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Effects of Leukotriene Inhibition on Pulmonary Morphology in Rat Pup Lungs Exposed to Hyperoxia

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Assisted ventilation is necessary for treating preterm infants with respiratory distress syndrome. Unfortunately, high and prolonged concentrations of oxygen associated with assisted ventilation often lead to pulmonary changes, such as hemorrhage and inflammation. The resulting chronic pulmonary condition is known as bronchopulmonary dysplasia. Pulmonary changes characteristic of this syndrome can be produced in rat pups exposed to high oxygen levels. We exposed 21-d-old rats to room air or continuous 95% oxygen for 7 d and then allocated them into 6 groups to evaluate whether treatment with zileuton and zafirlukast, 2 agents which decrease the effects of leukotrienes, lessened the pulmonary effects of short-term hyperoxia. After 7 d, lung tissue was collected for light and electron microscopy. Pulmonary changes including edema, hemorrhage, alveolar macrophage influx, and Type II pneumocyte proliferation were graded on a numerical scoring system. Compared with controls exposed to hypoxia and saline, rats exposed to hypoxia and treated with zileuton had significantly reduced levels of alveolar macrophage influx and Type II pneumocyte proliferation, but those exposed to hypoxia and treated with zafirlukast showed no significant reduction in any pulmonary changes. This study helps define pulmonary changes induced secondary to hyperoxia in rat pups and presents new information on the mechanisms of leukotriene inhibition in decreasing the severity of hyperoxic lung injury.

Abbreviations: 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; BPD, bronchopulmonary dysplasia; HS, hyperoxia and saline; HZa, hyperoxia and zafirlukast; HZi, hyperoxia and zileuton; LT, leukotriene; NS, normoxia and saline; NZa, normoxia and zafirlukast; NZi, normoxia and zileuton; RDS, respiratory distress syndrome

Respiratory distress syndrome (RDS) remains a dominant clinical problem in premature infants.[9] It is the most expensive inpatient condition billed by US hospitals; national statistics collected by the US Agency for Healthcare Research and Quality show costs averaging $68,000 for a mean stay of 24.6 d.[29] The treatment for RDS requires assisted ventilation, which often exposes immature lungs to prolonged and high concentrations of oxygen. In 1967, bronchopulmonary dysplasia (BPD) was first described as a serious pulmonary complication of premature infants with RDS.[16] BPD is the chronic inflammatory lung disease associated with RDS and is a leading source of morbidity, despite improved tolerance to oxygen exposure in newborns.[27] BPD has been reported to occur in 5% to 68%[33] of infants with RDS, although the exact incidence is not defined easily.

Although the causes and pathogenesis of BPD remain uncertain, reports demonstrate an association between elevated leukotriene levels and oxygen exposure with the development of this syndrome.[19,25,32] Leukotrienes (LTs) were first identified in 1938 and have a wide range of biologic effects on cells and tissues.[2,11] These potent mediators of inflammation result from the conversion of arachidonic acid by 5-lipoxygenase to produce 5-hydroperoxyeicosatetraenoic acid (5-HPETE). Nonpeptide LTs include LTA_4 and LTB_4, and the cysteinyl LTs include LTC_4, LTD_4, and LTE_4.[2] Further processing converts 5-HPETE into LTA_4, but it is unstable and rapidly converts to LTC_4 and LTB_4.[8] An earlier study identified cysteinyl LTs (LTC_4, LTD_4, LTE_4) in the lung lavage fluid of neonates with persistent pulmonary hypertension, humans with chronic obstructive pulmonary disease, and animals subjected to acute hyperoxia.[17,22,26,34] Several studies have shown that LTB_4 plays a crucial role in various neonatal disorders, including BPD, revealing that bronchoalveolar lavage from newborns with BPD had high levels of LTB_4.[7,33] We hypothesized that inhibition of LTs might decrease the lung damage seen with hyperoxia in human preterm infants. Two main features of hyperoxia-induced pulmonary disease in human infants are inflammation and edema, both of which correlate with the action of LTB_4.[25,33] Understanding the response of the lungs to hyperoxia is essential for determining a pharmacologic approach to decreasing the effects of BPD and improving respiratory function.[18,23,36]

The numerous therapies available for addressing BPD include steroids, indomethacin, inositol supplementation, vitamins A and E, cromolyn sodium, and bronchodilators. These treatments have not proven effective, and each is associated with a unique set of side effects.[9,10,13,14,16,17,37] Therefore, a treatment for BPD that decreases or eliminates the adverse effects of hyperoxia on the respiratory system and results in minimal secondary side effects would have a positive effect on human health.

