Protection against Acute and Chronic Hyperoxic Inhibition of Neonatal Rat Lung Development with the 21-Aminosteroid Drug U74389F

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ABSTRACT. Normal lung development involves septation of the large air saccules present at birth to form smaller diameter alveoli with a much increased surface area for respiratory exchange. This process in the newborn animal is markedly inhibited by hyperoxia, and the altered lung morphology that results may be permanent. We tested whether treatment of neonatal rats with the new 21-aminosteroid (21-AS) drug, U-74389F (15 mg/kg/d), could protect against O₂-induced inhibition of normal lung development. By morphometric analysis after 10 d in >95% O₂, the lungs of the animals treated with this potent iron chelator and inhibitor of lipid peroxidation showed a substantial protective effect—with reduced mean air space diameter and significantly increased internal surface area compared with O₂ control pups. [Air control mean air space diameter = 47.4 μ m, internal surface area = 1014 cm²; O₂ controls = 61.0 μ m ($\uparrow$ 29%), 769 cm² ($\downarrow$ 24%); O₂ 21-AS = 53.4 μ m ($\uparrow$ 13%), 919 cm² ($\downarrow$ 9%); $p < 0.05$ between O₂ groups.] Similarly, inhibition of lung elastin deposition (involved in septation process) during hyperoxia was significantly ameliorated by 21-AS treatment. In addition, follow-up studies of young adult rats demonstrated permanently enlarged lung alveoli and reduced surface area after neonatal high O₂ exposure. These chronic morphologic effects were also significantly reduced by neonatal 21-AS treatment. The right ventricle hypertrophy (2° to pulmonary hypertension) in the O₂ controls reaching adulthood [right ventricle/left ventricle + septum weight = 0.496 ($\uparrow$ 55% versus air control value = 0.320)] was markedly reduced in the adult 21-AS animals exposed to hyperoxia as neonates [0.382 ($\uparrow$ 19%)]. Thus, 21-AS treatment had a substantial protective effect against both the acute and chronic lung as well as cardiac morphologic changes that early life exposure to hyperoxia can produce. (*Pediatr Res* 33: 632–638, 1993)

Abbreviations

21-AS, 21-aminosteroid (U74389F)

L_M, mean linear intercept (mean lung air space diameter)

ISA, internal surface area

AOE, antioxidant enzyme

Neonatal animals are generally much more resistant to the severe lung-damaging effects of prolonged exposure to hyperoxia than adult animals. Whereas adult animals of many tested species die within 3 to 5 d in >95% O₂ with severe lung pathology (pulmonary edema, hemorrhage, and inflammatory cell influx), most newborn animals survive similar hyperoxic exposure for >7 to 10 d with much less evidence of acute lung injury (1–3). However, a unique consequence of hyperoxic exposure in the newborn is marked inhibition of the rapid lung growth and development that normally occurs in the early days of life in most newborn species. In the neonatal rat lung, for example, between postnatal d 4 to 14, there normally occurs a very dynamic septation process by which the large immature terminal air spaces (saccules) present at birth are subdivided into many smaller mature terminal air spaces (alveoli), with a resultant several-fold increase in lung surface area for respiratory exchange (4–6). Exactly how hyperoxic exposure during this so-called “critical period” of lung development inhibits the coordinated process of alveolarization and surface area expansion of the newborn’s lung is unknown.

However, the biochemical mechanism of cellular O₂ toxicity is by now quite well established. Hyperoxia results in a marked increase in the normal rate of intracellular production of partially reduced and highly reactive O₂ species that are by-products of aerobic metabolism. When produced in excess of the cell’s capacity to detoxify them, these reactive O₂ species are capable of causing diffuse cellular damage (7, 8). It is also well established that ionic iron (or other transition metals) is a major factor in promoting both O₂ free-radical production as well as the subsequent cytotoxic interactions of O₂ free radicals with their cellular lipid, protein, and nucleotide target molecules (9, 10).

