyperoxia-exposed mice.

MATERIALS AND METHODS

Experimental animals. All animal experiments were performed according to protocols approved by the University of Michigan Institutional Animal Care and Use Committee. Timed pregnant C57BL/6J mice were either purchased from Jackson Laboratories (Bar Harbor, ME) or bred at the University of Michigan animal facility. Newborn pups from six different litters were subdivided into four groups within 24 h of birth. At 3 days of age, pups were exposed to normoxia (21% O₂), hyperoxia (75% O₂), carbon monoxide (CO 250 ppm) + normoxia, or CO (250 ppm) + hyperoxia for 7, 10, 14, or 21 days. Selected experiments employed B6.129S4-Scy/Lod CCL2-null mice (Jackson Laboratories).

In addition, we obtained HO-1-null mice (C57BL/6J background) from Dr. Mu-En Lee (Harvard School of Public Health) (54). Colonies were maintained by breeding HO-1−/− males with HO-1+/− females. Offspring were genotyped at the time of birth using PCR (16). For all experiments, HO-1−/− and HO-1+/− mice were age matched at postnatal day 3. Each litter consisted of 8–10 pups per group to control for the effects of litter size on nutrition and growth. Animals were either exposed to room air or various gas mixtures for up to 3 wk.

To achieve 75% O₂, 100% O₂ was delivered at a flow rate of 0.5 l/min into a custom-made 1.5-m³ plastic chamber. Oxygen concentrations were monitored continuously with an oxygen analyzer (Neutronics, Exton, PA), gas lines were filtered with activated charcoal to remove excess ammonia, and calcium chloride was used to maintain CO₂ levels below 0.05%. Animals were exposed to 250 ppm CO at a flow rate of 10 l/min for 1 h twice daily. A CO analyzer (Niton model 1100; Neutronics, Exton, PA) was used to monitor CO levels continuously in the chamber. Dams were rotated between normoxia and hyperoxia conditions, or CO + normoxia and CO + hyperoxia conditions daily to minimize oxygen toxicity to the dams and to remove potential confounders of milk production induced in the various environments. Relative humidity in the chamber was maintained at 45–50%. Dams and pups were given food and water ad libitum and maintained on a 12-h:12-h light:dark cycle. Mice were euthanized with an overdose of ketamine and xylazine followed by thoracotomy after exposure for the indicated time periods and experimental conditions.

Histological analysis. Lungs were perfused with PBS with 20 mM EDTA through the right ventricle at a constant pressure of 20 cm H₂O for 3–5 min. Lungs were then intratracheally inflated with 10% formalin, fixed overnight at 4°C, then stored in 70% ethanol before use. Tissues were embedded in paraffin and 4-μm sections were cut. Paraffin sections were stained with hematoxylin and eosin. Stained sections were visualized under light microscopy, and images were captured using a Nikon Eclipse microscope, and images were captured using MetaMorph version 7.0r3 software (Molecular Devices, Sunnyvale, CA) on an Eclipse TE2000-E microscope (Nikon Instruments, Melville, NY). Morphometric analysis. To assess alveolarization, mean alveolar diameter was determined by measurement of mean linear intercept (MLI). MLI, equal to the mean interalveolar distance, was measured by dividing the total length of a line drawn across the lung section by the number of intercepts encountered, as described (7). Briefly, three representative lung sections for each animal were photographed at ×200 magnification. A grid with parallel lines spaced at 60 μm was overlaid onto the image, and the length of each cord was defined by the intercept with the alveolar walls. Fifteen nonoverlapping fields for each section were examined. MetaMorph image analysis software was used for morphometric analysis.

Bronchoalveolar inflammatory and differential cell counts. Bronchoalveolar lavage fluid (BAL) fluid was obtained by intratracheal instillation of 3 × 1 ml ice-cold PBS and filtered via a 70-μm cell strainer to exclude epithelial cells. Erythrocytes were lysed using RBC Lyse (BD Biosciences, San Diego, CA). BAL fluid was centrifuged at 2,000 revolution/min for 15 min, and supernatants were removed and stored in −80°C until further analysis. The cell pellet was resuspended in PBS, and total infiltrating cells (neutrophils and macrophages) were counted using a Hemavet 950FS cell hemocytometer (Drew Scientific, Dallas, TX).