Several species have served as models for hyperoxic experiments: baboons, rabbits, guinea pigs, hamsters, rats, and mice.[12,16,19,25,27] Pneumotoxic effects of oxygen are analogous among species and, with the use of similar doses, it is feasible to make comparisons between species.[35] Belik and colleagues[1] reported that after 14 d of chronic exposure to 60% oxygen levels,

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Table 1. Group comparisons of scores for pulmonary changes

	Group				P		
	NS	HS	HZi	HZa	NS versus HS	HS versus HZi	HS versus HZa
No. of animals in group	5	7	7	7	not applicable	not applicable	not applicable
Edema	0 (0,0)	2 (1,3)	0 (0.0)	2 (1,3)	0.0013	0.0408	0.6999
Hemorrhage	0 (0,0)	1 (0,3)	0 (0,0)	0 (0,1)	0.0278	0.021	0.2127
Alveolar macrophage influx	1 (1,2)	3 (2,3)	2 (1,2)	2 (1,3)	0.0025	0.0034	0.0862
Type II pneumocyte proliferation	0 (0,0)	3 (2,4)	1 (1,2)	2 (1,4)	0.0013	0.0023	0.7535
Total group scores	1 (1,2)	8 (6,13)	3 (2,5)	7 (5,9)	0.0013	0.0006	0.2348

Data are represented as median (range).

newborn rats develop histologic lung changes compatible with human bronchopulmonary dysplasia and hypertension. Moreover, Denis and colleagues[6] found that exposure of newborn rats to moderate hyperoxia (50%) induces airway responsiveness and histiologic changes similar to those reported in human infants with bronchopulmonary dysplasia.

In the present studies, we investigated 2 LT inhibitors, zileuton and zafirlukast, in a rat pup model of hyperoxic lung injury. A novel class of antiasthmatic drugs, LT modifiers are unique hybrids between anti-inflammatory agents (antagonizing the proinflammatory activities of LTs) and bronchodilators (antagonizing LT-induced smooth-muscle bronchoconstriction).[21] Zileuton prevents LT formation by inhibiting 5-lipoxygenase activity and thus disrupts the formation of LTB_4 and LTC_4 and neutrophil chemotaxis.[8] In addition, zileuton precludes LTC_4- and LTD_4-induced smooth muscle constriction, eosinophil migration, and edema formation.[8] Zafirlukast, a cysteinyl LT receptor antagonist, inhibits the actions of only LTC_4, LTD_4, and LTE_4,[8] which mediate smooth muscle constriction, eosinophil migration, and edema formation.

Materials and Methods

Animals. The present study used 39 specific pathogen-free male and female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing an average of 50 g at the beginning of the study. Rats were received as adult females with 2-wk-old litters. The rat pups from multiple litters were acclimated for 1 wk, weaned, separated from the dams, and arbitrarily assigned into hyperoxic or normoxic groups (n = 5 to 7 per group). The normoxic groups of rats were housed in microisolation caging measuring 0.0908 m^2 (Allentown, Allentown, NJ) and containing hardwood bedding (Sani-chips, Harlan Teklad, Indianapolis, IN). The hyperoxic groups—each in its own cage—were placed in a 161-l acrylic chamber (DNA Enterprises, Memphis, TN) with a continuous flow of oxygen. Delivery of oxygen was maintained at a minimum of 95% and a flow rate of approximately 16 l/min. All groups had access to water bottles with long sipper tubes and pelleted rodent chow (8640 Harlan Teklad 22/5 Rodent Diet, Harlan Teklad, Madison, WI) ad libitum. Cages were changed twice weekly; animals were on a 12:12-h light:dark cycle; and health checks were done twice daily. The animal holding room was maintained under environmental conditions of 15 air changes hourly and an average of 47% relative humidity. All procedures were performed in an animal facility accredited by the Association for Assessment and Accreditation for Laboratory Animal Care, International, with the approval of the institutional animal care and use committee.

Experimental and treatment groups. A hyperoxic chamber was set up to simulate a high-oxygen environment similar to what a premature human infant experiences. We evaluated 6 experimental groups to determine whether the drug-treated hyperoxic groups developed less severe histopathologic pulmonary changes than did untreated hyperoxic groups.