Based on the assumption that O₂-induced inhibition of neonatal lung development is similarly mediated by reactive O₂ species, a variety of antioxidant and free radical scavenger agents have recently been tested in an attempt to prevent or ameliorate O₂-induced lung growth inhibition, with varying degrees of success (11–13). The same rationale prompted our present studies using a prototype of a new class of compounds—the 21-AS, or lazaroids. These drugs have several relatively unique properties that in theory would make them well suited to counter O₂ free radical-induced toxicity: 1) being lipophilic, they should easily penetrate cell membranes reaching the intracellular sites of O₂ radical production and attack; 2) as potent iron-chelators, they should be able to reduce the Fe⁺⁺ available for Fe-dependent O₂ free-radical reactions; and 3) as reportedly effective inhibitors of lipid peroxidation, they should be able to block O₂-induced cell membrane oxidative damage (14, 15). We report herein a beneficial effect of treatment with this theoretically promising agent, U74389F, against both the acute and chronic effects on lung growth/developmental changes that early life exposure to hyperoxia can produce. We also discuss the possible implications of such treatment in the O₂-requiring premature infant, whose lung

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is likely susceptible to the same type of early developmental inhibition by prolonged exposure to high fraction of inspired O₂ levels.

MATERIALS AND METHODS

Animals and treatments. Adult male and female Sprague-Dawley albino rats to be used as breeders were obtained from Sprague-Dawley (Wilmington, MA). Rats were maintained in the Animal Care Facility at the University of Miami, fed standard laboratory food and water *ad libitum*, and kept on a 12-h light/dark cycle. Rat pups were obtained from our own long-established rat breeding program. For each experiment, 4-d-old pups from four or eight litters were pooled and then randomly redistributed to the dams (10 to 12 pups/litter). The pups were treated with either daily s.c. injections of the 21-AS drug, U-74389F (kindly supplied by Dr. David Zimmerman, Upjohn, Kalamazoo, MI) in citrate vehicle (0.02 M citric acid, 0.0032 M sodium citrate, 0.077 M sodium chloride) at a dose of 15 mg/kg in a volume of 5 μ L/g body weight or equivolume vehicle alone (designated the control group). Half of the 21-AS-treated and control litters were placed in hyperoxia, and half remained in room air for the 10-d experiments. Every 24 h, the respective dams were rotated from the O₂-exposed litters to the air-exposed litters to prevent O₂ toxicity and resultant poor nurturing by the dams. Litters of pups were weighed daily to rule out poor nursing (comparatively poor pup weight gain) by any of the dams, but no dam replacements were needed throughout the course of these studies.

Exposures to hyperoxia were conducted as previously described (16) in 3.5-ft³ plastic isolettes with regular monitoring of O₂ (>95% O₂) and CO₂ concentrations (<0.40%), temperature (23 to 25°C), and humidity (40 to 60%). Exposures were continuous except for a daily 20- to 30-min interval to allow for animal maintenance, dam switching, and pup weighing and injection. NIH guidelines for humane animal treatment were followed in all experimental procedures, and the protocol was preapproved by the University of Miami Animal Welfare Committee.

Lung morphometry. For the morphologic studies, rat pups were killed by an overdose of pentobarbital at the end of the 10-d period in >95% O₂ or room air. Lungs were fixed with 10% buffered formalin via a tracheal cannula, using a constant inflation pressure of 20 cm H₂O (fixative), maintained for 48 h before sectioning. No correction was made for tissue shrinkage. Lung volumes were measured by water displacement (17) and specific lung volumes (mL/100 g body weight) were calculated. From all lungs, similarly oriented sections from similar portions of the left lung and the right middle and lower lobes were stained with hematoxylin and eosin and initially examined to eliminate any sections with evidence of inadequate preparation (atelectasis). Morphometry was performed using a standard integrating eyepiece with a grid matrix composed of seven equal lines and 42 intercept bars (Zeiss). The coded slides were examined at 400 $\times$ magnification, with 30 random fields counted per slide. We counted the number of times the intercept bars fell on lung tissue per field and the number of times the lines were crossed by tissue septa per field. L_M was calculated using the formula (18):

$$L_M = \frac{\text{Length of line} \times \text{no. of lines} \times \text{no. of fields}}{\text{Total no. of tissue intercepts}}$$

where length of line is 0.21 mm, number of lines is seven, and number of fields is 30. L_M is a measure of the average air space diameter and decreases during the period of early postnatal lung maturation. ISA was calculated using the formula (19):

$$ISA = \frac{4 \times \text{lung volume}}{L_M}$$

ISA is essentially a measure of the amount of lung surface area available for respiratory exchange function, and ISA normally

increases several-fold during the period of early postnatal lung maturation.