Immunohistochemistry and fluorescence microscopy. Paraffin blocks were sectioned at 500-μm intervals at a thickness of 5 μm. After deparaffinization, rehydration, and antigen retrieval, endogenous peroxidase was blocked using a rodent serum block. Serial sections were probed with fluorophore-labeled mouse anti-α-smooth muscle actin (clone 1A4, Sigma-Aldrich), Alexa Fluor (AF)-conjugated donkey anti-goat IgG (Molecular Probes, Portland OR), rat anti-mouse F4/80 (AbD Serotec, Raleigh, NC), anti-CD11b (R&D Systems, Minneapolis, MN), and rat anti-mouse Gr-1 (R&D Systems). Slides were subsequently incubated with secondary antibody, ABC reagent (Vector Laboratories, Burlingame, CA) and diamobenzidine. Nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) (Molecular Probes), and immunofluorescence (DAPI). In addition, sections were incubated with AE55-conjugated goat anti-mouse CXCL2 (R&D Systems), AF633-rat anti-mouse CD68 (AbD Serotec), or AF-conjugated isotype control IgGs. To examine endogenous HO-1 staining, we used a rabbit polyclonal to rat HO-1 (StressGen; Victoria, BC, Canada). Images were visualized using an Axiovert 100M inverted fluorescent microscope (Carl Zeiss, Thornwood, NY).

Focused gene array analysis of neonatal mouse lung lysates. Lung RNA was subjected to a targeted PCR array examining 84 mouse inflammatory cytokines (SA Biosciences, Frederick, MD). Total RNA extraction was performed using the Qiagen Animal Spin protocol (Valencia, CA). Total RNA was quantified using the Nanodrop N-1000 (Agilent Biosystems, Santa Clara, CA). After verification of RNA quality and integrity, first-strand cDNA synthesis was performed on an Eppendorf Mastercycler using a Reaction Ready First-Strand cDNA Synthesis kit (SA Biosciences). The cDNA template was combined with RT² Real-Time SYBR Green/ROX Master Mix (SA Bioscience) and RNase-free water. A final reaction volume of 25 μl was added to each well of PCR array. Finally, pathway-focused mRNA was amplified on a 7300 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Genes were plotted as heat maps by hierarchical clustering using the correlation coefficient as a distance measure and the average of each cluster for cluster formation of the genes using Amap software (Bioconductor, Seattle, WA). Expression values are visualized with color ranging from red (high expression) over white (intermediate expression) to blue (low expression).

RNA isolation and real-time RT-PCR. Quantitative RT-PCR was performed on eight genes with increased or decreased expression following the different exposure conditions in C57BL/6J mice (n = 10–18 samples/condition). Lung total RNA was extracted using an RNAeasy Mini kit (Qiagen, Gaithersburg, MD). First-strand cDNA (500 ng) was reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed with an ABI PRISM 7000 sequence detection system with TaqMan Universal PCR Master Mix and Assay-on-Demand gene expression probes (Applied Biosystems). We measured the following mRNAs: IL-1β, TNF-α, IL-10, IL-13, IL-6, CCL2, CXCL1, and CXCL2. β-Actin served as housekeeping gene. All primers were purchased from Applied Biosystems. All samples were analyzed in quadruplicate. mRNA expression was normalized to β-actin level by the 2−ΔΔCt method.

Protein extraction and cytokine expression. Whole lungs were homogenized with lysis buffer containing 1 mM Tris-HCl, pH 7.4, 0.5 M EDTA, 5 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 10% SDS supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail; Roche, Indianapolis, IN). Supernatants were analyzed for IL-1β, IL-6, IL-10, IL-13, TNF-α, CCL2, CXCL1, and CXCL2 protein abundance with a multiplex mouse
were exposed to 95% O2 or 250 ppm CO at 37°C. Cells were grown to 60–80% confluence and rendered quiescent.

RESULTS

Lung tissues were harvested, and single-cell suspensions were obtained by digestion with collagenase IV (Gibco Life Technology, Grand Island, NY) for 1 h at 25°C while being shaken. FACSLyse buffer (BD Bioscience) was used to lyse erythrocyte contaminants. Total lung cell counts were assessed by Hemavet hemocytometer (Drew Scientific) and reconfirmed by flow cytometry analysis using FITC-anti mouse CD45 antibody (BD Bioscience) and flow cytometry absolute count standard beads (BD Bioscience). Viable cells were identified by the absence of DAPI (Molecular Probes) staining. Nonspecific antibody staining was inhibited using Fc Block (BD Bioscience).

Analysis of whole lung total alveolar leukocytes was performed using APC-anti mouse CD11b and phycoerythrin (PE)-anti-mouse Ly6G/Ly-6C (Gr-1) (both from BD Bioscience). To detect infiltrating alveolar macrophages, cells were purified and identified in four stages. First, the leukocyte population was gated using FITC-conjugated anti mouse CD45. Second, infiltrating monocyte/macrophages double positive for staining with APC-Cy7-conjugated anti-mouse F4/80<sup>high</sup> and PE-conjugated anti-mouse CD11c were then positively gated. Third, cells were gated using PerCy5.5-conjugated anti-mouse CD11b (all from BD Bioscience). For calculation of total cell numbers in tissue, normalization to weight of tissue was performed. Data were acquired on a BD Bioscience FACS Canto flow cytometer and analyzed with FlowJo v.9.4.11 (Tree Star, Ashland, OR).