Rats were divided arbitrarily into 6 groups: normoxia and saline (NS; n = 5), hyperoxia and saline (HS; n = 7), normoxia and zileuton (NZi; n = 6), hyperoxia and zileuton (HZi; n = 7), normoxia and zafirlukast (NZa; n = 7), and hyperoxia and zafirlukast (HZa; n = 7). The NS, NZi, and NZa groups were kept in 21% oxygen (normoxia), whereas the HS, HZi, and HZa groups were maintained in a 95%-oxygen acrylic chamber. The experimental design for this study included 2 types of controls, whereby the NS rats provided the negative control and the HS rats served as the positive control. Zileuton was administered by gavage at a dose of 20 mg/kg once every 24 h for 7 d to rats in the NZi and HZi groups. The NZa and HZa groups were given 40 mg/kg zafirlukast once every 24 h for 7 d by gavage. The NS and HS groups were given saline by gavage in a similar volume to that of the treatment groups. The oxygen chamber housed the HS, HZi, and HZa groups throughout the length of the experiment. The percentage of oxygen was kept constant with an oxygen monitor (Multigas Monitor 9100, BCI International, Waukesha, WI).

After 7 d, the rats were deeply anesthetized with intraperitoneal ketamine (87 mg/kg)–xylazine (13 mg/kg). The lung lobes were perfused with saline through the left ventricle, carefully removed, and inflated via the trachea with either 10% formalin or 2.5% glutaraldehyde (Tousimis Research Corporation, Rockville, MD) at a pressure of 20 cm H_2O for 15 min. Once the lungs were fully expanded, they were placed in either formalin or glutaraldehyde, and then either the right middle lobe or the left lung lobe was processed for light and transmission electron microscopy. The right middle lung lobe was used most often, but when this lung lobe failed to expand fully, the left lung lobe was used to ensure consistency and quality of fixation.

Light microscopy. Tissues placed in cassettes were fixed in neutral buffered 10% formalin and prepared in a standard manner for histologic examination. Sections (thickness, 4 to 5 µm) cut from paraffin-embedded blocks of lung tissue were stained with hematoxylin and eosin. Sections of plastic-embedded lung tissue (thickness, 1 µm) from all 6 groups were stained with toluidine blue and examined by light microscopy. A pathologist blinded to the original experimental protocol reviewed all lung sections. For scoring of edema, hemorrhage, and Type II pneumocyte proliferation, scores from 0 to 4 were assigned: 0, no change; 1, mild change; 2, moderate change; 3, marked change; 4, massive change. Alveolar macrophage influx was scored as follows: 0, no or only occasional cells present; 1, 3 or fewer macrophages per high-power field; 2, 4 to 6 macrophages per high-power field; 3, 7 to 9 macrophages per high-power field; 4, 10 or more macro-