Percentage of air space was calculated by:

$$\% \text{ Air space} = P_A / (P_A + P_T) \times 100$$

where P_A is the mean number of intercept bars per lung field hitting air and P_T is the mean number of intercept bars per lung field hitting tissue, with (P_A + P_T) = 42 intercept bars per lung field.

The same lung sections used for quantitative morphometry were also stained with orcein to highlight lung elastin (black fibers). With coded slides, using the same seven-line grid matrix eyepiece at 400 $\times$ magnification, we estimated the relative-length density of elastin fibers in lung parenchymal tissue using a technique for length-density measurement as described by Niewoehner and Kleinerman (20). Each line of the test system represented a plane perpendicular to the plane of the section. The section was screened parallel to each of the seven lines, and the number of intersections of the lines with black elastin fibers was recorded. Each of these intersections corresponds to a fiber transected by the test plane. No attempt was made to quantitatively estimate the dimension of the test plane perpendicular to the plane of the section (a few μ m), but because this was constant for all sections scanned, the number of transections yielded relative values rather than absolute values of length density. Thirty random fields per lung were counted (omitting fields in which large vessels or airways with circumferential elastin fibers were present), and the mean number of transections per lung field was calculated for each coded slide.

Similar methods of lung preparation and morphometric analysis were used for the follow-up (chronic) studies of 63-d-old rats that were exposed to hyperoxia (or room air) at age 4 to 14 d of life and thereafter kept in room air. In addition, to assess for right ventricular hypertrophy in these chronic animals, the right ventricle was carefully dissected away from the left ventricle and septum, and both portions were weighed to the nearest mg (11).

Finally, to assess for intraalveolar edema in the neonatal rats exposed to hyperoxia for 10 d, the coded lung sections used for morphometric analysis were scanned for the presence of pink-staining material within the lumen of the air spaces (proteinaceous edema fluid).

Lung biochemical analyses. Lung elastin content was assayed by the method of Naum and Morgan (21). After homogenization of the lungs in 0.15 M NaCl and centrifugation, 1.0 mL of 5 M guanidine HCl, pH 7.0, was added to the pellet. Guanidine extraction continued for 24 h at room temperature in a shaking water bath. After centrifugation and a second 24-h extraction with guanidine, the residue was suspended in 1.0 mL H₂O and then autoclaved at 15-lb pressure for 45 min. After centrifugation, the precipitate was mixed with 1.0 mL of H₂O and 1.0 mL of elastase solution (0.1 mg of purified elastase in 0.1 M veronal acetate buffer, pH 8.8) and incubated for 30 min at 37°C in a shaking water bath. Aliquots were then assayed for protein (elastin) content by the method of Lowry (22). Purified standards of elastin were treated as above for generation of a standard assay curve.

Two assays of lipid peroxidation products were performed for lung malondialdehyde content (left lungs) and for conjugated dienes (right lungs). Lung malondialdehyde concentrations were assayed by measuring thiobarbituric acid-reactive substances extracted into butanol (23). After perfusion and homogenization in 1.15% KCl (10:1, vol/wt), 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid, pH 3.5, 2 mL of 0.8% thiobarbituric acid, and 10 μ L of butylated hydroxytoluene solution to equal 0.01% butylated hydroxytoluene were added to 0.3 mL of homogenate. The well mixed suspension was heated at 95°C for 60 min. After cooling, 5 mL of butanol was added. After vigorous mixing followed by centrifugation at 4000 rpm for 15 min, the absorbance of the organic layer was read at 532 nm and compared to a standard curve of tetramethoxypropane.

Conjugated dienes were determined by a modification of the method of Buege and Aust (24). After homogenization in 0.8 mL of H₂O/g lung tissue, 6.0 mL/g lung tissue of chloroform:methanol (2:1) heated to 45°C were added to the homogenized samples. After vigorous mixing, an additional 2 mL of chloroform and 2 mL of acidified water, pH 2.5, were added to the homogenate. After centrifugation, the lower chloroform:H₂O layer was removed by aspiration and dried under a flow of nitrogen gas. The residue was suspended in heptane, and the OD at 233 nm was compared to a heptane blank. Purified reagents for each of these biochemical assays were obtained from Sigma Chemical Co., St. Louis, MO. For each assay, the nonperfused accessory lobe of the lung was weighed before and after drying to constant weight in an 80°C oven to determine wet/dry weight ratios in the air and O₂ treated and control groups. In addition to using the ratio as an index of pulmonary edema, it was used for expression of the assay results as absorption units or nmol/g lung dry weight.

