Biochemical Pharmacology, Vol. 56, pp. 259–268, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00138-5

Dexamethasone Enhancement of Hyperoxic Lung Inflammation in Rats Independent of Adhesion Molecule Expression

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ABSTRACT. Infants and adults on oxygen often are treated with glucocorticoids in an attempt to reduce lung inflammatory injury. However, glucocorticoids hasten the development of hyperoxic lung injury in some animal models. The purpose of this study was to test the hypothesis that dexamethasone alters the lung inflammatory responses to hyperoxia exposure. We studied male Sprague–Dawley rats, placing them in >95% oxygen immediately after administration of 0, 0.1, 1, or 10 mg/kg of dexamethasone. At 0, 24, or 48 hr of exposure to hyperoxia, extravascular lung water contents were measured, and lung inflammatory responses were assessed by lung myeloperoxidase activities, lung neutrophil counts, and lung expression of E-Selectin and intercellular adhesions molecule-1 (ICAM-1). Dexamethasone, independent of exposure to hyperoxia, led to marked increases in lung neutrophil counts, without increases in lung myeloperoxidase activities or increases in the expression of the adhesion molecules. Hyperoxia exposure also enhanced lung neutrophil accumulation, and extravascular lung water increased earlier in animals exposed to hyperoxia and dexamethasone than in those exposed to hyperoxia alone. In conclusion, the increase in lung neutrophils in dexamethasone-treated rats without enhanced expression of E-Selectin or intracellur adhesions molecule-1 suggests that dexamethasone leads to lung neutrophil accumulation by its effect on neutrophils. The more rapid development of hyperoxic lung injury associated with earlier lung neutrophil accumulation suggests that dexamethasone-induced lung neutrophil sequestration primes the lung for the development of hyperoxic lung injury and supports further the conclusion that lung inflammation contributes significantly to the development of hyperoxic lung injury. BIOCHEM PHARMACOL **56**;2:259–268, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. lung injury, oxygen toxicity, adhesion molecule, lung inflammation, animal model

Many prematurely born infants require treatment with supplemental oxygen; however, this exposure to hyperoxia may cause acute lung injury, which in turn, may contribute to the development of chronic lung diseases and their associated mortality and morbidity [1, 2]. The administration of dexamethasone, an anti-inflammatory glucocorticoid, is a common clinical strategy in the care of patients with lung disease. The reported clinical benefits of dexamethasone in infants include an induction of the maturation of the surfactant and glutathione systems in fetal lungs [3–5], and an improvement of pulmonary function, with findings that were interpreted as indicating reductions in lung inflammation [6–8]. Based on the apparent role of hyperoxic lung injury in the development of chronic lung disease in infants as well as adults, mechanisms for the development of hyperoxic lung injury have been investigated extensively in adult and newborn animals, and the effect of dexamethasone on the progression of hyperoxic lung injury has been studied [9–12].

The reported effects of dexamethasone administration on the consequences of exposure of experimental animals to hyperoxia have varied dramatically, with either an increased tolerance to hyperoxic exposure in newborn animals [9], or an augmentation of hyperoxic lung damage in mature animals [10, 11]. In addition, dexamethasone can be either detrimental or protective toward hyperoxic lung injury, depending on the dosing schedule of dexamethasone administration relative to exposure to hyperoxia [10–12]. The enhanced susceptibility to oxygen-induced lung injury observed with early administration of dexamethasone has been associated with decreased lung antioxidant functions, while the diminished susceptibility to oxygen-induced lung injury observed with late administration of dexamethasone has been associated with attenuation of neutrophil accumulation [11]. However, the mechanisms of action of dexamethasone remain ill-defined, and very little attention has been paid to the effect of dexamethasone on the progression of hyperoxia-induced lung inflammation or on

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Received 8 October 1997; accepted 4 March 1998.

the mechanisms by which dexamethasone may alter the course of lung inflammation.

Morphological studies of lungs of animals exposed to hyperoxia have shown that neutrophil accumulation occurs, and this inflammatory response may contribute to injury of the capillary endothelium and alveolar epithelium [13–16]. Some studies of the effects of hyperoxia have noted an attenuation of hyperoxia-induced lung injury in animals in which neutrophil pools were depleted [17], whereas other studies report no effect of neutrophil depletion [18]. More recent animal studies on the contributions of neutrophil accumulation to oxygen-induced lung injury have focused on possible interventions to the process of adhesion to prevent neutrophil accumulation, activation, and tissue damage. Levels of E-Selectin and ICAM-1,§ two pro-inflammatory adhesion molecules that when induced may be crucial for neutrophil adhesion and migration [19, 20], increase in the lungs of animals exposed to hyperoxia [21–24]. Attenuation of hyperoxia-induced lung injury has been observed in animals treated with monoclonal antibodies that target ICAM-1 or the leukocyte β_2 integrin molecule that binds to ICAM-1 [25, 26]. However, mice in which basal expression of ICAM-1 was diminished through genetic homologous recombination were more susceptible to hyperoxia-induced lung inflammation and injury [27]. These complexities suggest that the inflammatory response that normally accompanies hyperoxic lung injury is mediated by multiple mechanisms that may be compensatory as well as redundant. Some components of the inflammatory response probably are injurious, although other responses may be beneficial. Despite years of active investigation, the mechanisms regulating neutrophil accumulation and the specific contributions to hyperoxic lung injury remain largely undefined.

The present study focuses on the effect of a single dose of dexamethasone administered to adult rats on lung inflammatory responses in hyperoxia. We, therefore, tested the hypotheses that dexamethasone alters hyperoxia-induced lung inflammation, and that alterations in lung inflammatory responses were associated with alterations in pulmonary expression of E-Selectin and ICAM-1.

