Oxygen Toxicity: Comparison of Lung Biochemical Responses in Neonatal and Adult Rats

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Summary

Neonatal rats (4–7 days old) and adult rats (approximately 80 days old) were continuously exposed to either 96–98% oxygen or air. Examination of the lungs of neonatal rats, who survived 5 days of oxygen exposure with no evidence of respiratory distress, showed significant increases in the pulmonary superoxide dismutase (SOD) activity (peak value: 144% of air-exposed controls), glutathione peroxidase (GP) activity (126%), glutathione reductase (GR) activity (122%), reduced glutathione (GSH) level (176%), and glucose-6-phosphate dehydrogenase activity (151%). Adult rats, most of whom succumbed within 3 days of oxygen exposure, did not show any significant increase in the activities of pulmonary SOD, GP, GR, and the level of GSH as compared to the air-exposed adult animals. Glucose-6-phosphate dehydrogenase was significantly elevated in the 72-hr oxygen-exposed adult rats. It is concluded that increases in the lung complement of SOD, GR, GP, and GSH in the neonatal rat during oxygen challenge may provide the mechanism(s) for their increased tolerance to hyperoxia-induced lung injury as compared to the adults.

Speculation

Further knowledge about the role of the antioxidant defense mechanism(s) of the neonatal lung may have important implications in the pursuit of the etiology of neonatal respiratory disease and in the identification of agent(s) that may minimize pulmonary toxicity associated with oxygen therapy.

The toxic consequences of oxygen therapy have long been a subject of intensive clinical and experimental concern (5, 14, 38). Considerable evidence exists which links oxygen to the development of two neonatal diseases: retrolental fibroplasia (14) and bronchopulmonary dysplasia (26, 39). Although past research efforts have concentrated mainly on the characterization of the histopathology of oxygen toxicity, recent work has been directed towards the understanding of the biochemical mechanism of oxygen toxicity. It is now generally believed that oxygen-induced cell toxicity is the result of the formation of highly reactive free radicals (9, 35).

Superoxide anion (O_2^-), a highly reactive free radical formed in the cell by univalent reduction of oxygen, has generally been suspected as a prime causative agent in oxygen-induced cell toxicity (9, 35). During prolonged high oxygen exposure, superoxide anion is thought to react with polyunsaturated lipids in the cell, particularly those in cell membranes, thereby initiating a chain reaction of lipid peroxidation, which subsequently leads to destruction of essential cellular components and disruption of normal cellular functions (4, 9). Superoxide anion and its toxic byproducts may further upset the homeostasis of the cell

either by denaturation of intracellular enzymes and/or nucleoproteins (20) or by some other cytotoxic reaction. Other reactive secondary products of superoxide anion, hydroxyl radical (OH·), peroxy radical (HOO·), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), have also been suggested to participate in oxygen-induced cell damage (9, 17).

These free radical-induced cytotoxic reactions, however, do not proceed uncontrolled. It is believed that oxygen-metabolizing cells possess antioxidant protective mechanisms which minimize the toxicity of free radicals and lipid peroxides by converting them to less toxic substances (6, 9, 15, 35). Of all the cells in the body, the greatest challenge to these protective systems would occur in the cells of the lung, since these cells are exposed to the highest concentrations of oxygen in the body during either normoxic or hyperoxic conditions.

There are at least two potential protective antioxidant defense mechanisms present in the cells of the lung that may offset oxygen-induced cell injury. One mechanism involves the enzyme, SOD (EC 1.15.11). SOD catalyzes, by dismutation, the reaction:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$
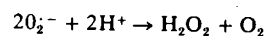
SOD has been proposed to play an important role in the detoxification of oxygen both in the microorganism (24, 35) and in mammalian species (1, 6, 7, 11, 15, 33, 37). As the production of superoxide anion exceeds the capacity of the defense mechanism, *i.e.*, SOD, some superoxide anion may escape dismutation and initiate lipid peroxidation with the resultant formation of toxic lipid peroxides.