Cell culture and ex vivo CO exposure. Murine type II alveolar epithelial cells were isolated from C57BL/6J mice as described previously (8). Briefly, after proteolytic digestion of lung tissue, single-cell suspensions of lung tissue were depleted of macrophages by negative selection in a magnetic separator using anti-CD32 and anti-CD45 antibodies. The negative cells were plated overnight in 100-mm culture plates. The type II alveolar epithelial cells comprised the nonadherent population. These cells were recovered for cell culture and maintained in humidified incubators (5% CO<sub>2</sub>-95% air) at 37°C. Cells were grown to 60–80% confluence and rendered quiescent in medium containing serum-free medium. Selected cultures were exposed to 95% O<sub>2</sub> or 250 ppm CO + 95% O<sub>2</sub> in an incubator chamber (Biospherix, Lacona, NY). Control cells were cultured in standard tissue culture conditions (95% air-5% CO<sub>2</sub>).

Statistical analysis. All results were expressed as means ± SE, with the number of experiments performed provided in the figure legends. Results were compared using a one-way ANOVA or Mann-Whitney U test, as appropriate, using GraphPad Prism software (La Jolla, CA). To pinpoint differences between specific groups, Newman-Keuls test was used. Values were considered significantly different when P < 0.05.

RESULTS

CO partially mitigates the effects of hyperoxia-induced lung injury on body weight. Prolonged neonatal exposure to hyperoxia adversely affected somatic growth (Fig. 1A). Hyperoxic exposure resulted in a significantly decreased weight at the end of the exposure period compared with air-exposed littermates. This was true for all experiments under hyperoxic conditions despite rotation of dams. Intermittent exposure to CO at 250 ppm during hyperoxic exposure tended to increase body weight, with the difference reaching statistical significance on day 21 of exposure (P < 0.05).

CO mitigates hypoalveolarization in hyperoxia-exposed neonatal mice. Premature mice are born at the saccular stage of lung development. Sacculi are transformed into alveoli within the first 2 wk after birth by septal thinning and secondary septation. Histological analysis in untreated postnatal 3-day (p3) normoxia-exposed mice revealed markedly underdeveloped lungs at the saccular stage of development (Fig. 1B). After 7–21 days of exposure (postnatal days 10–24), hyperoxic mice exhibited a significant structural delay in alveolar development compared with age-matched normoxia-exposed counterparts. Alveolar simplification in hyperoxia-exposed mice was accompanied by increased collagen deposition in the alveolar walls (not shown). Lungs of mice intermittently exposed to 250 ppm CO for 1 h twice daily were structurally similar to normoxic control mice. Mice exposed to 75% oxygen and CO showed partial mitigation of hypoalveolarization compared with mice exposed to hyperoxia alone. To quantitate these differences, morphometric measurements of the MLI were compared between study groups (Fig. 1C). Compared with air-exposed mice, mean MLI for hyperoxia-exposed mice was significantly increased at 10, 14, and 21 days of exposure. When neonatal pups were exposed to 250 ppm CO in the setting of hyperoxia, we observed a significant decrease in MLI on day 10 of exposure compared with hyperoxia-exposed mice (P < 0.001). Similar decreases in MLI were observed at other time points although the differences were not statistically significant. Together, these data demonstrate that chronic neonatal hyperoxic exposure results in markedly decreased alveolarization, which is mitigated by intermittent CO treatment.

CO suppresses hyperoxia-induced lung leukocyte recruitment. We hypothesize that CO mitigates the effect of hyperoxia on the developing lung via an anti-inflammatory mechanism. We examined the inflammatory cell response in the BAL fluid. The number of BAL polymorphonuclear cells and monocyte/macrophages was significantly increased in hyperoxia-exposed pups at 7 days (Fig. 2A), reaching a peak at 14 days of hyperoxic exposure (Fig. 2B). Macrophages from hyperoxia-exposed mice showed a larger, foamy appearance, indicative of activation (data not shown), concordant with their previously reported appearance (49). CO treatment decreased the number of neutrophils and monocyte/macrophages in the BAL fluid. These findings indicate that CO treatment may confer significant protection against hyperoxia-induced pulmonary inflammation in developing lungs.

To further understand the role of HO-1/CO in neonatal lung injury, we measured lung HO-1 mRNA expression in hyperoxia-exposed mice. Hyperoxia significantly increases HO-1 expression (Fig. 3A). HO-1-null (HO-1<sup>−/−</sup>) mice exposed to hyperoxia showed exaggerated alveolar simplification compared with wild-type mice (Fig. 3B). Exaggerated hypoalveolarization was confirmed by MLI measurements (Fig. 3C). Immunohistochemistry showed that hyperoxia increases HO-1 expression in alveolar macrophages (Fig. 3D). As expected, HO-1 expression was not detectable in hyperoxia-exposed HO-1-null mice. Finally, compared with HO-1<sup>−/−</sup> hyperoxia-exposed mice, HO-1-null mice showed a significant increase in BAL macrophages (Fig. 3E). There was no significant increase in BAL neutrophils (Fig. 3F). Together, these data demonstrate that HO-1 deficiency is sufficient to exacerbate the effects of hyperoxia on the newborn lung.