Statistical analysis. Hyperoxic survival rates of the treated *versus* control rat pups were compared by χ^2 testing. Analysis of variance testing followed by Duncan's multiple-range testing was used for comparison of lung biochemical values and morphometric values, using $p < 0.05$ as the level of significant differences between treatment groups (25).

RESULTS

Hyperoxic survival, lung injury, and lung biochemistry. There was no evidence for any serious toxic effects of 21-AS in the 4- to 14-d-old rat pups. All the 21-AS-treated rats maintained in room air for 10 d survived, as did all the control pups. Similarly, weight gain remained comparable in both groups of room-air pups.

The data in Table 1 indicate that, although the 10-d survival rates of the 21-AS-treated animals was greater than the hyperoxia-exposed control pups (79 *versus* 70%), these differences did not reach statistical significance ($p = 0.08$). However, there was evidence for decreased O₂-induced lung injury in the 21-AS *versus* the control-group survivors, with reduced pulmonary edema by lung wet:dry weight ratios and, perhaps more definitively, by the histologic finding of intraalveolar edema in 39% of the O₂-control-group lung sections *versus* only 18% of the O₂-21-AS animal lung sections.

Lung lipid peroxidation was assessed by two methods, as generally recommended because of the vagaries of any single assay for *in vivo* lipid peroxide products (23). The results (Table 2) indicate that, after 3 d in hyperoxia, the 21-AS group had a significantly smaller increase in malondialdehyde than the O₂

control group (+16 *versus* +45%) when compared with the air controls. The conjugated diene levels at 3 d in hyperoxia, however, were lower in both O₂-exposed groups than in the air-exposed animal lungs—the O₂ control value was 17% lower, whereas the 21-AS group value was 57% lower than the air control lung value. At 6 d in O₂, malondialdehyde values were again higher in the O₂ control ($\uparrow 20\%$ *versus* air control) than the 21-AS group ($\downarrow 21\%$). The conjugated diene values at 6 d were elevated in both O₂ groups *versus* the air control values, with slightly less of an increase in the 21-AS animal lungs ($\uparrow 31\%$) than in the O₂ control lungs ($\uparrow 40\%$). Thus, the consistent finding for the lipid peroxidation results was lower malondialdehyde and conjugated diene levels in the 21-AS O₂ rats compared with the O₂ controls at both time points examined. However, because statistically the only significant difference between the O₂-exposed group values was for the conjugated dienes at 3 d, the lipid peroxidation data are not easy to interpret definitively.

Lung morphometry (acute and chronic). In each of three separate morphometric studies, there was evidence of a significant protective effect of 21-AS treatment against hyperoxia-induced inhibition of normal alveolarization and lung surface-area expansion ($p < 0.05$ difference *versus* O₂ control values for L_M, ISA/100 g for each study). The composite data are illustrated in Figure 1, which demonstrates the significantly smaller L_M, the significantly expanded lung ISA, and, more physiologically meaningful, the expanded ISA/100 g body weight in the O₂-21-AS-treated rat lungs compared with the lungs of the control O₂-exposed group. Compared with the normal values for these three lung developmental parameters in the pooled air-exposed animal lungs, the mean values for the O₂ 21-AS *versus* the O₂ control group, respectively, were as follows: L_M $\uparrow 13\%$ *versus* $\uparrow 29\%$; ISA, $\downarrow 9\%$ *versus* $\downarrow 24\%$; and ISA/100 g, $\downarrow 9\%$ *versus* $\downarrow 21\%$ ($p < 0.05$ for all comparisons between O₂ groups).

Table 3 lists the comparative lung elastin data in the four groups of study rat pups, both the biochemical values and the morphometric results. Increased lung elastin deposition is integral to the normal postnatal restructuring (septation and alveolarization) of the newborn lung. By both measures of elastin in Table 3, it is apparent that the 21-AS-treated rat pups were significantly protected from hyperoxic inhibition of normal elastin deposition compared with the O₂ control group. Figure 2 shows representative photomicrographs that reflect the elastin data in Table 3 as well as the morphometric data in Figure 1.