To further protect the lung from injury by lipid peroxides, the glutathione system, which consists of a battery of enzymes, functions as an additional antioxidant defense mechanism in the lung. The first enzyme in this system, GP (EC 1.11.1.9.), is generally thought to reduce the toxic lipid peroxides to the correspondingly less toxic hydroxy fatty acids, utilizing GSH as a cofactor (3, 15). Recent evidence, however, suggests that GP may exert its primary effect by preventing free radical attack on polyunsaturated membrane lipids rather than by reducing membrane lipid peroxides to hydroxy fatty acids (22). A second enzyme, GR (EC 1.6.4.2), is necessary to regenerate the pool of GSH by reducing oxidized glutathione at the expense of NADPH. The depleted NADPH may then be replenished by one of several cellular enzymatic reactions including glucose-6-phosphate dehydrogenase (G-6-PD, EC 1.1.1.49).

It has long been recognized that the immature animal, although not totally immune to oxygen toxicity, is nevertheless more resistant to oxygen-induced lung damage than the adult (2, 5, 12, 18, 29, 40). We have previously observed that exposure of young animals to only 24 hr of 85% oxygen produced a significant increase in pulmonary SOD activity which was not seen in adult animals exposed under identical

conditions (11). We postulated that the rapid increase in pulmonary SOD activity in young animals during hyperoxic exposure may be an important factor accounting for their reduced susceptibility to pulmonary hyperoxic injury compared to the older animal (11). To further test this hypothesis, and to extend the study to the glutathione system, we have examined in the present study the response of both these antioxidant defense mechanisms in the lungs of neonatal and adult rats during continuous exposure to 96–98% oxygen.

MATERIALS AND METHODS

Neonatal animals used for these studies were Sprague-Dawley albino rats bred and raised in the Animal Care Center at The University of Iowa. In each experiment, paired litter mates from several litters were randomly assigned to either the oxygen-exposed or air-exposed control groups. A foster mother was then provided for each mixed litter. Unless otherwise specified, male and female rats of age 4–7 days were used. Male Sprague-Dawley albino rats (275–325 g), obtained from BioLab Corporation (St. Paul, MN), were used in the adult studies.

Both neonatal and adult rats were continuously exposed to either oxygen or room air in identical stainless steel exposure chambers (11). The oxygen and CO_2 levels in the chamber were monitored continuously with Beckman model OM-11 and LB-2 gas analyzers (Beckman Instruments, Inc., Schiller Park, IL). The oxygen concentration was maintained at 96–98% and the CO_2 concentration was less than 0.4% throughout the exposure period. The temperature of the chamber was 24–26° and the vapor pressure 10–15 mm Hg. Animals were fed Wayne Lab-Blox pellet diet and given water *ad libitum*. The foster mothers were interchanged daily between litters exposed to oxygen and room air in order to avoid oxygen intoxication of the mothers and to minimize differences in nursing abilities. The chambers were opened daily for 15 min for cleaning, to change food and water, and to remove animals to be killed.

Animals were killed daily by decapitation and their lungs were carefully perfused with ice-cold isotonic buffer (0.1 M potassium phosphate, 0.15 M KCl, pH 7.4) through the pulmonary artery. The lungs were then trimmed of extraparenchymal trachea-bronchial and vascular tissue and homogenized for 2 min in a 1:15 (w/v) dilution with ice-cold 0.005 M phosphate buffer, pH 7.8, using a Sorvall Omnimixer (Ivan Sorvall Inc., Newtown, CT). For neonatal animal samples, lung tissue was pooled from four to eight rat pups. SOD, GSH, and DNA levels were determined in the lung homogenates and GP, GR, and G-6-PD activities were determined in the 15,000 × g lung supernatant. SOD activity was determined by the standard determination of the inhibition of the xanthine and xanthine oxidase-catalyzed reduction of ferricytochrome *c* (23). DNA was determined according to the method described by Richards (32). GSH was determined by the enzymatic method of Klotsch and Bergmeyer (16) as modified by Delucia *et al.* (8). GP activity of the cytosol fraction was determined by coupling peroxide reduction to NADPH oxidation through GR using H_2O_2 as substrate (28). Cytosol GR activity was determined by following NADPH disappearance (31). G-6-PD activity was determined by the method as described by Langdon (19). Lung enzyme activities, DNA, and GSH levels were expressed on a per lung basis as percentage of control values.