MATERIALS AND METHODS

Materials

We purchased zinc formalin (ANATECH), 0.5% bovine serum albumin (Life Technologies), goat serum and avidin–biotin complex (Vector Laboratories), Hemo–De (Fisher Scientific), and rabbit anti-human myeloperoxidase antibody (DAKO Corp.). Additional materials included monobromobimane (Calbiochem), powdered heparin (Fisher Scientific), and soda lime (Sodasorb; Dewey & Almy Chemical Division, Grace & Co.). All other chemicals and reagents were purchased from the Sigma Chemical Co. Male Sprague–Dawley rats were obtained from Harlan Industries. Phosphor Screens and the ImageQuant Analyzer for quantification of blot signals were from Molecular Dynamics. A computer-driven CODE-ON system (Instrumentation Laboratories), Optimas imaging software (Optimas Corp.), and an Olympus VANOX microscope (Optimas Corp.), were used for immunohistochemical analyses. A Technicon H-1 analyzer (Technicon Instruments Corp.), was used to determine circulating neutrophil counts.

Experimental Protocol

The study was approved by the Baylor College of Medicine Animal Protocol Review Committee for the care of animals, and the care and handling of the animals were in accord with National Institutes of Health guidelines for ethical animal research. Male Sprague–Dawley rats weighing 180–200 g were studied. Hyperoxia-exposed rats were placed in a Plexiglas-covered chamber that allowed them free access to food and water, while exposing them to $>$ 95% oxygen continuously by administration of pure oxygen at 5 L/min. Excess carbon dioxide was removed with soda lime. A second group of rats was maintained in room air. Immediately prior to their exposures, rats were treated with either 0.1, 1, or 10 mg/kg of dexamethasone or vehicle (0.2 mL of ethanol) intraperitoneally. The dose of dexamethasone to be administered to the animals was based on previous reports of dexamethasone administration to experimental animals varying from 0.4 to 10 mg/kg [10, 12]. Therefore, in these studies we sought to determine whether a dose–response effect could be detected in response to the dexamethasone administration, in combination with hyperoxia exposure. During the exposure of the animals to hyperoxia, the oxygen concentrations in the chamber were measured twice daily, and the animals were monitored for signs of illness or any unexpected deterioration in their conditions.

At either 24, 48, or 60 hr of hyperoxia-exposure, the rats were anesthetized with 200 mg/kg of i.p. pentobarbital, a thoracotomy was performed to expose the intrathoracic contents, and whole blood samples were obtained via cardiac puncture with a syringe containing powdered heparin. A 16-gauge catheter was placed in the trachea and secured with a suture. The right lung was isolated by placing a suture around the hilum, followed by excision distal to the suture, and the excised tissue was snap-frozen in liquid nitrogen. Right lung samples were used to determine extravascular lung water contents, myeloperoxidase activities, and expression of ICAM-1 and E-Selectin. The left lungs were fully inflated with zinc formalin, excised, fixed, and embedded in paraffin for subsequent immunohistochemical analyses. Air-breathing rats served as controls in all studies, and samples were obtained and analyzed in parallel with those obtained from hyperoxia-exposed rats. It should be noted that animals exposed to dexamethasone or vehicle and exposed to room air were killed 24 and 48 hr after dexamethasone or vehicle dosing, and the data from

§*Abbreviation:* ICAM-1, intercellular adhesion molecule-1.

both time points were evaluated and presented as 0 hr of exposure to hyperoxia. In our initial study design, we included a 60-hr time point, but we experienced unexpected mortality between 48 and 60 hr of exposure to hyperoxia in animals given dexamethasone. In these initial studies in which we measured extravascular lung water, lung myeloperoxidase activities, and adhesion molecule expressions, we included the 60-hr time point in the appropriate figures in the vehicle-treated rats to demonstrate what happened in the control group. There was no 60-hr time points provided for animals exposed to hyperoxia and dexamethasone.

Extravascular Lung Water

As one measure of lung injury, extravascular lung water contents were determined by a modification [28] of the wet-to-dry determination described by Pearce *et al.* [29].

Lung Tissue Myeloperoxidase Activities

Lung tissue myeloperoxidase activities were measured as reflections of pulmonary neutrophil accumulation, using techniques described by Goldblum and co-workers [30]. Briefly, lungs were homogenized in 0.5% hexadecyltrimethylammonium bromide in 50 mM of phosphate buffer (pH = 6.0, approximately 5.0 mL of hexadecyltrimethylammonium bromide/g of tissue), followed by sonication, three freeze–thaw cycles, and centrifugation (3000 *g*, 30 min) at 4°. The myeloperoxidase activities were determined in the sample supernatants by measuring the rate of change in A_{460} resulting from oxidation of *o*-dianisidine in the presence of added H_2O_2. Myeloperoxidase activities were expressed as units/lung.

Lung ICAM-1 and E-Selectin Expression

Protein concentrations were determined on the lung samples using the technique described by Bradford [31]. Details of the western blotting techniques used for determination of lung tissue expression of ICAM-1 and E-Selectin in rats have been described previously [22]. For ICAM-1 detection, 20 μg of protein were electrophoresed on 10% acrylamide denaturing gels, and ICAM-1 levels were evaluated using the 1A29 mouse anti-rat monoclonal antibody. For E-Selectin determinations, gels and samples were prepared similarly except that 2-mercaptoethanol was omitted. E-Selectin was detected using the CL-37 mouse anti-human monoclonal antibody, which is cross-reactive with rat E-Selectin. Signal intensities for ICAM-1 and E-Selectin were quantified with a Phosphor Screen and the ImageQuant Analyzer software.