The data in Table 4 demonstrate the chronic consequences of early postnatal inhibition of lung development by hyperoxia. By early adulthood, the lungs of the O₂ control group animals still demonstrate much-enlarged terminal air spaces and significantly reduced ISA/100 g compared with the lungs of normal adult animals. An ameliorative effect of 21-AS treatment during early postnatal hyperoxic exposure on these chronic lung alterations is clearly demonstrated by the data in Table 4.

Finally, the detrimental effects of early hyperoxic exposure on normal pulmonary vascular development and the subsequent pulmonary hypertension and right ventricular hypertrophy that results from early hyperoxic exposure are indicated in Table 5. In the O₂ control group, early hyperoxic exposure resulted in significantly increased right ventricular mass evident by each means of expression in Table 5. Treatment with 21-AS during early O₂ exposure had a substantial protective effect against these chronic cardiopulmonary consequences—reducing right ventricular enlargement by 50 to 67% depending on the means of expressing this late sequel of early neonatal hyperoxia.

DISCUSSION

It is puzzling why newborn animals resist severe pulmonary O₂ toxicity (florid pulmonary edema, hemorrhage, lethality) so well yet remain so susceptible to severe O₂-induced inhibition of normal lung development. It has been rather well established that the biochemical basis for the comparatively marked O₂

Table 1. Comparative survival rates and lung edema: 10 d in hyperoxia*

Treatment group	Survival rate (%)	Lung wet/dry wt (ratio)	Histologic intraalveolar edema (no. with edema/no. of lung sections)
Air controls	68/68 (100)	5.13 $\pm$ 0.15	0/40 (0)
Air 21-AS	67/67 (100)	5.14 $\pm$ 0.20	0/42 (0)
O ₂ controls	51/73 (70)	6.05 $\pm$ 0.49 ($\uparrow 18$)	19/49 (39)
O ₂ 21-AS	55/70 (79)	5.77 $\pm$ 0.37 ($\uparrow 12$)†	10/55 (18)†

* Four-d-old rat pups were exposed to $>95\%$ O₂ or room air for 10 d and treated with either 21-AS (15 mg/kg/d) or vehicle. Values are mean $\pm$ 1 SD; $n = 15$ /group for wet:dry ratios. For histologic sections, three to four sections per animal lung were examined, 12 to 16 animals per group, for presence or absence of pink-staining proteinaceous edema within the alveolar air spaces.

† Numbers in parentheses are percentages. $p < 0.05$ between O₂ controls and O₂ 21-AS groups.

Table 2. Comparative measures of lung lipid peroxidation in air- and O₂-exposed rat pups*

Treatment group	3 d		6 d	
	Malondialdehyde (nmol/g dry wt; n = 4)	Conjugated dienes (OD/g dry wt; n = 4)	Malondialdehyde (nmol/g dry wt; n = 4)	Conjugated dienes (OD/g dry wt; n = 3-4)
Air controls	17.2 ± 1.8	23.4 ± 1.1	17.7 ± 2.0	15.1 ± 2.8
Air 21-AS	15.5 ± 1.9	20.8 ± 2.4	15.0 ± 2.7	14.5 ± 3.4
O ₂ controls	25.0 ± 2.1† (↑45%)	19.5 ± 0.7 (↓17%)	21.2 ± 5.3 (↑20%)	21.1 ± 1.9† (↑40%)
O ₂ 21-AS	19.9 ± 1.8 (↑16%)	10.0 ± 1.3‡ (↓57%)	14.0 ± 1.0§ (↓21%)	19.8 ± 1.5 (↑31%)

* Four-d-old rat pups were exposed to >95% O₂ or room air for 3 or 6 d and treated with either 21-AS (15 mg/kg/d) or vehicle. Values are mean ± 1 SEM. Values in parentheses are mean percentage change for O₂ group value compared with air control group value (↑ or ↓).

† $p < 0.05$ (> air groups).

‡ $p < 0.05$ (< air groups); $p < 0.05$ (< O₂ control).

§ $p < 0.05$ (< air control).