For histologic studies, three adult rats alive at the end of 72 hr of oxygen exposure were killed. The thoracic cavity was opened, the trachea was cannulated, and 10% buffered formalin was instilled via the airway at the perfusion pressure of 25–30 cm water. Three neonatal animals were also killed at the end of 5 days of oxygen exposure and their lungs were fixed by immersion in buffered formalin. Various areas of the lung were embedded in paraffin and stained with hematoxylin and eosin for light microscopic evaluation of lung morphology.

Another group of neonatal rats was exposed to either air or 96–98% oxygen for a total period of 4 days. At the end of the

exposure period, the animals were killed and the activity of the following enzymes in the lung were measured: alkaline phosphatase (EC 3.1.3.1) (21) and β-glucuronidase (EC 2.3.1.31) (10) were determined in the lung homogenate; glutamic dehydrogenase (EC 1.4.1.3) (36), fructose-1,6-diphosphate aldolase (EC 4.1.2.13) (30) and catalase (EC 1.11.1.6) (13) were analyzed in the 15,000 × g lung supernatant.

Student's *t*-test and paired *t*-test were used for statistical analysis of results from adult and newborn animals, respectively. *P* values equal to or less than 0.05 were considered significant (two-tailed).

RESULTS

In a preliminary study to assess the effect of age on oxygen toxicity, the mortality was determined among rats of varying ages (newborn to adult) exposed to 72 hr of 96–98% oxygen. The results are shown in Figure 1. Under the conditions of these studies, all rats of age 4 weeks or younger survived the 3 days of oxygen exposure. However, only 25% of rats over 7 weeks of age were alive at the end of this exposure period.

Experiments were then carried out to compare the pulmonary biochemical responses of the two selected age groups of rats, namely the neonatal rat (4–7 days old) and the adult rat (approximately 80 days old), to 96–98% oxygen exposure. Continuous exposure of the adult rats to 96–98% oxygen was terminated at the end of the third day because of the already high mortality rate at this time. The neonatal rat experiment was continued for a total period of 5 days, at which time all the neonatal rats were still alive.

The lung weight to body weight ratio was calculated to provide an index of pulmonary edema (Table 1). Neonatal animals showed no significant change in the lung weight to body weight ratio throughout the 120 hr of oxygen exposure, whereas the 72-hr oxygen-exposed adult rats showed a significant increase in this parameter over the adult air-exposed controls. The above findings were consistent with the gross autopsy and light microscopic examinations of the neonatal and adult rat lungs. The oxygen-exposed adults uniformly displayed fluid in their thoracic cavity, whereas none of the neonatal rats had any gross evidence of pulmonary edema at autopsy. Light microscopic characteristics of the 72-hr oxygen-exposed adult rat lung included thickened alveolar septa, fluid-filled alveolar spaces, focal areas of alveolar hemorrhage, and accumulation of alveolar macrophages within the alveolar space. Examination of 120-hr oxygen-exposed neonatal rat lungs revealed only minimal alveolar wall thickening and alveolar edema.

Table 2 indicates that the lung DNA content remained unchanged in adult rats throughout the oxygen exposure period.
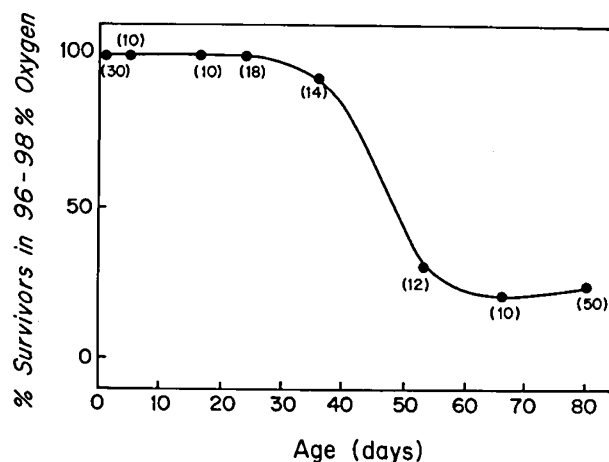

Fig. 1. Percentage of survival in rats of varying ages after exposure to 96–98% oxygen for 72 hr. Numbers in parentheses indicate the number of animals in each age group that were exposed to oxygen.