Lung Immunohistochemistry for Myeloperoxidase

Immunohistochemical analyses were performed on sections using a computer-driven CODE-ON system. Briefly, lung tissue sections were deparaffinized with a mixture of Hemo-De and xylene (3:1, v/v) and rehydrated in PBS buffer (pH 7.3) containing 0.1% Tween 20. All reagents were prepared with a diluent that consisted of the PBS buffer/Tween supplemented with 0.5% bovine serum albumin. To minimize background staining, sections were blocked with normal goat serum at room temperature for 20 min prior to application of the primary antibody. Blocking serum was removed, a rabbit anti-human myeloperoxidase antibody was applied at a concentration of 1:3200, and sections were allowed to incubate in a moist chamber at room temperature overnight. The antibody used has been shown in a study by Pinkus and Pinkus [32] to be specific for granulocytes. Subsequently, sections were rinsed in the PBS/Tween buffer, and then were incubated at room temperature for 45 min with a biotinylated secondary antibody. Endogenous peroxidase activities were quenched with a combination of methanol and hydrogen peroxide. It was important to quench background peroxidase activity because the chromogenic reaction for immunoreactive myeloperoxidase is dependent on peroxidase conjugated to avidin in combination with hydrogen peroxide and diaminobenzidine. Sections were washed with the PBS/Tween buffer and incubated with avidin–biotin complex at room temperature for 45 min. Sites of antigen expression were visualized by incubating tissue sections for 5 min with a chromagenic solution consisting of diaminobenzidine supplemented with 1% nickel chloride for signal enhancement, after which the sections were counterstained with eosin and mounted.

Neutrophil Counting with Computer-assisted Image Analysis

Analyses were performed using Optimas imaging software on a Gateway 486/33 personal computer and a Summagraphics digitizing tablet. A color video camera was attached to an Olympus VANOX microscope that utilized a 20X objective lens with a 6.7X photo eyepiece. Live microscopic images were captured in a 24 bit true color frame grabber buffer, and the frozen images were displayed and analyzed on twin Sony monitors. Prior to image analysis, the system was calibrated with a stage micrometer to provide data in millimeters. The calibration was saved to the system's configuration file.

An interactive macro was written to automate data acquisition and export the data to a Microsoft EXCEL spreadsheet for analysis. The macro employed the software's capability of thresholding on user-defined cellular or histologic structures, based on the structure's gray scale value, which created screen objects that differentiated foreground from background. Alveolar areas (mm^2) and alveolar lengths (mm) were determined after setting threshold values to ensure that the areas bounded by alveoli were foreground objects. For area determination, real number values were extracted from the area screen objects in calibrated units squared. The perimeter that bounds the

area is a real number that is extracted from the area screen object, which gives the boundary length in calibrated units. The macro also tallied the number of screen objects created, and the total was exported. Using the macro, the user was able to edit luminal structures such as vessels, or large airways, to ensure that only alveolar measurements were included for analysis.

Neutrophils were counted automatically by thresholding on the relatively low gray scale value of immunohistochemically positive cells. As above, the macro provided the user with editing options to delete false positives and to add cells that were clearly immunoreactive. Neutrophil counts were expressed relative to alveolar area.

The number of neutrophils present in each of ten random high power fields were counted in each section, and the alveolar surface areas of the regions assessed were measured. Neutrophils were only counted in fields in which the alveolar space was greater than or equal to 60% of the area of the field of interest so that neutrophil counts would be determined in areas with comparable levels of inflation. The mean number of neutrophils per alveolar surface area was determined for each animal.

Myeloperoxidase Activity/Neutrophil

White blood cell counts were obtained from the whole blood samples and the differential counts were determined using a Technicon H-1 analyzer, which was programmed to analyze rat whole blood samples. The myeloperoxidase activities were assayed spectrophotometrically by the method described by Christensen and Rothstein [33]. Briefly, 50 μL of whole blood was added to 2 mL of Hanks' balanced salt solution. Erythrocytes were lysed using 6 mL of distilled water for 2 min followed by the addition of 2 mL of 3.5% NaCl. The resulting solution was centrifuged at 1200 *g* for 5 min, and 0.5 mL of 0.5% hexadecyltrimethylammonium bromide was added to the supernatants. After adding hexadecyltrimethylammonium bromide, 0.1 mL of the solution was added to 2.9 mL of 50 mM of phosphate buffer, pH 6.0, containing 0.167 mg/mL of *o*-dianiside and 0.0005% hydrogen peroxide, and the rates of change of absorbance at 460 nm were measured. The average myeloperoxidase activities per neutrophil were calculated from the measured neutrophil counts and the myeloperoxidase activities in the samples of whole blood.

Statistics

All data are expressed as means ± SEM. Two-way ANOVA was used to assess the effects of treatment and duration of hyperoxic exposure, and possible interactions between these two variables. When ANOVA detected a significant treatment effect, modified *t*-tests were used to determine differences at each duration of exposure. When there was a significant effect of hyperoxia exposure, a one-way ANOVA with a post-hoc Newman–Keuls test was used to determine differences in hyperoxia-exposure time within a given treatment group. The statistical analyses were carried out on SPSS for windows (SPSS version 6.0, Inc.). Statistical significance was attributed to $P < 0.05$.

RESULTS

In our previous studies of hyperoxic lung injury with adult male Sprague–Dawley rats, marked lung inflammation and injury were observed between 48 and 60 hr of exposure. Therefore, our studies were designed initially to expose animals to hyperoxia for up to 60 hr. In our initial experiment, we determined the effect of dexamethasone administration on extravascular lung water (lung injury), lung myeloperoxidase activities (lung inflammation), and adhesion molecule expressions in hyperoxic animals through 60 hr of exposure to hyperoxia. However, in the initial study, three of the four rats given dexamethasone and exposed to hyperoxia died between 48 and 60 hr. As a result of this mortality, the 60-hr time point was eliminated in subsequent studies. In those measurements in which a 60-hr time point was available in vehicle-treated control rats, we have included the data to provide a perspective of the time course in control animals. The figures in which a 60-hr time point in control animals is included are extravascular lung water (see Fig. 1), lung myeloperoxidase activities (see Fig. 2A), and adhesion molecule expressions (see Fig. 4). Although no further mortality was encountered, we consistently observed higher levels of respiratory distress, as evidenced by tachypnea and intercostal retractions, in the dexamethasone-treated rats than in the vehicle-treated rats exposed to hyperoxia. In rats exposed to hyperoxia for up to 48 hr, there were no instances in which early killing was necessary due to unexpected deterioration. The observed responses in all studies were similar in the dexamethasone-treated rats, regardless of whether the rats received 0.1, 1, or 10 mg/kg of dexamethasone. The data presented in this manuscript are from the 1 mg/kg dosage.

