Neonatal Red Cell Superoxide Dismutase Enzyme Levels: Possible Role as a Cellular Defense Mechanism against Pulmonary Oxygen Toxicity

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Summary

Red cell superoxide dismutase (SOD) enzyme activity was determined in 58 cord blood specimens obtained from infants over a range of gestational ages. An inverse relationship between red cell SOD activity and gestational age was demonstrated. Red cell SOD activity showed a progressive fall from 263.1 ± 30.5 units/mg non-hemoglobin protein (NHP) in infants of less than 29 weeks of gestation to 168.9 ± 21.3 units/mg NHP in infants of more than 36 weeks of gestation ($P < 0.05$). Infants treated for RDS showed an increase in red cell SOD activity which reached significance at 72 hr when compared to cord blood levels from the same population ($P < 0.05$). No similar significant difference could be demonstrated in gestational age-matched control subjects over the same time period. However, initial cord blood SOD enzyme levels were lower in premature infants with RDS (229.5 ± 30.6 units/mg NHP) than in premature infants without RDS (264.0 ± 38.0 units/mg NHP). When infants with RDS were examined for oxygen toxicity and survival, red cell SOD levels were noted to decrease over 24 hr in four of five infants who died, three of whom developed bronchopulmonary dysplasia. In the surviving infants, red cell SOD levels showed a significant increase by 48 hr ($P < 0.05$). None developed bronchopulmonary dysplasia and all survived.

Speculation

Increases in red cell superoxide dismutase activity in response to hyperoxic stress may prevent toxic effects of O_2^- radicals and oxygen toxicity to the lung.

Oxygen is fundamental to survival of aerobic systems. However, in excess, molecular oxygen has been associated with toxic tissue effects as noted in bronchopulmonary dysplasia (4, 8, 33, 38) and retrolental fibroplasia (22). The complete reduction of 1 molecule of oxygen yields intermediates which include O_2^- (superoxide radical), $OH\cdot$ (hydroxyl radical), and H_2O_2 (24). Fridovich (12, 13, 17, 18, 25, 40) and others (32) have recently proposed that a major part of oxygen toxicity at the cellular level may be mediated by the superoxide radical. All oxygen-metabolizing cells so far examined have been found to contain enzymes that catalyze the reaction: $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. These enzymes, which have been characterized as metalloproteins, are designated superoxide dismutases (12).

Mammalian studies have only recently been attempted in an effort to delineate the relationship of SOD enzyme levels and the occurrence of pulmonary oxygen toxicity (3, 8, 14). Decreased levels of lung SOD have been demonstrated in those infants dying from RDS (4, 38). Induction of enzyme activity has been observed in rat lungs after exposure to increased oxygen concentrations (3, 8, 14) and seems to be dependent upon the presence

of an unidentified factor in plasma for induction to be demonstrated (3).

The red cell provides an accessible source of human SOD. Although the relationship of SOD to bronchopulmonary dysplasia is not clearly understood, measurement of red cell SOD activity in red cells may reflect a response to intrapulmonary exposure to O_2^- radicals and provide a mechanism for their removal while the red cell is in close proximity to the alveolar wall. Oxygen induced lung damage has been associated with oxygen-induced hemolysis (44). In the present study, we have (1) compared cord blood levels of red cell SOD over a range of gestational ages and (2) compared the SOD response of premature infants with RDS exposed to oxygen over 72 hr of therapy with gestational age-matched premature infants without RDS over a similar 72-hr period; and (3) evaluated the relationship of red cell SOD enzyme activity over a 72-hr period to the development of pulmonary oxygen toxicity in infants treated for RDS.

METHODS

Fifty-eight cord blood specimens were obtained for analysis. Gestational age was determined by the method of Dubowitz *et al.* (10) and by the mother's expected date of confinement. An additional study was performed over a 72-hr period on 22 infants: 11 premature infants with RDS and hyperoxic exposure and 11 premature infants of similar gestational age and birth weight without RDS maintained in room air. Mean birth weight of the non-RDS group was 1619 g. Those with RDS were provided sufficient oxygen (FIO_2) to keep their PaO_2 between 50-70 mm Hg. Continuous positive airway pressure (CPAP) was used when required FIO_2 was greater than 0.60, whereas mechanical ventilation (volume-cycled) with positive end-expiratory pressure (PEEP) was used when FIO_2 of greater than 0.80 was required and/or apnea with hypercarbia ($PaCO_2 > 65$) developed. FIO_2 was measured with a Beckman oxygen analyzer and PaO_2 was determined in infants with RDS with a Radiometer Copenhagen acid-base blood gas analyzer.