Table 1. *Effect of 96-98% oxygen exposure on lung weight to body weight ratio in neonatal and adult rats*

Treatment	Lung wt/body wt ($\times 10^{-2}$)[1]	
	Neonate (n = 4)	Adult (n = 6-10)
24 hr		
Air	1.63 ± 0.04	0.455 ± 0.004
O_2	1.61 ± 0.15	0.441 ± 0.019
48 hr		
Air	1.62 ± 0.01	0.474 ± 0.040
O_2	1.74 ± 0.04	0.553 ± 0.050
72 hr		
Air	1.63 ± 0.04	0.452 ± 0.012
O_2	1.66 ± 0.05	0.882 ± 0.046[2]
96 hr		
Air	1.61 ± 0.11	
O_2	1.52 ± 0.05	
120 hr		
Air	1.53 ± 0.20	
O_2	1.47 ± 0.11	

[1] Values shown are means ± SE.

[2] Significantly different from the respective air-exposed control values ($P < 0.05$).

Table 2. *Effect of 96-98% oxygen exposure on DNA content in neonatal and adult rat lungs*

Treatment	Lung DNA content (mg/lung)[1]	
	Neonate (n = 4)	Adult (n = 6-10)
24 hr		
Air	1.6 ± 0.2	7.3 ± 0.5
O_2	1.6 ± 0.2	7.7 ± 1.0
48 hr		
Air	1.7 ± 0.1	7.5 ± 0.8
O_2	1.6 ± 0.2	7.0 ± 0.5
72 hr		
Air	2.1 ± 0.2	9.5 ± 0.5
O_2	1.7 ± 0.2[2]	8.8 ± 0.5
96 hr		
Air	1.8 ± 0.2	
O_2	1.5 ± 0.1	
120 hr		
Air	2.4 ± 0.1	
O_2	1.7 ± 0.2[2]	

[1] Values shown are means ± SE.

[2] Significantly different from the respective air-exposed control values ($P < 0.05$).

Lung DNA content in the 72- and 120-hr oxygen-exposed neonatal rats was, however, significantly lower than the respective air-exposed controls whose levels increased with age.

The lung biochemical responses of the oxygen-induced neonatal and adult rats are shown in Figures 2-6. Twenty-four hours after the start of oxygen exposure, SOD activity in the neonatal rat lung increased to 144% of control (Fig. 2). This initial increase in SOD activity declined to about 12% on day 2 and remained between 10 and 15% higher than control activity up to the end of the fifth day of oxygen exposure. The oxygen-exposed adult rat lung, in contrast, consistently showed a progressive decrease in SOD activity.

A similar pattern was seen with the effect of oxygen on GP (Fig. 3), GR (Fig. 4), GSH (Fig. 5), and G-6-PD (Fig. 6) activities. The lungs of the oxygen-exposed neonatal rats showed a progressive increase in activity levels which plateaued after 3-4 days of exposure. Peak values (expressed as a percentage of air control) in oxygen-exposed neonatal lungs were; GP (126%), GR (122%), GSH (176%), and G-6-PD (151%). The oxygen-exposed adult rats failed to show any increase in GP and GR
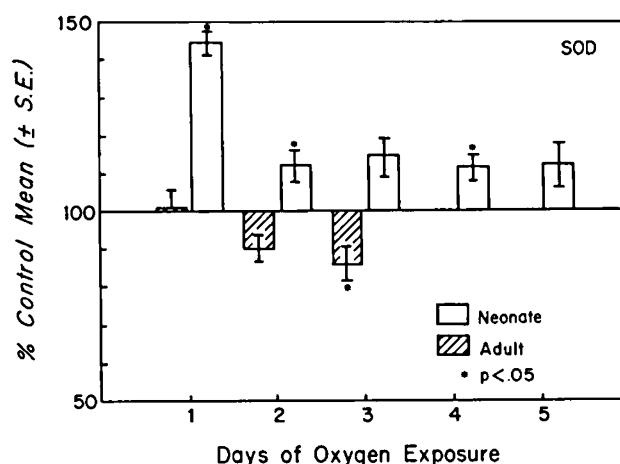