Lung injury, as measured by the accumulation of extravascular lung water, characteristically is seen late in the course of exposure to hyperoxia [15, 16]. The extravascular lung water contents following exposure to hyperoxia increased in both the dexamethasone-treated and vehicle-treated rats (Fig. 1). Increases in extravascular lung water contents were observed in the dexamethasone-treated rats after 48 hr, whereas the extravascular lung water contents increased only after 60 hr of exposure to hyperoxia in the vehicle-treated rats.

The shift in the onset of hyperoxic lung injury to an earlier time point in the dexamethasone-treated rats was associated with a similar change in the onset of lung inflammation, which was observed uniformly prior to the accumulation of extravascular lung water. Lung inflammation, as measured by increases in lung myeloperoxidase activities, was observed after 24 hr of exposure to hyperoxia in the dexamethasone-treated animals, rather than after 48 hr, as was observed in the vehicle-treated animals (Fig.

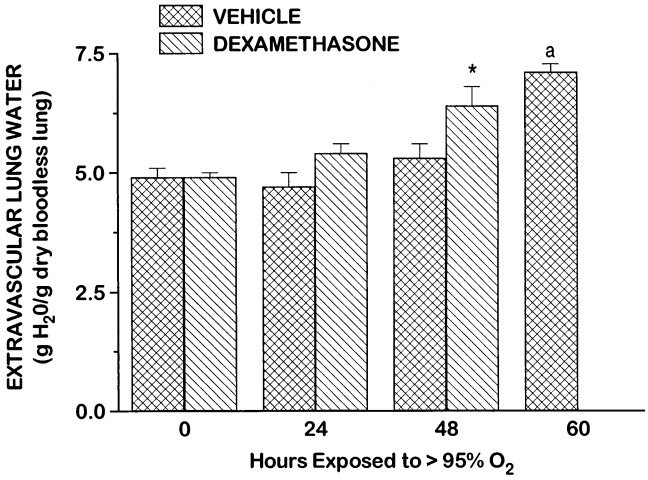

FIG. 1. Effects of dexamethasone administration and exposure to hyperoxia on extravascular lung water contents. Rats were given dexamethasone or vehicle and exposed immediately to >95% oxygen or room air (0 hr exposure to hyperoxia). At the times of exposure to hyperoxia indicated, extravascular lung water contents were determined. In an initial experiment, 3 of 4 animals treated with dexamethasone and exposed to hyperoxia did not survive for 60 hr. In addition, 6/6 rats given dexamethasone and exposed to hyperoxia had pleural effusions, while 0/6 rats given vehicle and exposed to hyperoxia had pleural effusions at 48 hr. Data are expressed as means ± SEM, N = 6–9 in each group. As determined by two-way ANOVA, extravascular lung water contents were increased by both hyperoxia-exposure and dexamethasone treatment, and there was no interaction of the two parameters. Key: (⋆) $P < 0.05$, different from saline-treated animals similarly exposed to hyperoxia; and (a) $P < 0.05$, different from saline-treated animals exposed to 0, 24, or 48 hr of hyperoxia.