To further characterize the inflammatory response to hyperoxic exposure and CO treatment, lung cells were subjected to flow cytometry. More than 90% of the isolated whole lung inflammatory cells were CD45-positive cells. First, we examined cells of the monocyte/macrophage lineage. By day 7, the...
Fig. 1. Low-intermittent-dose CO partially mitigates the effects of hyperoxia on body weight and alveolar arrest in neonatal mice. A: group mean body weight at 3, 7, 10, 14, and 21 days of life under conditions of normoxia (n = 167), hyperoxia (n = 106), CO + normoxia (n = 52), and CO + hyperoxia (n = 64). Hyperoxic exposure decreased body weight compared with normoxia. On day 21 of exposure, mice of the hyperoxia group were significantly larger than mice of the hyperoxia group (*P < 0.05, ANOVA). B: representative images of hematoxylin and eosin-stained lungs of neonatal mice exposed to normoxia (21% oxygen), hyperoxia (75% oxygen), intermittent exposure to 250 ppm carbon monoxide plus compressed air for 1 h twice daily (normoxia + CO), or 75% oxygen and 250 ppm CO for 1 h twice daily (hyperoxia + CO). Compared with air-exposed mice, hyperoxia-exposed mice demonstrated increased alveolar size indicative of alveolar development arrest. CO-treated mice exposed to hyperoxia showed attenuated alveolar simplification compared with hyperoxia-exposed mice. CO-treated mice exposed to normoxia showed similar alveolar architecture as normoxia-treated mice. Solid bar scale = 200 μm, and all panels are ×200. C: corresponding group mean linear intercepts of all groups. Data are reported as means ± SE. n = 17–24 per group, *different from hyperoxia, P < 0.05. All data were assessed by 1-way ANOVA.
percentage of cells expressing the monocyte/macrophage cell surface markers F4/80+ and CD11c+ (49) significantly increased under hyperoxic exposure. The total number of F4/80+ and CD11c+ double-positive cells peaked at 14 days of hyperoxic exposure (data for day 14 are shown in Fig. 4A). CO treatment tended to decrease the accumulation of cells infiltrating in the hyperoxic-exposed lungs. On the other hand, hyperoxia-exposed HO-1-null mice showed increased F4/80+ and CD11c+ double-positive macrophages.

Recent studies suggest that, unlike resident alveolar macrophages, freshly recruited macrophages express low levels of CD11c and high levels of CD11b, which is required for their successful migration to inflamed tissues (20, 43). We therefore analyzed CD45-positive lung cells for both CD11c and CD11b (data for day 14 are shown in Fig. 4A). CO treatment tended to decrease the accumulation of cells infiltrating in the hyperoxic-exposed lungs. On the other hand, hyperoxia-exposed HO-1-null mice showed increased F4/80+ and CD11c+ double-positive macrophages.

Fig. 2. Episodic CO treatment suppresses leukocyte trafficking to the lungs of hyperoxia-exposed neonatal mice. A and B: bronchoalveolar lavage fluid (BALF) from 3-day-old pups exposed to normoxia or hyperoxia in the absence or presence of CO for 7 (A) or 14 days (B). Total BALF cells (left), monocyte/macrophages (middle), and neutrophils (right) were measured by Hemavet. n = 12–17 per group, *P < 0.05, ANOVA.

Next, we used flow cytometry to examine lung neutrophils. We employed antibodies against Gr-1 (Ly6C/G) and CD45 to identify infiltrating neutrophils (CD45<sup>high</sup> Gr-1<sup>+</sup>). Following hyperoxic exposure, the number of CD45<sup>high</sup> Gr-1<sup>+</sup> cells increased compared with normoxic littermates, and exogenous CO decreased infiltrating neutrophils at all time points (data from day 14 are shown in Fig. 5A). Immunofluorescent staining confirmed that hyperoxia increased lung Gr-1<sup>+</sup>positive neutrophils compared with normoxic littermates (Fig. 5B, bottom). Exogenous application of CO to hyperoxic neonatal mice attenuated the infiltration of neutrophils. Similarly, CD11b-positive (red), F4/80-positive (green; colocalization, yellow) macrophages were found in the alveolar septae and alveolar spaces of lungs from hyperoxia-exposed mice (Fig. 5B, top). Intermittent CO exposure during hyperoxia significantly decreased macrophage infiltration into the alveolar space.