Cord blood and neonatal specimens were anticoagulated with acid-citrate-dextrose and stored at 4°. Preparation of the red cell SOD fraction was performed using a modification of the methods of McCord and Fridovich (26) and Deutsch (20, 21, 41, 42). A 1.0×4 cm DEAE-cellulose column (DE-52 Whatman Chemical Co.), previously equilibrated with Na phosphate buffer, pH 7.25, was used to separate hemoglobin from non-hemoglobin protein.

Non-hemoglobin protein was then eluted from the column with 4 ml 300 mM Na-acetate buffer. The resulting eluent was stored at -10° until assayed for SOD enzyme activity.

SOD enzyme activity was determined by its ability to inhibit the autoxidation of colorless epinephrine to colored adrenochrome according to the method of Fridovich *et al.* (30). This was detected spectrophotometrically using a Gilford kinetic

spectrophotometer at 480 nm. One unit of SOD enzyme activity was defined as the concentration which inhibited adrenochrome formation by 50% of control rate. Pure bovine erythrocyte superoxide dismutase (Truett Laboratory, 45) was used as a standard. Hemoglobin contamination of the nonhemoglobin fraction was measured by the standard clinical benzidine method (7). Non-hemoglobin protein in the column eluant was determined by subtracting hemoglobin protein from total protein determined by the biuret reaction (16). Units of SOD activity are expressed per mg non-hemoglobin protein.

RESULTS

ASSAY RELIABILITY

Pure bovine SOD in varying concentrations inhibited autoxidation of epinephrine under assay conditions (0.3 mM epinephrine, 0.1 mM EDTA, 50 mM Na carbonate buffer, pH 10.2) corresponding to the work of Fridovich (30). When the reciprocal of percentage of inhibition $\times 10^2$ was plotted against the reciprocal of the fractional dilution of the sample, a straight line relationship was obtained (15). Hemoglobin autoxidation of epinephrine in the range present in eluted samples (0.005–0.040 mg/ml) accounted for less than 10% of total autoxidation of epinephrine. This is acceptable for assay conditions (15). Mean level for non-hemoglobin protein for all samples tested equaled 0.97 ± 0.13 mg/ml. Range of values on 39 samples tested varied from 0.78 to 1.30 mg/ml and did not significantly affect calculated SOD results when expressed in units/mg NHP.

CORD RED CELL SUPEROXIDE DISMUTASE ENZYME ACTIVITY

Cord red cell samples were examined for a relationship between red cell SOD enzyme activity and gestational age. The data indicates an inverse relationship between red cell SOD activity and increasing gestational age (Fig. 1). Independent *t*-test analysis between groups demonstrated statistical significance for cord blood red cell SOD enzyme activity from samples

Fig. 1. Cord blood superoxide dismutase enzyme activity as a function of gestational age. Superoxide dismutase activity is expressed as units per mg non-hemoglobin protein (NHP). Mean and standard error range are shown for each group. Sample size is shown in parentheses. A straight line relationship was obtained by the method of least squares.

Fig. 2. Superoxide dismutase enzyme activity in red cell samples from infants with and without idiopathic respiratory distress syndrome (RDS) as a function of time postnatally. Mean and standard error are shown for each time period. Zero time refers to cord blood levels. ●—●: RDS group; ○—○: non-RDS group. Superoxide dismutase activity is expressed as units per mg non-hemoglobin protein (NHP).

obtained from infants less than 29 weeks of gestational age versus samples obtained from infants of more than 36 weeks of gestational age ($T_1 = 2.22$, $P < 0.05$).

COMPARISON OF RED CELL SOD ENZYME ACTIVITY IN PREMATURES WITH AND WITHOUT RDS

Eleven infants in each group were included whose cord blood samples had been obtained prior to subsequent samples at 4, 24, 48, and 72 hr. Sample size in each time period varied as some of the infants, particularly those in the RDS, treated group, received packed red cell transfusions, eliminating any further sampling. Although independent *t*-test analysis revealed no significant differences when comparing red cell SOD enzyme levels between groups for each time period (Fig. 2), paired *t*-test analysis for mean SOD levels at different time periods compared to cord blood levels within each group demonstrated statistical significance by 72 hr in the RDS group ($T_0 = 2.49$, $P < 0.05$). This may be explained by noting that initial mean cord red cell SOD enzyme levels were lower in premature infants with RDS (229.5 ± 30.6 units/mg NHP) than levels obtained from the group without RDS (264.0 ± 38.0 units/mg NHP, in agreement with a previous report (4).