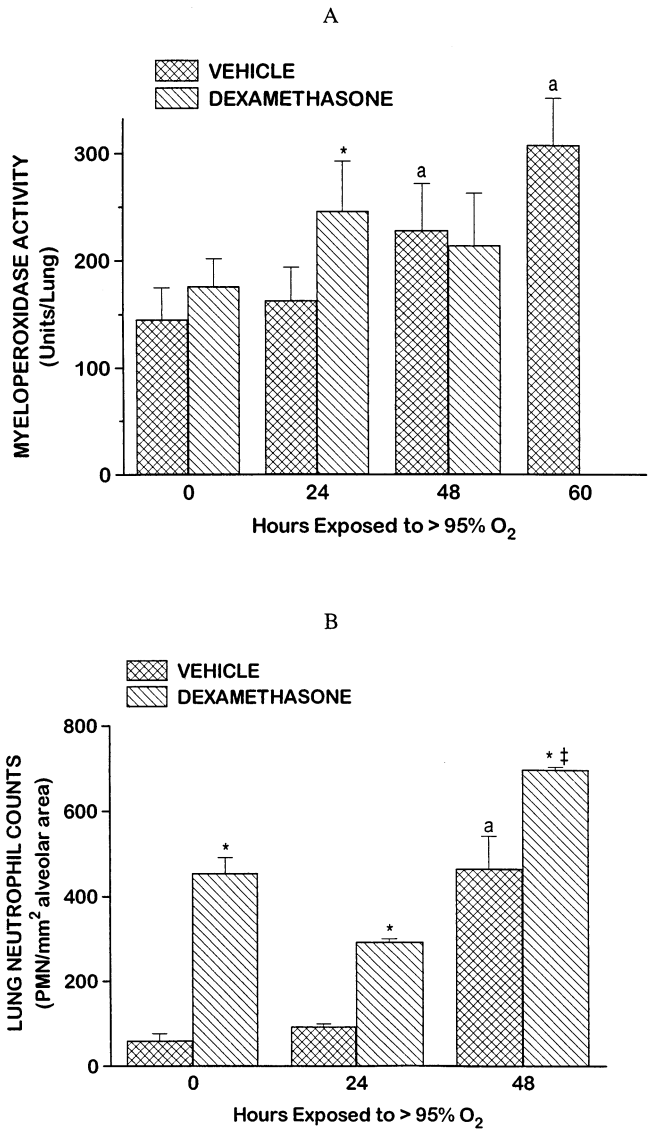

FIG. 2. Effects of dexamethasone administration and exposure to hyperoxia on lung myeloperoxidase activities and lung neutrophil counts. Rats were given dexamethasone or vehicle and exposed immediately to >95% oxygen or room air (0 hr exposure to hyperoxia). At 0, 24, or 48 hr of exposure to hyperoxia, lung myeloperoxidase activities were measured in homogenates of the right lungs (A) and lung neutrophil counts were determined in the left lungs (B). Data are expressed as means ± SEM, N = 4–6 in each group. Two-way ANOVA indicated that dexamethasone and hyperoxia exposure increased lung myeloperoxidase activities (A) and increased lung neutrophil counts (B), and there was no interaction. PMN = polymorphonuclear leukocytes. Key: (⋆) $P < 0.05$, different from saline-treated animals similarly exposed to hyperoxia; (a) $P < 0.05$, different from saline-treated animals at 0 and 24 hr of hyperoxia exposure; and (‡) $P < 0.05$, different-from dexamethasone-treated animals at 0 and 24 hr of hyperoxia exposure.

2A). Exposure to hyperoxia increased the number of neutrophils within the lungs of both the dexamethasone-treated and vehicle-treated rats by 48 hr (Fig. 2B). The most striking increase in the number of neutrophils per lung was observed in the dexamethasone-treated rats not exposed to hyperoxia. Dexamethasone alone led to lung neutrophil counts that were roughly eight times those observed in vehicle-treated air-breathing animals (Fig. 2B), although lung myeloperoxidase activities were not increased in the dexamethasone-treated air-breathing animals (Fig. 2A).

The effects of exposure to hyperoxia and dexamethasone on lung inflammation and injury also were observed by immunohistochemistry of lung sections (Fig. 3). Panels A and B of Fig. 3 are representative lung sections in which the effects of dexamethasone administration are compared with vehicle administration in the lungs of animals maintained in room air. The effects of exposure to hyperoxia following administration of dexamethasone or vehicle are illustrated in Fig. 3, panels C and D, respectively. The administration of dexamethasone resulted in higher neutrophil counts than were observed in the respective vehicle-treated animals, both in the air-breathing controls and in the animals exposed to hyperoxia (Fig. 2B).

To examine further the mechanisms of dexamethasone's effects on lung inflammatory responses to hyperoxia, we assessed lung tissue expression of the adhesion molecules E-Selectin and ICAM-1. Lung E-Selectin (Fig. 4A) and ICAM-1 (Fig. 4B) expressions increased in the dexamethasone-treated animals after 48 hr of hyperoxia, rather than after 60 hr, as was observed in the vehicle-treated animals.