Exogenous CO administration regulates the expression of pro- and anti-inflammatory cytokines/chemokines under hyperoxic conditions. To determine the cytokines responsible for hyperoxia-induced macrophage/monocyte lung infiltration, we performed a gene array focusing on inflammatory markers. Lung mRNA from day 3 normoxia-exposed mice, day 14 normoxia-exposed mice, day 14 normoxia-exposed mice, day 14 hyperoxia-exposed mice, and day 14 hyperoxia + CO-exposed mice was subjected to a targeted PCR array examining mouse inflammatory cytokines (Fig. 6). Hierarchical clustering revealed a group of 12 genes, which were downregulated during normal development, increased by hyperoxic exposure, and decreased by concomitant CO exposure. This group of genes included Ccl2, Cxcl11, and Iil1b. We therefore examined lungs from HO-1-null mice showed increased lung macrophages, in particular CD11c<sup>low</sup> CD11b<sup>high</sup> cells.
Fig. 3. Heme oxygenase (HO-1) knockout exacerbates hypoalveolarization and increases leukocyte infiltration in hyperoxia-exposed neonatal mice. A: whole lung HO-1 mRNA expression from wild-type mice exposed to either normoxia ($n = 20–26$ mice per group) or hyperoxia ($n = 19–23$ mice per group) for up to 21 days. Hyperoxic exposure increased mRNA expression, as measured by qPCR ($*P < 0.05$, ANOVA). B: 3-day-old HO-1$^{+/+}$ or HO-1-null (HO-1$^{-/-}$) mice were exposed to air or 75% oxygen for 14 days. Hyperoxia-exposed HO-1$^{-/-}$ mice showed alveolar arrest with large airspaces. Hyperoxia-exposed HO-1-null mice showed exaggerated alveolar simplification compared with wild-type mice. C: group mean chord length data ($n = 4–9$ per group, $*P < 0.05$, ANOVA). D: hyperoxia-induced HO-1 expression is observed in lung alveolar macrophages (arrows). Immunohistochemistry was performed with an antibody against murine HO-1 (scale bar = 100 μm). E and F: hyperoxia-induced inflammatory cell influx in neonatal mouse lungs is exacerbated by HO-1 deletion. BALF monocytes (E) and neutrophils (F) were counted 14 days after exposure to room air (21% O$_2$) or hyperoxia (75% O$_2$). Compared with HO-1$^{+/+}$ mice, HO-1-null mice showed a significant increase in monocytes and a trend toward increased neutrophils ($n = 4–7$ mice per group, means ± SE, $*$different from hyperoxia-exposed group, $P < 0.05$, ANOVA).
the mRNA and protein levels of these and other cytokines by qPCR and multiplex immune assay, respectively. Lung samples from 14 days postexposure were studied (Fig. 7, A–F).

Expression of mRNAs encoding the proinflammatory cytokines IL-1β, TNF-α, IL-6, the monocyte chemoattractant CCL-2, and the neutrophil chemoattractants CXCL1 and CXCL2 were significantly greater in the lungs of hyperoxia-treated mice compared with lungs from air-exposed mice. Hyperoxia slightly decreased β-actin copy number on days 14 and 21 of exposure, which may have led to an underestimation of cytokine induction (not shown). Similar increases were observed on the protein level. CO treatment under hyperoxia effectively suppressed all proinflammatory markers measured.

In addition to the examination of proinflammatory cytokines/chemokines, experiments were also performed to determine the expression levels of two “anti-inflammatory” cytokines, IL-10 and IL-13 (Fig. 7, G and H). IL-10 and IL-13 were decreased in the lungs of hyperoxia-treated mice. Concomitantly, the protein levels of both cytokines were also decreased after hyperoxic exposure. CO tended to increase IL-10 and significantly increased IL-13 mRNA and protein levels during both air and hyperoxic exposure.

CCL2 is required for hyperoxia-induced macrophage/monocyte infiltration and hypoalveolarization. On the basis of the strong induction of CCL2 demonstrated by our microarray data, we chose to evaluate in depth the functional consequences of its expression in the development of BPD. CCL2 is a critical monocyte chemoattractant protein, which binds to its cognate ligand CCR2 expressed on monophagocytes, recruiting them to sites of inflammation. To further characterize hyperoxia-induced CCL2 expression, we performed immunofluorescence staining of lung sections for CCL2 and CD68, a macrophage marker (Fig. 8A). Hyperoxia increased the trafficking of macrophage subpopulations into the neonatal lung, and CO treatment attenuated macrophage recruitment. HO-1−/− mice showed increases lung macrophages.