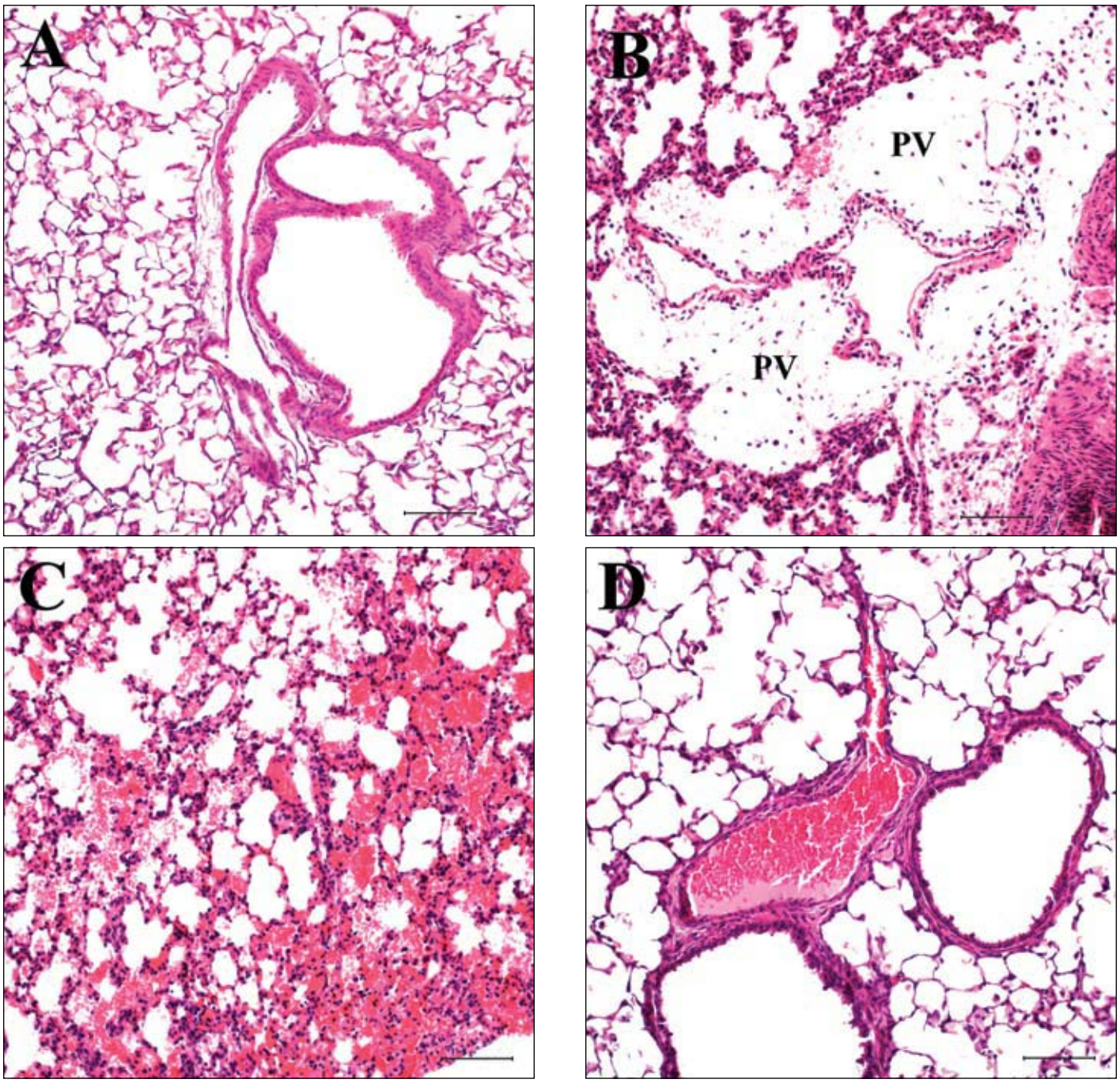

Figure 1. (A) Lung of NS rats (negative control) reveal no evidence of vascular leakage. (B and C) Lungs of HS (positive control) rats show marked perivascular edema (B) and intraalveolar hemorrhage (C). (D) Lung of HZi rats reveal minimal evidence of vascular leakage. This finding is similar to that for NS rats and is significantly different from that of HS rats. PV, perivascular edema. Hematoxylin and eosin stain; bar, 100 µm.

phages per high-power field.

Electron microscopy. Lung samples collected for transmission electron microscopy were fixed in 2.5% gluteraldehyde in Millonig's phosphate buffer, postfixed in 1% OsO_4 in phosphate buffer, dehydrated though a graded ethyl alcohol series, carried through propylene oxide, and embedded (Araldite, Electron Microscopy Sciences, Fort Washington, PA). Additional ultrathin sections were cut, mounted on uncoated copper grids, stained with uranylacetate in 25% methanol and lead citrate, and examined by using transmission electron microscopy (LED Electron Microscopy, Carl Zeiss SMT AG, Thornwood, NY). The EM slides helped to substantiate the findings seen with light microscopy and were not used primarily for scoring pulmonary changes.

Statistical analysis. The 2-sided Wilcoxon-Mann-Whitney test was used to individually compare group median scores (Table 1) of NS and HS groups, HS and HZi groups, and HS and HZa groups for each of the pulmonary changes: edema, hemorrhage, alveolar macrophage influx, and Type II pneumocyte proliferation. In addition, the total score for all the pulmonary changes in each group was calculated and compared between the previously