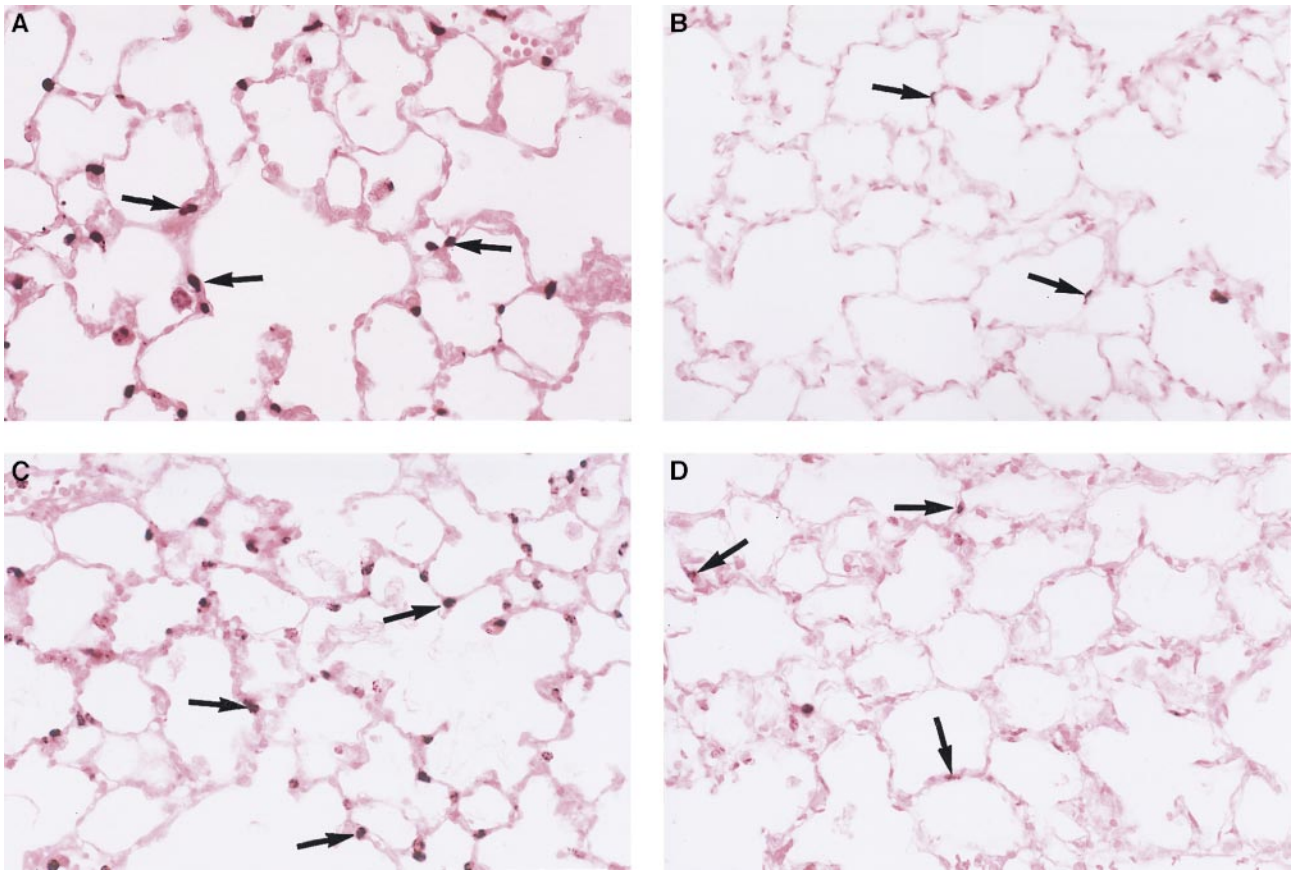

FIG. 3. Representative lung sections for myeloperoxidase immunohistochemistry from rats exposed to room air or 48 hr of hyperoxia, after administration of dexamethasone or vehicle. Paraffin-embedded tissues were sectioned, mounted, and incubated with anti-myeloperoxidase antibody and additional reagents, as described in Materials and Methods, to show myeloperoxidase-staining cells to be purple against a pink eosin counterstain. In the lungs of rats treated with dexamethasone and maintained in room air, there was a remarkable accumulation of myeloperoxidase-reactive cells in alveoli (A, see arrows for representative myeloperoxidase-reactive cells). A similar inflammatory response was not observed in the lungs of rats given vehicle and left in room air (B, see arrows for representative myeloperoxidase-reactive cells). After 48 hr of exposure to hyperoxia, the lungs of rats treated with dexamethasone (C, see arrows for representative myeloperoxidase cells) or vehicle (D, see arrows for representative myeloperoxidase-reactive cells) showed accumulation of myeloperoxidase-reactive cells, although the rats treated with dexamethasone showed greater sequestration of reactive cells. In addition, note that neutrophils accumulating in the lungs of dexamethasone-treated animals are spherical in shape (arrows in A and C), whereas neutrophils accumulating in animals exposed to hyperoxia and vehicle appear to be more deformed (arrows in D). The accumulation of debris in the air spaces of animals exposed to hyperoxia (C and D) also indicates lung injury.

Circulating neutrophil counts increased late in the course of exposure to hyperoxia, and were higher in animals given dexamethasone than in the vehicle-treated rats (Fig. 5A). Interestingly, the mean myeloperoxidase activities per neutrophil within the circulation were lower in air-breathing rats treated with dexamethasone than in vehicle-treated rats. Myeloperoxidase activities of neutrophils also were lower after 48 hr of exposure to hyperoxia in the dexamethasone-treated rats than in vehicle-treated rats (Fig. 5B).

DISCUSSION

The contributions of neutrophil accumulation and actions in hyperoxic lung injury remain controversial. Subtle evidence of lung injury precedes lung neutrophil accumulation [14], suggesting that lung neutrophil accumulation is not the most proximal effector of injury. Rapid deterioration in lung function is associated temporally with lung neutrophil accumulation, although the cause-and-effect relationship between neutrophil accumulation and acceleration of lung injury is still open to debate. Because early glucocorticoid administration hastens the development of hyperoxic lung injury [10, 11] and in view of the reported anti-inflammatory effects of glucocorticoids, a dissociation of the normal temporal relationship between lung injury and lung neutrophil accumulation might have been expected in the present studies, and such a dissociation would have lessened the enthusiasm for a significant role for lung inflammation in the pathogenesis of hyperoxic lung injury. The enhanced lung neutrophil accumulation that we observed with dexamethasone administration was somewhat surprising based on the known anti-inflammatory effects of glucocorticoids. This pro-inflammatory effect of dexamethasone in the lung is a new and important finding in that glucocorticoids are often administered to patients with significant cardiopul-