**Fig. 4.** Hyperoxic exposure induces alveolar monocyte/macrophage infiltration into neonatal lungs that is ameliorated by CO-induced treatment. Representative flow cytometric scattergrams of monocyte/macrophage subpopulations within the lungs of wild-type (WT) and HO-1-null mice exposed to normoxia and hyperoxia for 14 days. Wild-type mice were also exposed to normoxia + CO or hyperoxia + CO. Each dot plot is representative of 4 independent experiments. A: CD45+, CD11c+, F4/80+ macrophages. B: gating scheme used in subsequent flow cytometric analysis. C: whole lung live CD45+ cells were analyzed for expression of CD11c and CD11b. D–F: comparison of cell counts for F4/80 and CD11c double-positive macrophages (D), CD11chigh CD11bhigh exudative macrophages (subpopulation B, E), and CD11cint CD11bhigh inflammatory monocytes (subpopulation C, F). Cell counts represent the number of cells per 10⁶ events. Hyperoxia increased the trafficking of macrophage subpopulations into the neonatal lung, and CO treatment attenuated macrophage recruitment. HO-1−/− mice showed increases lung macrophages. n = 4–10 per group, means ± SE, *compared to hyperoxia alone, P < 0.05, ANOVA.
infiltration. CCL2 expression was also increased, primarily in CD68-negative cells of the airway and alveolar epithelium. CO administration decreased hyperoxia-induced epithelial cell CCL2 expression. We tested whether hyperoxia increases respiratory epithelial CCL2 expression in vitro. Murine type II alveolar epithelial cells were cultured in an incubator chamber containing a normoxic or hyperoxic atmosphere in the absence or presence of 250 ppm CO (Fig. 8B). Hyperoxia caused a rapid increase in CCL2 mRNA expression, which was attenuated by CO treatment. Taken together, these data suggest that CO attenuates hyperoxia-induced lung inflammation by decreasing respiratory epithelial cell CCL2 expression.

To test the hypothesis that CCL2 is required for hyperoxia-induced macrophage/monocyte infiltration and hypoalveolarization, wild-type and CCL2-null mice were exposed to air or hyperoxia, as described above. As shown previously, hyperoxia blocked alveolar development, as evidenced by an increase in MLI (Fig. 9, A and B). In contrast, CCL2-null mice showed no increase in mean chord length. Hyperoxia-exposed CCL2-null mice showed a trend toward reduced BAL monocytes and, interestingly, a reduction in BAL neutrophils (Fig. 9, C and D). In CCL2-null mice, flow cytometry showed a reduction in F4/80+ CD11c+ macrophages, CD11c\textsuperscript{high} CD11b\textsuperscript{high} (population B) exudative macrophages, and CD11c\textsuperscript{low} CD11b\textsuperscript{high} (population C) inflammatory monocytes (Fig. 10, A–E).

**DISCUSSION**

In this report, we show for the first time that 1) brief episodic exposure to low-dose CO significantly mitigates the deleterious effects of hyperoxia on alveolar development, 2) low-dose CO inhibits hyperoxia-induced lung neutrophil and macrophage infiltration, and 3) HO-1 deficiency exacerbates hyperoxia-induced hypoalveolarization. Taken together, these studies indicate that, in hyperoxia-exposed neonatal mice, inhalation of the HO-1 product CO suppresses inflammatory cell trafficking, proinflammatory cytokine expression, and alveolar simplification. We also found that CO attenuates respiratory epithelial cell CCL2 expression and that CCL2 is required for hyperoxia-induced alveolar simplification, consistent with the notion that CO works in part by suppressing hyperoxia-induced CCL2.

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**Fig. 5.** Hyperoxic exposure induces neutrophilic infiltration into neonatal lungs that is ameliorated by CO-induced treatment. A and B: high Gr-1+ CD45+ cells, representing infiltrating neutrophils, were measured by flow cytometry. Treatment of hyperoxia-exposed mice with exogenous CO decreased the number of CD45 high Gr-1+ cells. Data from 14-day exposures are shown (A, number of cells per 10⁶ events; B, percentage of CD45+ cells). C: immunofluorescent stains of 14-day lung sections from normoxia-, hyperoxia-, CO + normoxia-, and CO + hyperoxia-exposed mice. Top: neutrophil panels show Gr-1 (green, arrows) and α-actin (red). Bottom: macrophage staining panels include F4/80 (green) and CD11b (red). Colocalization (yellow) indicates CD11b-positive macrophages (arrows). Nuclei were counterstained with DAPI (black). Original magnification is ×200, scale bar = 10 μm. Insets: high-magnification views (×400).
Previous studies have examined the role of neutrophils and macrophages in the pathogenesis of neonatal hyperoxic lung injury. In neonatal rats, hyperoxia induces neutrophil influx and hypoalveolarization, which is attenuated by neutralizing antibodies against the neutrophil chemoattractants CXCL1 (2) or CXCL2 (9). Neutrophilic inflammation is also prevented by a selective chemical antagonist of CXCR2 (55). Similar to our results, neutralizing antibodies against CCL2, a monocyte chemoattractant, also decrease lung hypoalveolarization, as well as lung macrophage and neutrophil counts, in hyperoxia-exposed rats (50). However, the monocyte subpopulations involved have not previously been characterized.