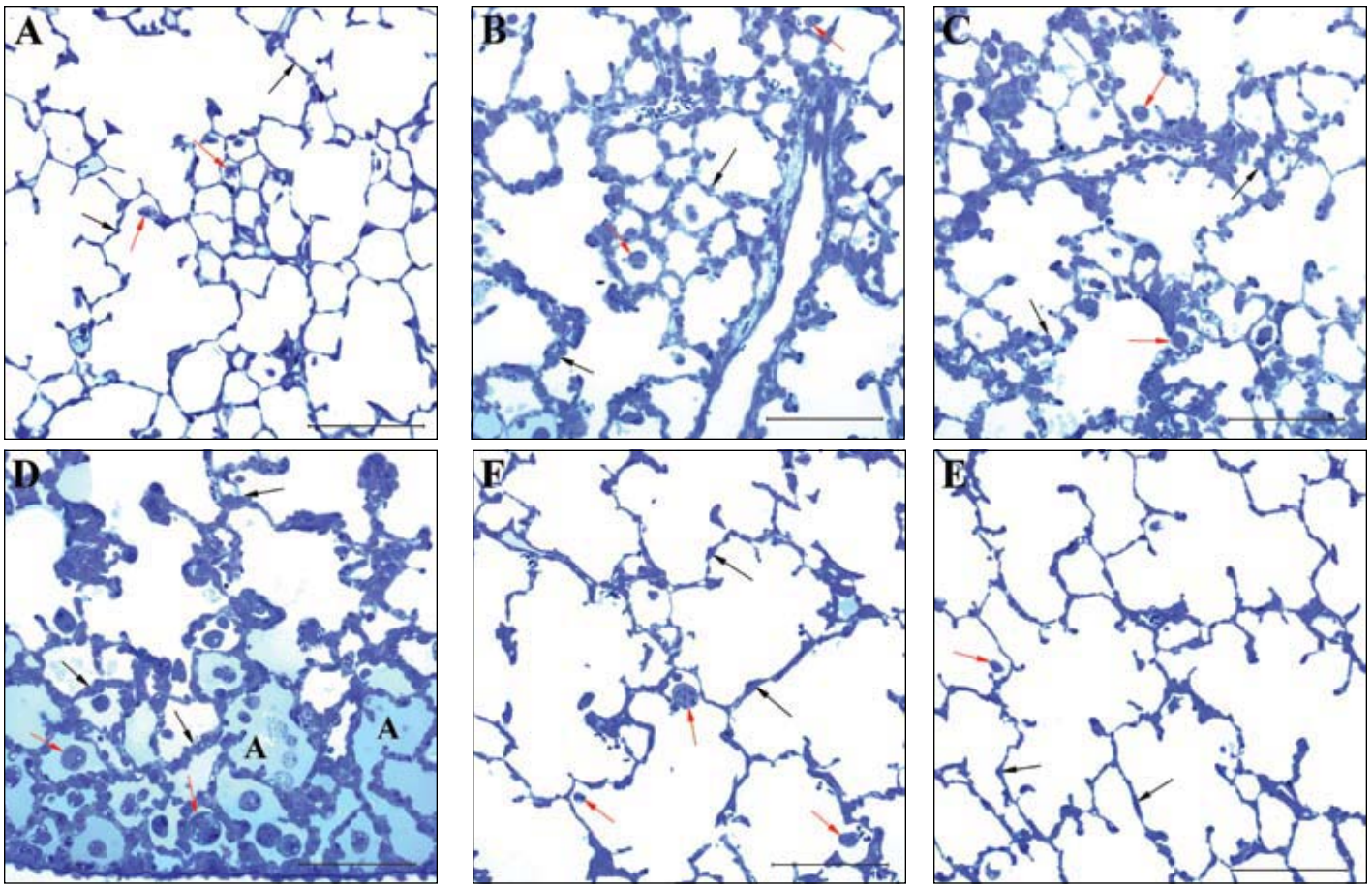

Figure 2. Alveolar region of rat lungs. (A) NS rat. Only occasional alveolar macrophages and Type II pneumocytes were present. (B, C, and D) HS rats. Marked thickening of the alveolar septae, due primarily to Type II pneumocyte proliferation; numerous large alveolar macrophages; and intraalveolar edema frequently were present. (E and F) HZi rats. The incidence of Type II pneumocyte proliferation and presence of alveolar macrophages was much less than those of HS rats. In addition, vascular leakage (characterized by edema and hemorrhage) was apparent only occasionally and much less severe in intensity than that in HS rats. Red arrows, alveolar macrophages; black arrows, alveolar septae; A, intra-alveolar edema. Section thickness, 1 µm. Toluidine blue stain; bar, 100 µm.

listed groups All testing procedures were performed with StatXact-5 software (Cytel Software Corporation, Cambridge, MA) implemented by using SAS version 9 (SAS Institute, Cary, NC). Because 15 statistical tests were performed, to preserve an overall experimental error rate of 0.05, an adjusted P value of 0.0035, based on the Bonferroni correction, was considered the threshold for statistical significance.