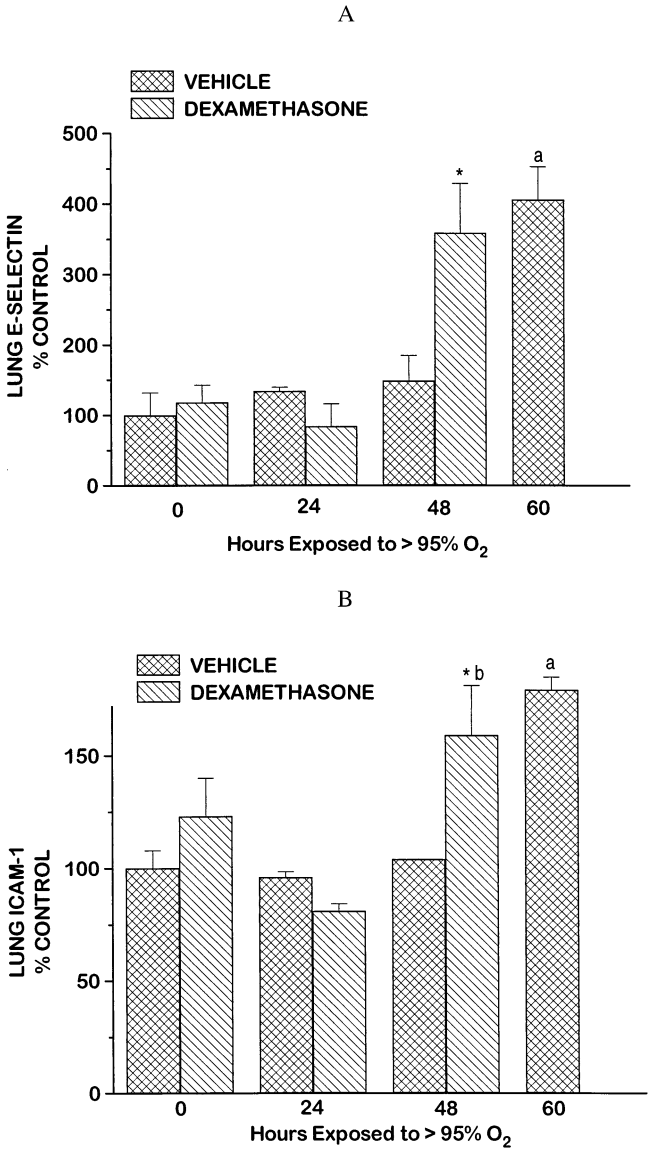

FIG. 4. Effects of dexamethasone administration and exposure to hyperoxia on E-Selectin and ICAM-1 expression in the lung. Rats were given dexamethasone or vehicle and exposed immediately to >95% oxygen or room air (0 hr exposure to hyperoxia). At 0, 24, 48, or 60 hr of hyperoxia exposure, lung E-Selectin (A) and ICAM-1 (B) expressions were determined by Western blotting, with subsequent quantification. In panel A, data are expressed as means ± SEM, N = 4 in each group. In panel B, data are expressed as means ± SD; N = 4 in each group except for the vehicle 48-hr column where the mean of 2 values is given. As determined by two-way ANOVA, dexamethasone and hyperoxia exposure led to increases in lung E-Selectin (A) and ICAM-1 (B) expressions, and there was no interaction. Key: (⋆) $P < 0.05$, different from saline-treated animals similarly exposed to hyperoxia; (a) $P < 0.05$, different from saline-treated animals at 0, 24, and 48 hr of hyperoxia exposure; and (b) $P < 0.05$, different from dexamethasone-treated animals at 0 and 24 hr of hyperoxial exposure.

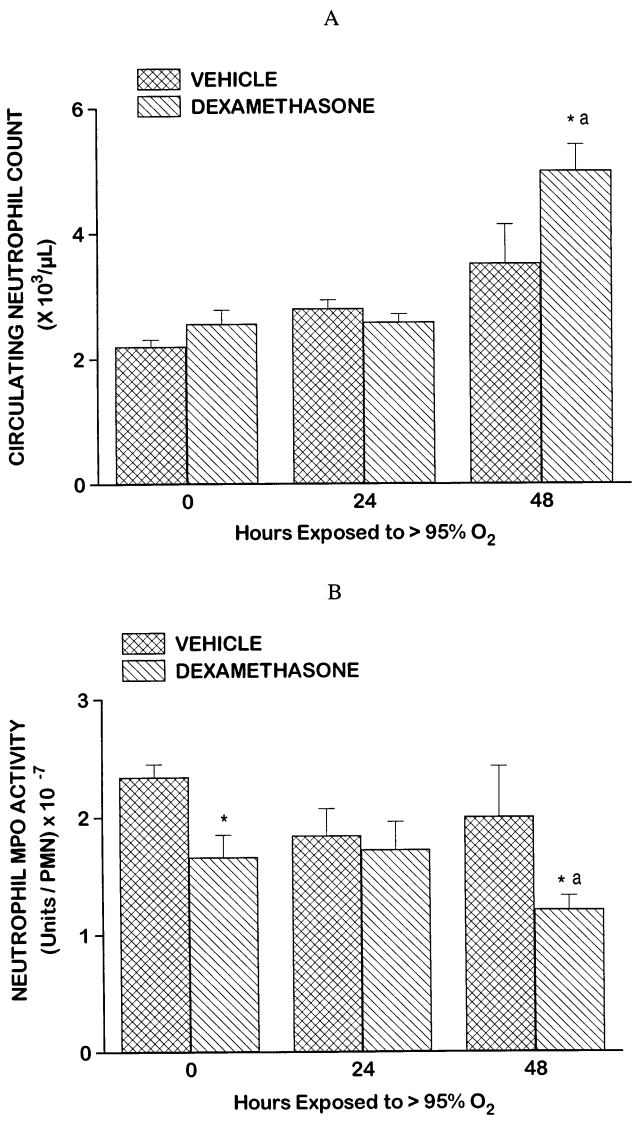

FIG. 5. Effects of dexamethasone administration and exposure to hyperoxia on circulating neutrophil counts and myeloperoxidase activities in circulating neutrophils. Rats were given dexamethasone or vehicle and exposed immediately to >95% oxygen or room air (0 hr exposure to hyperoxia). At 0, 24, or 48 hr of exposure to hyperoxia, circulating neutrophil counts (A) and myeloperoxidase (MPO) activities (B) were determined. Data are expressed as means ± SEM, N = 6–9 in each group. Two-way ANOVA indicated that dexamethasone and hyperoxia exposure led to increases in circulating neutrophil counts, and decreases in myeloperoxidase activities in circulating neutrophils. In neither panel A nor panel B was there a significant interaction. Key: (⋆) $P < 0.05$, different from saline-treated animals similarly exposed to hyperoxia; and (a) $P < 0.05$, different from dexamethasone-treated animals at 0 and 24 hr of hyperoxia exposure.

monary disease. Furthermore, the association between the enhanced neutrophil sequestration in rats given dexamethasone and the earlier onset of hyperoxic lung injury is consistent with the inflammatory response contributing significantly to the tissue damage. The sequestered neutrophils presumably contribute to injury through their actions on the vascular endothelium or other tissue structures through the release and actions of proteolytic enzymes, myeloperoxidase, or reactive oxygen species such as hypochlorous acid [34–36].