Fig. 2. Effect of 96-98% oxygen exposure on SOD activity in neonatal and adult rat lungs. Each bar represents the percentage of control mean (±SE) of n = 4 for neonatal and n = 6-10 for adult studies. Mean control SOD activities (units per lung) were: 2020 ± 140 for adult rat; 240 ± 58 for neonatal rat at day 5.
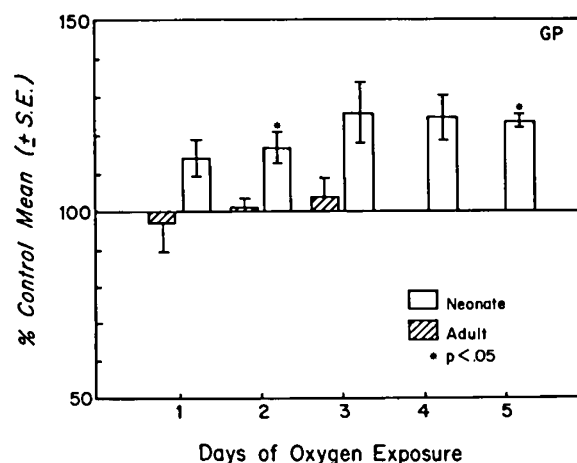

Fig. 3. Effect of 96-98% oxygen exposure on GP activity in neonatal and adult rat lungs. Each bar represents the percentage of control mean (±SE) of n = 4 for neonatal and n = 6-10 for adult studies. Mean control GP activities (micromoles of NADPH oxidized per min per lung) were: 16.6 ± 1.1 for adult rat; 1.02 ± 0.29 for neonatal rat at day 5.

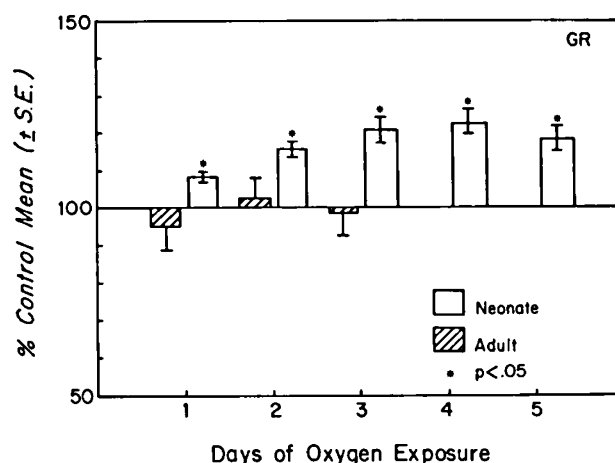
Fig. 4. Effect of 96-98% oxygen exposure on GR activity in neonatal and adult rat lungs. Each bar represents the percentage of control mean (±SE) of n = 4 for neonatal and n = 6-10 for adult studies. Mean control GR activities (micromoles of NADPH oxidized per min per lung) were: 2.49 ± 0.06 for adult rat; 0.234 ± 0.024 for neonatal rat at day 5.
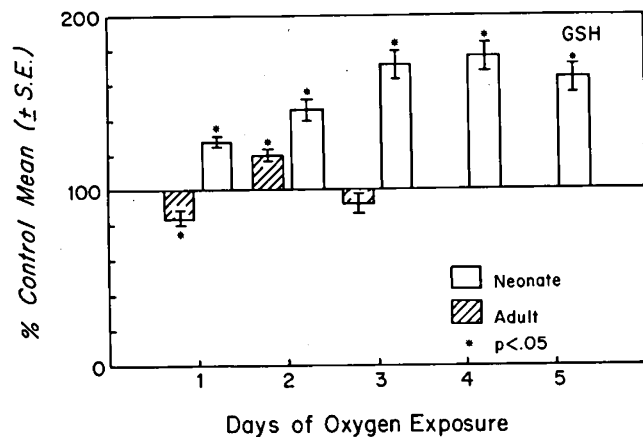
Fig. 5. Effect of 96–98% oxygen exposure on GSH level in neonatal and adult rat lungs. Each bar represents the percentage of control mean (±SE) of $n = 4$ for neonatal and $n = 6$–10 for adult studies. Mean control GSH levels (micromoles per lung) were: 1.45 ± 0.03 for adult rat; 0.249 ± 0.046 for neonatal rat at day 5.
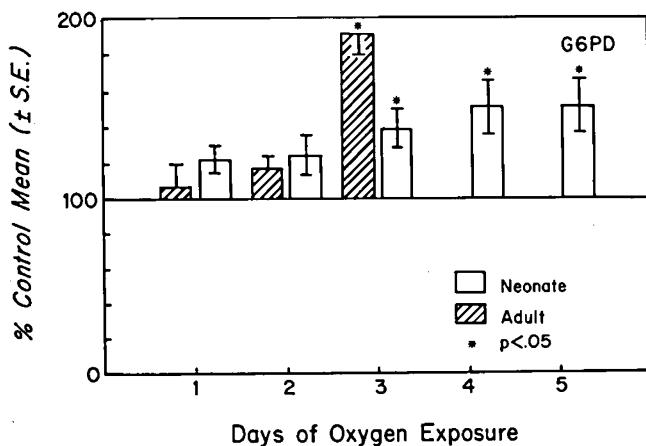