Fig. 1. Effects of 10-d exposure to >95% O₂ on lung morphometric parameters. LV, lung volume. Data are mean values ± 1 SD bar for 12-15 rat lungs/group. The 21-AS group received daily 15-mg/kg injections during O₂ or air exposure (age 4-14 d); controls received equivolume diluent injections. (L_M normally decreases between 4 and 14 d of life, whereas ISA increases). *, $p < 0.05$ vs air control and air 21-AS values; t, $p < 0.05$ between O₂ control and O₂ 21-AS values.

Table 3. Comparative lung elastin concentrations: 10 d in hyperoxia*

Treatment group	Biochemical elastin concentration (mg/g dry lung wt)	Morphologic elastin relative length density (elastic fiber transections/hpf)
Air controls	58.5 ± 5.8	17.9 ± 1.5
Air 21-AS	58.3 ± 10.8	18.1 ± 1.7
O ₂ controls	43.6 ± 9.6 (↓25%)†	12.1 ± 1.4 (↓33%)†
O ₂ 21-AS	50.6 ± 7.6 (↓13%)	15.4 ± 1.6 (↓14%)†‡

* Four-d-old rat pups were exposed to >95% O₂ or room air for 10 d and treated with either 21-AS (15 mg/kg/d) or vehicle. Values are mean ± 1 SD for 12 samples/group for elastin biochemical assay and 10 lung sections examined/group for elastin morphometric study. hpf, high-power field (×400).

† $p < 0.05$ vs air controls and air 21-AS.

‡ $p < 0.05$ between O₂ controls and O₂ 21-AS groups.

tolerance of neonatal *versus* adult animals is the ability of the newborn lung to respond to hyperoxic challenge with significantly increased pulmonary AOE levels, an adaptive response that adult animals seem unable to manifest during >95% O₂

exposure (1-3, 26). Yet, despite this endogenous biochemical response, the newborn is apparently unable to protect its growing lung from hyperoxia-associated inhibition of normal lung development. Even when additional AOE elevations could be stimulated by prenatal dexamethasone treatment (27) or by exogenous AOE administration (28), the hyperoxia-induced inhibition of alveolarization persisted.

One possible explanation for this puzzling aspect of hyperoxic challenge in the newborn is the concept of site-specific O₂ free-radical damage (29, 30). This concept maintains that a normal or even an augmented antioxidant defensive system may not be able to completely detoxify O₂ free radicals at specific intracellular sites where these defenses are not very concentrated. The major AOE defenses are primarily cytosolic (CuZn-superoxide dismutase, glutathione peroxidase), mitochondrial (Mn-superoxide dismutase), and peroxisomal (catalase) in location. Other important nonenzymatic antioxidant components are also cytosolic (vitamin C, β-carotene, glutathione) or integrated into lipid membranes of the cell (vitamin E) (1, 29, 30). There are very sparse experimental data about intranuclear antioxidant capacities (29-31), and it has been suggested that O₂ free radicals generated in or in close proximity to the nucleus may readily instigate damaging reactions with vital nuclear components

Fig. 2. Comparative light-level micrographs of representative lung sections from air- and hyperoxia-exposed rat pups age 14 d (exposure period age 4–14 d). Orcein stain to emphasize elastin fibers. A, Air, original magnification 160 $\times$; B, O₂, 160 $\times$; C, air, 100 $\times$; and D, O₂, 100 $\times$. Note the smaller and more homogeneous alveoli size in the air- (A) vs the O₂-exposed rat lungs (B) and the more circumferential arrangement of the dark-staining elastin fibers. After hyperoxia (B), not only is the relative length density of fibers reduced and the circumferential arrangement around the alveoli lacking, but the fibers themselves in the O₂-exposed lungs appear either aggregated or frayed in areas (*). Note again in the air lung at lower magnification (C) the rather homogeneous small alveoli, some showing circumferential elastin fibers. Mouths of alveoli coming off terminal airway or alveolar duct tend to have dense elastin deposits (X). In the O₂-exposed lung (D), the alveoli are again much larger and more heterogeneous in diameter, and frayed elastin fibers (*) and more haphazard elastin deposition than in the air-exposed normal 14-d rat lung are evident.