Previous studies have shown that adhesion molecules on the vascular endothelium act sequentially with leukocyte receptors to initiate neutrophil adhesion and transendothelial migration [19, 37]. Increases in the expression of E-Selectin, P-Selectin, and ICAM-1 have been observed in the lungs of animals exposed to hyperoxia, and the up-regulated expressions have, in general, preceded evidence of lung inflammation [21, 22]. The adhesion molecule expression studies and the evidence that antibody directed against ICAM-1 exerts a protective effect in hyperoxia-exposed animals [4] suggest that increased expression of the adhesion molecules is responsible for at least a portion of the lung inflammation, and that therapeutic agents, such as dexamethasone, may exert their effects by altering adhesion molecule expressions. However, in the present study the expression of E-Selectin and ICAM-1 was not altered by treatment with dexamethasone (Fig. 4), which suggests that lung neutrophil accumulation induced by dexamethasone was independent of altered adhesion molecule expressions, and is important in that investigations into the lung pro-inflammatory effects of dexamethasone should probably not focus on its effect on adhesion molecule expression. The lack of an up-regulation of E-Selectin or ICAM-1 concomitant with the enhanced sequestration of neutrophils in the lungs of rats treated with dexamethasone was similar to the findings of Kaiser *et al.*, who observed no effects of glucocorticoid pretreatment on the expression of E-Selectin or ICAM-1 by human umbilical vein endothelial cells stimulated with tumor necrosis factor, interleukin-1β, and interleukin-4 [38], suggesting that glucocorticoids affect inflammatory cell recruitment at alternative steps in the inflammatory process. However, our findings add to the results of Kaiser *et al.*, in that we found a pro-inflammatory effect of dexamethasone *in vivo*, while the purpose of the study of Kaiser *et al.* was to determine the mechanisms for anti-inflammatory effects of glucocorticoids *in vitro*. Our findings do not rule out the possibility that dexamethasone administration altered adhesion molecule expression in a lung-cell or lung-region specific pattern that was physiologically significant, but not measurably different in total lung homogenates.

One of the most striking findings in the present set of studies is that lung neutrophil counts and lung myeloperoxidase activities are not correlated tightly in that lung myeloperoxidase activity measurements do not correspond with assessments of lung neutrophil accumulation in tissue sections detected by immunohistochemical methods. The divergence in lung myeloperoxidase activities and lung neutrophil counts suggests that in some treatment groups the lung neutrophils have exocytosed significant fractions of their myeloperoxidase, probably as a result of neutrophil activation, and this exocytosed enzyme has lost assayable activity. Such a degranulation process is difficult to detect in *in vivo* studies, but is the reason why we are working to determine the specific chemical nature of molecular transformations occurring in hyperoxia *in vivo* [39, 40], as some of these transformations may be "footprints" for neutrophil degranulation. The evidence that dexamethasone administration affects neutrophils directly, probably through activation responses, is that myeloperoxidase activities per neutrophil in the circulating neutrophils are lower in animals given dexamethasone, and that myeloperoxidase activities per neutrophil probably are further reduced in neutrophils sequestered in the lung. This possibility is particularly noteworthy because the processes most likely responsible for enzyme inactivation are the reactions that also are more likely to cause tissue damage.

The mean myeloperoxidase activities that we calculated in this study are comparable to the activities determined by Christensen and Rothstein [33], who reported myeloperoxidase activities in the range of $2–6 \times 10^{-7}$ U/neutrophil, and with substantially lower activities in the neutrophils from experimental animals with lethal infections. Pember and Kinkade [34] reported that differences in cell-associated myeloperoxidase activities following stimulation of purified peripheral blood neutrophils with *N*-formyl-methionyl-leucyl-phenylalanine were related to selective exocytosis of enzymatically distinct forms of myeloperoxidase. Bradley *et al.* [41] proposed that the decrease of cell-associated myeloperoxidase activities in circulating neutrophils from infected animals occurred secondary to neutrophil activation and myeloperoxidase exocytosis during transit in the blood. Interestingly, in experimental animals with lower mean neutrophil myeloperoxidase activities in the peripheral blood, further decreases of neutrophil myeloperoxidase activities were observed in the neutrophils localized to the sites of infection, with additional decreases of up to 48% of the myeloperoxidase activities observed in the uninfected animals [34]. Therefore, the fact that lung myeloperoxidase activity measurements do not closely reflect lung neutrophil counts using immunodetection of the myeloperoxidase enzyme is most likely a result of lower myeloperoxidase activities per cell in the pool of circulating neutrophils, as well as degranulation by neutrophils sequestered in the lung, with extrusion and oxidative and proteolytic inactivation and degradation of the enzyme. Additionally, the neutrophils within the lungs of the dexamethasone-treated animals exhibited a more spherical shape than was observed in lung neutrophils of vehicle-treated animals, similar to histological observations on lung neutrophils in shock lung, presumably after neutrophil degranulation [35].

In conclusion, the observation that dexamethasone administration enhanced hyperoxic lung injury in parallel with, and subsequent to, enhanced neutrophil accumulation is further evidence that neutrophils contribute significantly to the development of hyperoxic lung injury. The present data suggest that the enhancement of hyperoxic lung injury in the dexamethasone-treated rats is mediated, at least in part, by the inflammatory responses. However, the mechanisms through which dexamethasone expresses its pro-inflammatory effects do not appear to involve the usual up-regulation and expression of E-Selectin and ICAM-1. It is important to note that we are not arguing

against the use of glucocorticoids in patients when appropriate, but rather that the classification of glucocorticoids as strictly anti-inflammatory agents is probably an oversimplification. Thus, studies to determine the specific effects of glucocorticoids on neutrophil function and neutrophil specific molecular transformations, both in conjunction with and independent of hyperoxia-exposure are crucial so that these two important therapies can be used with optimal efficacy and safety.

This work was supported by an American Academy of Pediatrics Resident Research Grant, and National Institutes of Health Grant 5 P30 HD27823.

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