Fig. 6. Effect of 96–98% oxygen exposure on G-6-PD activity in neonatal and adult rat lungs. Each bar represents the percentage of control mean (±SE) of $n = 4$ for neonatal and $n = 6$–10 for adult studies. Mean control G-6-PD activities (micromoles of NADPH reduced per min per lung) were: 2.13 ± 0.08 for adult rat; 0.180 ± 0.045 for neonatal rat at day 5.

Table 3. *Effect of oxygen exposure on activity of other pulmonary enzymes in neonatal rat*[1]

Enzymes	Air	Oxygen
Alkaline phosphatase (nmol p-nitrophenol liberated/hr/lung)	134 ± 11	127 ± 10
β-Glucuronidase (μmol phenolphthalein glucuronide hydrolyzed/hr/lung)	54.7 ± 3.9	60.1 ± 1.6
Glutamic dehydrogenase (nmol NADH oxidized/min/lung)	100 ± 10	78 ± 16
Fructose-1,6-diphosphate aldolase (nmol NADH oxidized/min/lung)	447 ± 30	510 ± 11
Catalase (IU/lung)[2]	620 ± 10	1020 ± 60[3]

[1] Neonatal rats (4–7 days old) were exposed to either air or 96–98% oxygen for 4 days (96 hr). Each value represents the mean ± SE of four to six determinations.

[2] One international unit (IU) is defined as the amount of enzyme which reacts with 1 μmol H_2O_2/min.

[3] Significantly different from the air-exposed control ($P < 0.05$).

activities or of GSH levels in their lungs with the exception of a small but significant increase of GSH level at the second day of oxygen exposure. G-6-PD was, however, significantly elevated in the 72-hr oxygen-exposed adult rats.

To determine whether the increased activity was confined to the pulmonary antioxidant enzymes, additional lung enzyme studies were conducted in oxygen-exposed neonatal rats (Table 3). Four days of oxygen exposure were chosen because the activity of most of the pulmonary antioxidant enzymes studies (Figs. 3–5) was found to peak at this time. Activity of pulmonary alkaline phosphatase, a ubiquitous, nonspecific phosphatase; β-glucuronidase, a drug-metabolizing enzyme; fructose-1,6-diphosphate aldolase, a glycolytic enzyme; and glutamic dehydrogenase, a transaminase which catalyzes the formation of amino acids from Kreb's cycle intermediates, in oxygen-exposed neonatal rats was not significantly different from that of air-exposed controls. Activity of catalase, an enzyme which sequesters H_2O_2 formed from the SOD dismutation reaction and can be considered as part of the pulmonary antioxidant defense system, was significantly increased in oxygen-exposed neonatal rat lung as compared to the air-exposed control.

DISCUSSION

Although oxygen-induced lung toxicity has been shown to be age-dependent (References 5, 18, 29, Fig. 1), the mechanism

to explain this phenomenon remains unclear. We propose that young animals, when challenged with oxygen, have a more rapidly adaptive pulmonary antioxidant protective capacity, *i.e.*, the SOD and the GSH systems, which provides a mechanism(s) for their increased tolerance to hyperoxia-induced lung injury as compared to the adults.