Table 4. Lung morphometry of adult rats exposed to hyperoxia as newborns*

Treatment group	Air space (%)	L _M (μ m)	ISA (cm ²)/100 g
Air controls (n = 8)	75.4 $\pm$ 1.3	53.3 $\pm$ 2.3	1483 $\pm$ 138
O ₂ controls (n = 5)	80.1 $\pm$ 1.0†	79.8 $\pm$ 3.0† ($\uparrow$ 50%)	1304 $\pm$ 59† ($\downarrow$ 12%)
O ₂ 21-AS (n = 5)	77.1 $\pm$ 1.2†‡	69.2 $\pm$ 3.7†‡ ($\uparrow$ 30%)	1424 $\pm$ 135 ($\downarrow$ 4%)

* Sixty-three-d-old rats were exposed to >95% O₂ (or room air) from age 4 to 14 d and then returned to room air. Rats were treated with 21-AS (15 mg/kg/d) or vehicle during exposure period in hyperoxia. Values are means $\pm$ 1 SD for n rats/group. Values for air-21-AS-treated group were similar to those for air-diluent-treated group and are pooled here as air control group.

† $p < 0.05$ vs air control value.

‡ $p < 0.05$ between O₂ 21-AS and O₂ control group.

(DNA) (29–31). Decreased cell proliferation is a well-known concomitant of hyperoxic exposure in the newborn (1, 8, 13, 16, 26, 32). Because the process of septation undoubtedly involves a very coordinated proliferation of specific lung cells, an inhibitor of DNA synthesis like hyperoxia, or disruption of DNA integrity

by O₂ free radicals, can easily be viewed as causing discoordination of this developmental process.

Because Fe²⁺ is believed necessary to initiate damaging DNA-O₂ radical interactions (1, 3, 9, 10, 29), Fe chelators may be an effective mode of treatment to “site-specifically” prevent oxidative DNA degradation. The 21-AS drugs are reportedly many-fold more potent Fe chelators than the commonly used agents deferoxamine, or o-phenanthroline, or EDTA, and more easily penetrate cells due to their lipophilic chemical nature (14, 33). The 21-AS drugs are also reportedly very effective inhibitors of lipid peroxidation by means of scavenging lipid peroxyl radicals (14, 33, 34). Lipid peroxidation is believed by many to be a key mechanism of cellular injury and cell death caused by O₂ radicals; and hyperoxic pulmonary edema is considered a reflection of altered lung cell membrane permeability due to oxidative attack on unsaturated membrane lipids (3, 8, 10, 13, 23, 24, 35). Very recent evidence from electron paramagnetic resonance studies suggests that the 21-AS drugs may also directly scavenge hydroxyl radicals in both *in vitro* and *in vivo* test systems (36).

Because of these multiple actions proposed for the 21-AS drugs—Fe²⁺ chelation, O₂ radical scavenging, and prevention of lipid peroxidation chain reactions—the prototype agent, U74389F, seemed to us to be a good treatment choice to test under hyperoxic conditions. The overall morphometric results would strongly suggest that U74389F treatment did indeed have

Table 5. Right heart changes in adult rats exposed to hyperoxia as newborns*

Treatment group	BW	RV/BW	RV/LV + septum	RV/RV + LV + septum (%)
Air controls (n = 8)	277 ± 22	0.065 ± 0.005	0.320 ± 0.023	24.2 ± 1.3
O ₂ controls (n = 5)	288 ± 24	0.093 ± 0.014† (↑43%)	0.496 ± 0.085† (↑55%)	33.0 ± 3.8† (↑36%)
O ₂ 21-AS (n = 5)	273 ± 16	0.079 ± 0.007†‡ (↑22%)	0.382 ± 0.054‡ (↑19%)	27.6 ± 2.7‡‡ (↑14%)

* Sixty-three-d-old rats were exposed to >95% O₂ (or room air) from age 4 to 14 d and then returned to room air. Rats were treated with 21-AS (15 mg/kg/d) or vehicle during exposure period in hyperoxia. Values are means ± 1 SD for n rats/group. All values for air 21-AS animals were similar to air-diluent-treated rats and are pooled here as air control group. RV, right ventricle wt; LV, left ventricle wt; BW, body wt.

† $p < 0.05$ vs air control value.