In our study, we extend the above observations by showing that hyperoxia induces the accumulation of different F4/80-positive macrophage subsets into the lung, specifically CD11c<sup>low</sup>CD11b<sup>high</sup> and CD11c<sup>high</sup>CD11b<sup>high</sup> cells. Previously, it was shown that, during influenza infection (29) and bleomycin exposure (47), CD11c<sup>-</sup>CD11b<sup>+</sup> inflammatory monocytes are recruited to the lung and develop into a CD11c<sup>high</sup>CD11b<sup>+</sup> activated macrophage population known as exudative macrophages. On this basis, we conclude that the CD11c<sup>low</sup>CD11b<sup>high</sup> cells (our population C) represent inflammatory monocytes and that CD11c<sup>high</sup>CD11b<sup>high</sup> cells (our population B) represent exudative macrophages, a major source of inflammatory cytokines and chemokines in the lung.

In addition to lung inflammation, hyperoxia increased the size of airspaces in the lung, indicating hypoalveolarization of the developing lung. Murine alveolar development begins on postnatal day 5, and saccular division is completed by the 30th postnatal day (53). This result is in agreement with previous work showing that exposure to supraphysiological levels of oxygen confers hypoalveolarization (18, 40, 41) and is consistent with the notion that oxygen exposure may be a contributing factor in the pathogenesis of BPD.

Next, we examined the effects of 1-h, twice per day CO treatment on lung development and inflammation. CO treat-
ment during hyperoxia significantly reduced hyperoxia-induced hypoalveolarization. In addition, CO decreased the number of exudative macrophages and inflammatory monocytes in the lung. Conversely, compared with wild-type mice, HO-1-null mice exposed to hyperoxia showed increased alveolar simplification and lung infiltration with CD11b(H11001)

In our previous work, we demonstrated that HO-1-deficient mice have significantly decreased survival upon ischemic lung injury and that CO can rescue these mice (16). Recent reports have demonstrated the cytoprotective role of HO-1/CO in in vivo models of acute lung injury. Intravenous hemoglobin, a potent inducer of HO-1 (a major heme-degradative enzyme), prevented lung injury in a rat model of sepsis (37). In a separate report, intratracheal administration of hemoglobin protected rats from hyperoxia-induced lung injury (45). It was subsequently shown that adenoviral gene transfer of HO-1 conferred protection against hyperoxic-induced lung injury (35). Inhaled CO at low concentrations (50–500 ppm) protected against hyperoxia-induced lung injury in adult rats (36). Recently, the effects of HO-1 overexpression and CO admin-
Fig. 8. Hyperoxia exposure is associated with an increase in respiratory epithelial cell CCL2 expression. Lung sections were stained for CCL2 (red) or CD68 (green); colocalization appears yellow. Compared with normoxia-exposed mice, hyperoxia-exposed mice show an increase in CCL2 expression primarily in the epithelium as well as an increase in CD68-positive macrophages. Episodic CO treatment the presence of hyperoxic exposure suppressed CCL2 expression. Lung sections from 7, 10, 14, and 21 days of exposure are shown (line segment = 50 μm, results are typical of 3 individual experiments).

B: murine type II alveolar epithelial cells were exposed to 250 ppm CO or room air in the absence or presence of high oxygen tension (≥95% O2) for a duration of 2, 6, or 12 h. CCL2 mRNA expression was increased by hyperoxia and attenuated by CO. Group mean data for CCL2 mRNA expression are presented (means ± SE, n = 3, *P < 0.05, ANOVA).
Fig. 9. CCL2 deletion decreases hyperoxia-induced alveolar simplification and inflammatory cell trafficking. A: representative lung sections from CCL2+/+ and CCL2-null mice exposed to normoxia or hyperoxia for 14 days were stained with hematoxylin and eosin. B: alveolar size, as measured by mean chord length, confirmed features noted on lung histology (n = 15–18 mice per group, *P <0.05, ANOVA). C: BAL cells were measured by Hemavet. Compared with hyperoxia-exposed CCL2+/+ mice, CCL2-null mice tended to show decreased neutrophils (C) and macrophages (D). (n = 5–8 mice per group, means ± SE, *different from hyperoxia-exposed group, *P <0.05, ANOVA).

istration (250 ppm for 1 h daily) were examined in a model of neonatal hyperoxic lung injury (15). HO-1 overexpression and CO each attenuated hyperoxic-induced arterial remodeling, right ventricular hypertrophy pulmonary edema, and hemosiderosis, while increasing blood vessel number. HO-1 overexpression and CO administration had modest effects on alveolar architecture and BAL, but the mechanisms driving inflammatory changes were not further explored.