COMPARISON OF RED CELL SOD ACTIVITY IN RDS GROUP WITH BRONCHOPULMONARY DYSPLASIA AND/OR DEATH VERSUS NO BRONCHOPULMONARY DYSPLASIA AND SURVIVAL

Table 1 tabulates the results of SOD activity in 11 infants treated for RDS. Bronchopulmonary dysplasia was identified clinically and radiographically in three of five infants who died during therapy. In four of five, not only did red cell SOD levels decline over 24 hr, but none survived. All five met criteria for mechanical ventilation with PEEP. In the remaining 6 of 11 who did not develop bronchopulmonary dysplasia or die, an increase in SOD activity was noted (Fig. 3). Three required mechanical ventilation with PEEP whereas the remaining three required only CPAP. Although mean PaO_2 over 48 hr (56.6 mm Hg

Table 1. Red cell superoxide dismutase enzyme activity in respiratory distress syndrome, treated group versus bronchopulmonary dysplasia and survival¹

Infant	BW	GA	Birth	4 hr	24 hr	48 hr	72 hr	Therapy	Mean PaO ₂ (48 hr)	Hours FIO ₂ >0.40	BPD	Survival	Died
1	1620	32	232.5		88.2	115.0		MV + PEEP	60.0	18	+	—	8 days
2	930	27	303.7	235.8	256.0			MV + PEEP	59.0	54	+	—	4 days
3	1670	32	265.2	217.0	175.0	130.5		MV + PEEP	66.0	106	+	—	5 days
4	980	26	485.6	300.0	374.0			MV + PEEP	47.0	9	—	—	36 hr
5	850	27	125.9	130.0	166.0			MV + PEEP	51.0	30	—	—	30 hr
Mean	1210	28.8	256.5	221.9	204.2	127.4			56.6	43.4			
± SE	±179	±1.32	±59.7	±43.0	±44.0	±5.0			±7.6	±17.3			
6	1730	32	147.0	133.0	250.0		544.5	MV + PEEP	71.0	49	—	+	
7	1235	31	221.0		246.7	152.0	343.4	MV + PEEP	56.5	48	—	+	
8	2670	36	164.2		173.0	257.9		MV + PEEP	52.5	39	—	+	
9	1830	32	221.0		220.0	230.2	277.4	CPAP	60.0	78	—	+	
10	1090	31	225.0		242.2	290.0	251.9	CPAP	59.0	24	—	+	
11	1155	30	136.5		150.5	472.0	473.0	CPAP	63.0	32	—	+	
Mean	1618	32	185.8	133.0	213.7	280.6	378.1		60.3	45.0			
± SE	±245	±0.86	±15.7	±0	±17.3	±49.5	±55.5		±6.3	±18.7			
Ratio											3/11	6/11	

¹ Superoxide dismutase activity expressed in units per mg non-hemoglobin protein. BW: birth weight; GA: gestational age; BPD: bronchopulmonary dysplasia; MV: mechanical ventilation; PEEP: positive end-expiratory pressure; CPAP: continuous positive airway pressure.

Fig. 3. Superoxide dismutase enzyme activity of infants with idiopathic respiratory distress syndrome with and without bronchopulmonary dysplasia and death as a function of time postnatally. Mean and standard error are shown for each group. Zero time refers to cord blood. ○—○: nonsurvivors; ●—●: survivors. Superoxide dismutase activity is expressed as units per mg non-hemoglobin protein (NHP).

versus 60.3 mm Hg) and mean duration of FIO₂ greater than 0.40 (43.4 hr versus 45.0 hr) did not differ significantly, comparison of levels between these two subgroups demonstrated a statistically significant difference at 48 hr ($T_1 = 2.98$, $P < 0.05$). No comparison could be made at 72 hr as no values were available in the group with bronchopulmonary dysplasia who died, but a further increase in red cell SOD enzyme activity was noted among five of six infants with values obtained at 72 hr who survived without bronchopulmonary dysplasia.