‡ $p < 0.05$ between O₂ 21-AS and O₂ control group.

a substantial protective effect against the acute inhibition of lung development associated with neonatal hyperoxia. The lipid peroxidation data give partial support to the expected effects of 21-AS treatment (Table 2). Due to the vagaries of any single assay for *in vivo* assessment of lipid peroxide products (23), both thiobarbituric acid-reactive products (malondialdehyde) and conjugated dienes were assessed. Postmortem lipid peroxidation or lipid peroxidation due to the preparative procedures for the assays should have been blocked by the addition of butylated hydroxytoluene to the assay solutions. The results consistently showed lower malondialdehyde and conjugated diene levels in the 21-AS-treated rat lungs in O₂ compared with the O₂ controls at both time points examined. (The relatively small number of lung samples tested may account for the lack of statistical significance in these single experimental studies.) The reported action of 21-AS as a potent Fe²⁺ chelator and as a direct antioxidant agent (scavenger of hydroxyl free radical) was not tested in these studies.

Elastin is considered to be integrally involved in the septation process as the electron microscopic demonstration of elastin near the tips of the secondary septa that subdivide the saccules into smaller alveoli would suggest (6). Hyperoxia is reported to inhibit lung elastin synthesis (37), and this may be another key means (in addition to inhibiting cell proliferation) by which hyperoxia disrupts the orderly septation/alveolarization process. Both the biochemical and morphometric elastin data (Table 3) support this effect of hyperoxia, as well as give evidence for a protective sparing of elastin development in the 21-AS treatment group.

Hyperoxic exposure during the first 2 wk of life [the so-called "critical period" for lung restructuring (38)] permanently alters adult lung morphology (1, 37–40). After early postnatal exposure to hyperoxia, the mean diameter of the terminal air spaces remains approximately similar to the lung saccule diameter at birth, and the alveoli in the adult animal are found to be much enlarged compared with the alveolar diameter in the normal adult animal, with the total number of alveoli significantly reduced (40). Additionally, with exposure to hyperoxia in early life, chronic vascular alterations occur manifested by extension of muscular walls into more distal and smaller lung arteries that are normally nonmuscularized, and pulmonary hypertension with increased pulmonary vascular resistance develops (41–44). Shaffer *et al.* (42) and Thibeault *et al.* (44) have demonstrated the consequences of these vascular changes in their studies of early postnatal hyperoxia, with resultant right ventricular hypertrophy (cor pulmonale) 2° to elevated pulmonary vascular pressures in the adult animals. The data in Tables 4 and 5 confirm these chronic changes. And, as with the acute lung changes after early life exposure to hyperoxia, 21-AS treatment was found to have a meaningful protective effect *versus* the chronic lung structural sequelae, as well as against right heart hypertrophic changes.

Although the period of rapid alveolarization in the newborn rat lung is very abbreviated compared with the human situation, the hyperoxic effects noted in the developing rat lung may have direct implications for the lung of the prematurely born human

infant. We suggest this because: 1) very low birth weight premature infants may require elevated F_iO₂ therapy for periods of months; and, 2) this hyperoxic treatment is occurring at a time when the fetal lung should normally be entering its most rapid phase of lung development and surface-area expansion (with the normal formation of an estimated 40 × 10⁶ alveoli between 26 wk gestation and term) (45, 46). In recent years, several morphometric studies of the lungs of human infants who died with chronic lung disease (bronchopulmonary dysplasia) have clearly illustrated much-enlarged terminal air spaces, markedly reduced total number of alveoli, and proportionately reduced lung internal surface area (47–49). These changes indeed appear quite similar to the findings of morphometric studies in the hyperoxia-exposed newborn animal. The recent pathologic study by Margraf *et al.* (47) also demonstrates the reduced elastin fiber deposition and altered (frayed) appearance of the elastin fibers in the lungs from premature infants who died from chronic lung disease after long-term O₂ treatment. The degree of inhibition of normal lung development in these O₂-requiring infants with bronchopulmonary dysplasia is dramatic, with a total lung alveoli complement of only 20 to 40 × 10⁶ *versus* 60 to 200 × 10⁶ for age-matched control infants and internal respiratory exchange surface areas totaling ≤1.0 m² *versus* the normal values of 2 to 6 m² (49).

Thus, in well-established experimental models, the development of effective agents to specifically counteract the vulnerability of the rapidly developing lung to the potent growth inhibitory effects of hyperoxic exposure may hold future promise for clinical application in the large population of O₂-requiring tiny premature infants who are at risk of developing chronic lung disease and a poor respiratory outcome.

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