We have previously reported that exposure of newborn and neonatal animals to 24 hr of 85% oxygen produced a significant increase in pulmonary SOD activity (1, 11). Similar findings were observed in the present study in neonatal rats exposed to 96–98% oxygen (Fig. 2). Adult animals, on the other hand, when exposed to sublethal concentrations of oxygen (85%) (34), did not show any increase in lung SOD activity 24 hr after hyperoxic exposure (11). When exposed to a lethal concentration of oxygen (96–98%) in this study, adult rats showed a significant decrease in the activity of lung SOD (Fig. 2), which correlates with their low survival rate under these conditions. The increase in SOD activity in the neonatal animal was particularly striking in the first 24 hr of oxygen exposure (Fig. 2). It is conceivable that this rapid early adjustment of pulmonary SOD activity in neonatal animals, not observed in adult animals, may be of paramount importance in preventing the initiation of lung damage early in the course of oxygen exposure (11). Catalase, an enzyme which detoxifies the H_2O_2 formed from the SOD dismutation reaction, was also found to be significantly increased in 96-hr oxygen-exposed neonatal rat lung (Table 3).

Examination of the glutathione system in the lungs of neonatal rats which survived 5 days of 96–98% oxygen exposure with no evidence of respiratory distress showed a progressive increase in the activity levels of pulmonary GP, GR, and GSH (Figs. 3–5). In accordance with the proposed protective role of the glutathione system (3, 15), adult rats, which succumbed within 3 days of oxygen exposure, did not show any significant changes in the activity levels of pulmonary GP, GR, and GSH with the exception of a small but significant increase in GSH levels after 48 hr of oxygen exposure. The potential contribution of G-6-PD in the protection against oxygen-induced lung injury is unclear, since G-6-PD was found to increase in both neonatal and adult rat lungs (Fig. 6).

The increase in pulmonary antioxidant enzyme activity in oxygen-exposed neonatal rat does not appear to be a generalized enzyme response since the activity of enzymes not related to the pulmonary antioxidant defense system remained essentially unchanged following the oxygen challenge (Table 3). It should be noted that the increase in protective enzyme activities and GSH levels in neonatal animals occurs at a time when the intracellular DNA synthesis appears to be inhibited (Table 2 and Reference 27). The increase in the production of these protective enzymes and GSH possibly at the expense of other

cellular constituents, may reflect a vital adaptive response of the neonatal animal to oxygen.

From the present data, it is reasonable to postulate that SOD comprises the major defensive factor in neonatal animals early in oxygen toxicity. As early as 24 hr after exposure, SOD activity in the neonatal lung is maximally increased (Fig. 2). As the course of oxygen exposure progresses, the increased production of superoxide anion may saturate the superoxide dismutase system. Excess superoxide anion and/or lipid peroxides may act as the stimulus to augment the activity of the glutathione system, which then assumes a major role in the defense against oxygen-induced pulmonary injury. The degree of oxygen-induced lung damage would thus appear to be governed by the sensitive balance in the lung cell between the generation of toxic intermediates of oxygen and the execution of the antioxidant defense apparatus. Pulmonary damage would occur only if the protective mechanism is overwhelmed by excess rates of generation of superoxide anion or other toxic intermediates, such as hydroxyl free radicals (9). During exposure to high concentrations of oxygen, the intracellular production of superoxide anion is believed to be increased (24, 25). Increases in the lung complement of SOD, GR, GP, and GSH in neonatal rat during oxygen challenge should permit a delay in onset and/or minimization of oxygen-induced lung injury in contrast to the situation in adult animals. Additional studies are warranted to more precisely define the cell types in the lung that account for the observed biochemical alterations and correlate them with the site and nature of oxygen-induced lung damage. The changes in enzyme activities and GSH level observed in the neonatal rat lung may be in reality of a much greater magnitude if the increase is found to occur in a specialized cell group.

A further understanding of the antioxidant defense mechanism in the lung has potentially great importance in neonatal respiratory disease and therapy. Neonatal respiratory disease may be aggravated or may even develop as a result of the lack of a normal response by the neonatal pulmonary protective systems to environmental stimuli as suggested previously for bronchopulmonary dysplasia (11). Identification of agents or drugs that may induce or augment these protective enzymes may provide a means to allow for the safer use of high oxygen therapy in neonatal intensive care.

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