Results

Statistical findings. Consistent with what has been described previously,[4,5,12,15,27,33] pulmonary changes such as edema, alveolar macrophage influx, and Type II pneumocyte proliferation were observed in all the untreated hyperoxic rat pups (HS) and were significantly ($P \leq 0.0035$) different from those in untreated normoxic animals (NS; Table 1).

The hyperoxic rat pups that received zileuton (HZi group) had significant decreases (Table 1) in the degree of alveolar macrophage influx and Type II pneumocyte proliferation ($P \leq 0.0035$ for both comparisons), compared with the HS rat pups. Among pups in the hyperoxic environment, there was no significant difference in any of the pulmonary changes between those that received zafirlukast (HZa group) and those that received saline (HS group; Table 1).

Striking differences in total scores for all pulmonary changes (edema, hemorrhage, alveolar macrophage influx, and Type II pneumocyte proliferation) were apparent between various groups (Table 1). These differences were most pronounced between the NS and HS groups and the HS and HZi groups ($P \leq 0.0035$ for both comparisons).

Histopathologic findings. Edema and hemorrhage, which are characteristic of vascular leakage, were not apparent on light and electron microscopic evaluation of the lungs from the NS rats (Figure 1 A); only occasional alveolar macrophages (Figure 2 A) and Type II pneumocytes (Figure 3 A) were present. The pulmonary findings for the drug-treated normoxic rats (NZi and NZa groups) were essentially the same as those described for the NS group. In contrast, morphologic changes in the HS group of rats were prominent and consisted of moderate perivascular (Figure 1 B and Figure 3 B) and intra-alveolar (Figure 2 D and Figure 3 C) edema and intra-alveolar hemorrhage (Figure 1 C); marked influx of alveolar macrophages (Figure 2 B, C, and D); and marked Type II pneumocyte proliferation (Figures 2 D and 3 C). Many of the Type II pneumocytes appeared to be degenerated with prominent blunting of the surface microvilli. Alveolar macrophages were engorged with cellular debris consistent with phagocytized pneumocytes (Figures 2 D and 3 C), especially Type II cells. Several of