DISCUSSION

Multiple assays have been developed to measure SOD enzyme activity from a variety of sources (1, 2, 6, 9, 26, 27, 31, 34, 36). In indirect assays, O₂⁻ is scavenged by an indicator molecule that becomes oxidized or reduced. SOD is detected by its ability to inhibit modification of the indicator scavenger by O₂⁻, in this case by its ability to inhibit oxidation of epinephrine to adrenochrome in the presence of O₂⁻. At elevated pH (10.2), epinephrine is autoxidized by a complex chain of electron transfers involving the O₂⁻ radical (12). Under these conditions, a separate source of O₂⁻ is not required and the enzyme can be assayed by its ability to inhibit autoxidation of epinephrine (30). Similarly, hemoglobin can cause both co-oxidation and peroxidation of epinephrine to adrenochrome, the former via the superoxide radical (31). This may be an alternative explanation for the presence of SOD within the red cell to prevent toxic exposure to O₂⁻ generated by hemoglobin autoxidation. We do not understand why red cell SOD activity is higher in the younger gestational age premature infants (Fig. 1). However, fetal tissues are exposed to oxygen from conception and the presence of intracellular SOD to prevent toxic exposure to O₂⁻ radicals may be required early in gestation. Although PaO₂ is constant throughout gestation (20–22 mm Hg) (39), a higher basal SOD level may be necessary to protect immature cell membranes during early gestation with decreasing dependence upon this mechanism as the fetus matures.

The pathophysiology of bronchopulmonary dysplasia has been well established (5, 23, 28, 29, 32, 33, 35, 37, 40, 43). We know that pulmonary complications may occur after only 24 hr of 100% oxygen exposure. The pathology includes abnormal permeability of pulmonary capillary endothelium, proliferation of pulmonary capillaries, metamorphosis of type II alveolar cells, necrosis of type I alveolar cells, necrosis of type I alveolar cells, changes in mucosal epithelium from columnar to squamous appearance, transudation of fluid and formed elements into alveolar spaces, and patchy atelectasis and hemorrhage, both alveolar and interstitial (40). The protective effect of SOD in preventing such cellular changes has been studied by several investigators. Superoxide dismutase has the ability to protect specifically against at least one means of peroxidative damage, that caused by O₂⁻. Fee and Teitelbaum (11) noted that peroxidative dialuric acid-induced hemolysis in vitamin E-deficient rat red cells was decreased by adding bovine SOD to the red cell

suspension. SOD was never found to have a protective effect by itself but in the presence of catalase, which prevents abnormal accumulation of H_2O_2 , protection was demonstrated. The necessary presence of both enzymes to protect against hemolysis where neither can protect alone is further evidence that an intermediate species other than O_2^- may be exerting the toxic effect. The hydroxyl radical, formed from the substrates of both enzymes by the reaction

may play such a role (19).

Within 2 hr postnatally, an infant's PaO_2 is greater than 80 mm Hg (39). Birth, therefore, represents an oxygen challenge. If red cell SOD enzyme induction is possible, an increase in central arterial PaO_2 may act as a stimulus to immature red cell in bone marrow to increase intracellular activity of SOD to prevent toxic exposure to O_2^- . However, if changes in activity depend upon changes in arterial PaO_2 , only small increases in red cell SOD would be anticipated in babies with RDS, since the aim of therapy is to keep central PaO_2 in a therapeutic range (50–70 mm Hg). However, the range of PaO_2 noted in several of the 11 infants studied indicates that such close control was not always possible. Measurement of red cell SOD may provide an indirect measure of intracellular response to alveolar hyperoxic stress. Data from our study indicates that both normal neonates and neonates with RDS exposed to hyperoxia demonstrated increased red cell SOD activity, especially after 72 hr of oxygen exposure (Fig. 2). The mechanism for these changes is not clear. Since mature red cells are devoid of protein-synthesizing capability (2), increases in SOD must reflect activation of SOD in red cells, synthesis of new enzyme by reticulocytes, or release into the circulation of a new population of red cells containing high SOD activity.

CONCLUSION

Our observation that prematures developing RDS tend to have lower red cell SOD enzyme activity than those who do not develop the disorder (Fig. 2) suggests that oxidative injury to alveolar membranes may have a causal relationship to RDS itself. Furthermore, the neonate's ability to respond to hyperoxia by increasing intracellular activity of SOD, both in red cells and pulmonary parenchyma, may well determine whether or not significant oxygen toxicity may develop. Our data (Fig. 3) demonstrate that increasing activity of red cell SOD correlates with absence of bronchopulmonary dysplasia and survival whereas failure to respond to hyperoxia and mechanical ventilation is associated with bronchopulmonary dysplasia and/or death. By increasing intracellular SOD activity in response to hyperoxic stress, toxic exposure to O_2^- radicals may be prevented and integrity of the cell membrane preserved.

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