To determine the mechanism of hyperoxia- and CO-mediated changes in lung inflammation, we examined the mRNA and protein levels of candidate cytokines by gene array, followed by qPCR and multiplex immune assay. Increases in lung leukocyte subpopulations with hyperoxia and mitigation of this response by CO were reflected in lung cytokine mRNA and protein levels. Lungs of hyperoxia-exposed mice showed increased levels of CCL2 mRNA and protein, which were reduced by CO treatment. We found a similar pattern for the type I cytokines IL-1β, TNF-α, CXCL1, and CXCL2. CCL2 is a chemoattractant for exudative macrophages (29). We therefore tested the hypothesis that respiratory epithelial cells produce CCL2 in response to hyperoxic exposure, leading to monocyte and macrophage infiltration of the lung. First, we found that hyperoxia increased epithelial CCL2 protein expression in vivo and that CO attenuated this response. Second, we exposed murine type II alveolar epithelial cells to hyperoxia in vitro. We found that hyperoxia increased murine type II alveolar epithelial cell CCL2 mRNA expression and that expression was inhibited by CO treatment. Finally, we exposed CCL2-null mice to hyperoxia in vivo and found that these mice were protected from alveolar simplification. In addition, the lungs of hyperoxia-exposed CCL2-null mice showed significantly reduced exudative macrophages and inflammatory monocytes, reproducing the effects of exogenous CO. Taken together, these data are consistent with the notion that inhalation of the HO-1 product CO suppresses inflammatory cell trafficking, proinflammatory cytokine expression, and alveolar simplification in part by attenuating CCL2 expression. However, other proinflammatory cytokines may also be involved. It is also possible that, in addition to targeting respiratory epithelial cells, CO has a direct inhibitory effect on leukocyte cytokine expression.

The precise mechanisms by which inflammation inhibits alveolar development are not completely known. Using a fetal lung explant model, it was recently shown that IL-1β-producing tissue macrophages mediate the inflammatory response to lipopolysaccharide and that macrophage activation inhibits airway morphogenesis (5). Conversely, macrophage depletion protected airway branching. Furthermore, tissue-specific macrophage activation induced by overexpression of IkB kinase inhibited lung development in vivo. We propose that CO protects against hyperoxia-induced alveolar simplification by blocking macrophage infiltration and activation. However, it is also possible that the neutrophilic inflammation plays a role in alveolar arrest. Both CO and the CCL2 knockout reduced neutrophil as well as macrophage infiltration, and recent studies have shown CD11b/CD11c-positive macrophages to be an important source of CXCL1 and CXCL2 (1, 39). Also, as noted above, treatment of hyperoxia-exposed neonatal lungs with antibodies or antagonists against neutrophil chemokines also prevents lung inflammation and alveolar simplification (2, 9, 55). Finally, it is also conceivable that hyperoxia retards the
growth of blood vessels in the lung needed for alveolar development (4, 26, 46) and that CO promotes angiogenesis (6, 28).

In summary, we have provided a detailed in vivo analysis of lung inflammation and development after hyperoxic exposure in neonatal mice. Our studies demonstrate a direct link between CO, the CCL2-dependent control of leukocyte migration into hyperoxic lung tissue, and alveolar growth. Inhibition of inflammatory cell infiltration by either CO or CCL2 gene dele-

Fig. 10. CCL2 deletion decreases alveolar monocyte/macrophage infiltration into hyperoxia-exposed neonatal lungs. Representative flow cytometric scattergrams of monocyte/macrophage subpopulations within the lungs of wild-type and CCL2-null mice exposed to normoxia and hyperoxia for 14 days. Each dot plot is representative of 4 independent experiments. A: CD45+, CD11c+, F4/80+ macrophages. B: gating scheme used for CD11c and CD11c staining was explained in Fig. 4 and is briefly shown here. Each dot plot is again representative of 4 independent experiments. Comparison of cell counts for F4/80 and CD11c double-positive macrophages (C), CD11c-high, CD11b-high exudative macrophages (subpopulation B, D), and CD11c-low,CD11b-high inflammatory monocytes (subpopulation C, E). Hyperoxia increased the trafficking of macrophage subpopulations into the neonatal lung, and CCL2 deletion attenuated macrophage recruitment. (n = 8–14 mice per group, means ± SE, *different from hyperoxic-exposed group, P < 0.05, ANOVA).
tion was associated with the attenuated alveolar simplification. Improved understanding of the mechanisms regulating macrophage recruitment into the developing lung could lead to new insights into the pathogenesis of BPD. Finally, these data suggest that additional studies exploring low-dose CO as a possible therapeutic option in neonatal lung injury are warranted.

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DISCLOSURES

Dr. Pinsky has an equity interest in Proterris, which is developing inhaled CO for clinical use, and potential patent-related royalties from Columbia University. No other conflicts of interest, financial or otherwise, are declared.

AUTHOR CONTRIBUTIONS


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