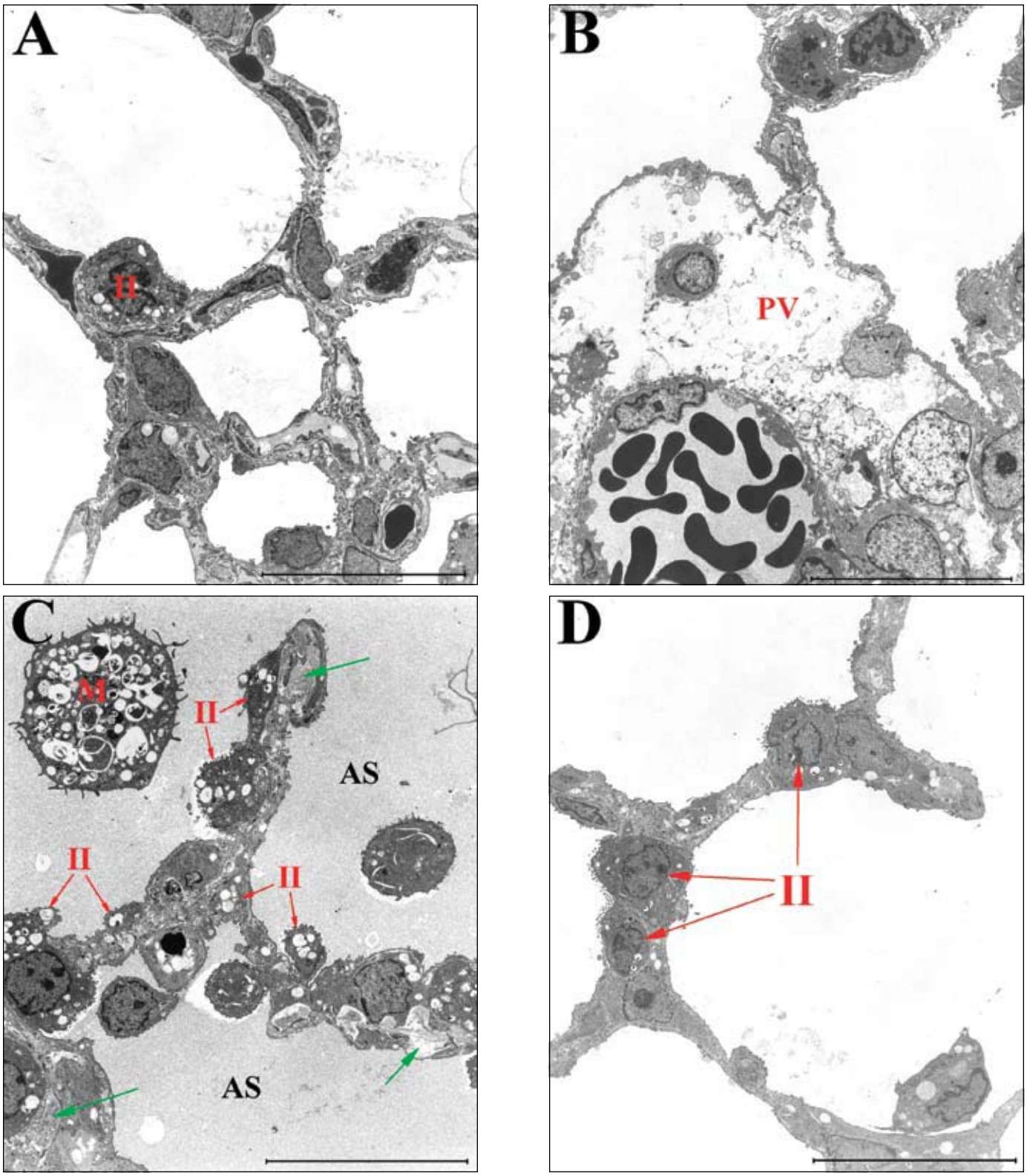

Figure 3. Electron micrographs of the alveolar region of rat lungs. (A) NS rats. Only an occasional Type II pneumocyte (II) was present. (B) HS rats. Marked intra-alveolar and perivascular (PV) edema were apparent in these rats. (C) HS rats. Marked Type II pneumocyte proliferation, with some cells appearing to be degenerated. A large debris-laden macrophage (M) is present within an edematous alveolar sac (AS). Much of the debris appeared to represent phagocytized Type II pneumocytes within the macrophage phagosomes. Multiple foci of increased interstitial matrix associated with apparent collagen deposition (green arrows) also were seen; this finding is consistent with early interstitial fibrosis. This feature was not present in either NS or HZi rats (D). HZi rats. The incidence of Type II pneumocytes was significantly less than that of the HS rats, and whatever Type II pneumocytes were present were essentially normal in appearance. Uranyl acetate and lead citrate stain; bar, 15 µm.

these rats also showed evidence of early pulmonary interstitial fibrosis (Figure 3 C).

The morphologic effect of the zileuton treatment on hyperoxic (HZi) rats is best summarized as the presence of only minimal of capillary leakage (Figure 1 D) and a marked decrease in the intensity of the alveolar macrophage influx (Figure 2 E and F) and Type II pneumocyte proliferation (Figure 3 D). Not only were fewer Type II pneumocytes present, but those present were basically normal in appearance.

The morphologic findings from the zafirlukast-treated (HZa) rats were very similar to those of the untreated hyperoxic animals (HS), indicating that the treatment had essentially no positive effect on preventing the pulmonary changes induced by the hyperoxic environment.

Discussion

In the 1960s, the ability to provide assisted ventilation for premature infants with RDS changed the expected course of the disease.[9] As the survival rate of the smaller and sicker infants increased, so did the incidence of the chronic lung damage now known as BPD.[9] Not only has successful intervention of BPD been limited, currently used therapies cause adverse side effects on numerous organ systems.[9,11,13,14,16,17] The connection between BPD and leukotriene increase is unclear. The basis for our study was the premise that modifying leukotriene formation would be expected to reduce the pulmonary damage that results in BPD. Namovic and coauthors[24] report that zileuton, a 5-lipoxygenase inhibitor, significantly reduces leukotriene levels in a dose-dependent fashion. Other studies[3] confirm zileuton as a highly effective inhibitor of 5-lipoxygenase after oral administration in the rat passive peritoneal anaphylaxis model. However, Carter and colleagues[3] also found in the mouse ear edema model that zileuton only partially prevented the edema caused by arachidonic acid; their results support other findings indicating that 5-lipoxygenase products are not the sole contributors to these inflammatory responses.

Our current study showed that treatment with zileuton significantly decreased pulmonary changes when compared with those of untreated and zafirlukast-treated hyperoxic animals. In our study, zafirlukast, a cysteinyl leukotriene receptor antagonist, had no effect on decreasing the pulmonary histopathologic effects of hyperoxia. One explanation might be differences in pharmacokinetics among animal species: for example, Savidge and colleagues[30] reported only 52% absorption of zafirlukast in rats at a dose of 5 mg/kg. In addition, Kertesz and colleagues[20] report that leukotriene E_4 appears during late stages of severe lung damage; in contrast, our study focused on the short-term effects of hyperoxia. This difference may explain to some extent why zafirlukast failed to ameliorate any of the hyperoxic-induced pulmonary changes.

Previous studies have shown that the main feature of respiratory disease in infants is pulmonary inflammation, characterized by neutrophil infiltration and edema.[8] We did not find increased levels of neutrophils or eosinophils in the lungs of any of the animals after hyperoxia. One likely explanation relates to the time of tissue collection. Other studies have shown that neutrophils may not play an important role in early sublethal hyperoxic injury to rabbit alveolar epithelium.[20,28] For example, Laughlin and colleagues[20] analyzed tissues at 64 h after hypoxia treatment;[20] in our study, animals were euthanized and tissues were analyzed at 168 h post-treatment. It is possible that the different outcome of our study compared with other studies relates to the elapsed time between injury and tissue collection. Another plausible explanation for the absence of neutrophils and eosinophils in any of the hyperoxic lungs of our animals might be related to the age of our animal model. Using weanling rats rather than premature or newborn rats may have been a confounding factor in some of the pulmonary changes evaluated, because age is reported to play an important role in how an animal's body responds to hyperoxia.[19]

Measuring leukotriene values between the different groups might have helped elucidate more of the role that leukotrienes play in hyperoxic-induced lung injury. This is especially important since the relationship between the appearance of leukotrienes and the severity of the lung injury is not well understood.[32] Another limitation of this study is the semi-quantitative nature of the experimental design. As BPD is a diffuse disease process, we evaluated only 1 lung lobe to aid in a more consistently measurable assessment.

Initially, there were 42 rats in our study, arbitrarily divided into 6 groups of 7 rat pups each. However, 3 of the rat pups, all from normoxic groups, died during the experiment due to gavage-associated trauma. Although the group sizes were not uniform because of these unexpected deaths, statistically significant results were still achieved in light of the conservative threshold for statistical significance provided by Bonferroni adjustment. Furthermore, we present this information as a pilot study.

In light of our findings, zileuton may have a protective effect against alveolar macrophage influx and Type II pneumocyte proliferation in hyperoxia-induced lung disease. Zileuton did not demonstrate a protective effect against edema and hemorrhage in hyperoxic-induced lung disease, possibly because the sample size was so small that the analyses did not have sufficient power. However, there were obvious histologic differences between the zileuton-treated and untreated hyperoxic groups, suggesting that zileuton did have a positive effect on decreasing all of the pulmonary changes observed, including hemorrhage and edema. It is noteworthy to consider the total scores for each of the groups and see the pattern among the group comparisons that reflect the experimental design. This pattern illustrates that zileuton alone had a significant effect on decreasing some of the pulmonary changes that are often observed in response to a hyperoxic environment. Crapo and colleagues[4,5] observed proliferation and hypertrophy of Type II epithelial cells in animals exposed to 85% and 100% oxygen environments. Several other studies reported similar findings of changes in Type II alveolar epithelial cells after hyperoxia was induced.[15,31] In addition, significant Type II pneumocyte proliferation occurred in all groups exposed to hyperoxia except the group that received zileuton.

It is interesting that zafirlukast, despite its similar mode of action to zileuton, showed no positive effect against hyperoxic-induced pulmonary changes. We speculate that this difference may be due to zileuton's activity against leukotrienes B_4, C_4, and D_4, whereas zafirlukast has action against leukotrienes C_4, D_4, and E_4. Therefore, leukotriene B_4 may be an important contributor to the pulmonary changes seen with hyperoxia. This unexpected result may provide better understanding into the role of leukotriene B_4 in hyperoxia-induced lung disease, but further investigation is needed to characterize this mechanism.

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