Superoxide Dismutase Improves Gas Exchange and Pulmonary Hemodynamics in Premature Lambs

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Rationale: Oxidant stress may increase the severity of respiratory distress syndrome (RDS) after premature birth by altering vasoreactivity and increasing lung edema, but the acute effects of superoxide dismutase (SOD) treatment on gas exchange, lung compliance (C_L), and pulmonary vascular resistance in premature animals with RDS are unknown. **Objective:** We studied the effects of intratracheal recombinant human SOD treatment (rhSOD) on gas exchange, C_L, and pulmonary hemodynamics in 46 premature lambs with RDS. **Methods:** After C-section delivery, lambs were randomly assigned to treatment with SOD (2.5–10 mg/kg) with or without inhaled nitric oxide (iNO, 5 ppm), and mechanically ventilated for 4 hours. At the end of the study, pressure–volume curves and wet–dry lung weights were measured to assess C_L and edema, respectively. **Main Results:** Despite an initial rise in Pa_{O₂}, Pa_{O₂} in control animals progressively declined over the 4-hour treatment period (Pa_{O₂} = 25.0 ± 7.5 mm Hg at 4 hours). In comparison with control animals, early treatment with SOD at 5 and 10 mg/kg improved Pa_{O₂} at 4 hours (167 ± 44 and 269 ± 33 mm Hg, respectively; *p* < 0.05 vs. control), but did not decrease lung edema or improve C_L. In contrast, late treatment with SOD did not improve Pa_{O₂}. Treatment with iNO increased Pa_{O₂} (196 ± 22 vs. 25 ± 8 mm Hg, control animals; *p* < 0.01), but the response to iNO was not augmented by combined therapy (SOD + iNO). After 4 hours of ventilation with F_{I_{O₂}} = 1.00, rhSOD treatment lowered pulmonary vascular resistance compared with control animals. **Conclusions:** Early intratracheal rhSOD treatment improves oxygenation in premature lambs with RDS and prevents the development of pulmonary hypertension.

Keywords: bronchopulmonary dysplasia; chronic lung disease; mechanical ventilation; pulmonary hypertension; surfactant

Acute lung injury in premature lambs contributes to the severity of respiratory distress syndrome (RDS), which is characterized by progressive deterioration in oxygenation, worsening lung compliance, and pulmonary edema (1). Multiple factors contribute to acute lung injury in RDS, but oxidant stress plays a critical role (1, 2). In experimental RDS in premature lambs, treatment with exogenous surfactant improves oxygenation and decreases lung injury (3–5). However, extremely premature lambs do not have sustained improvement in oxygenation despite repeated doses of surfactant (6), suggesting that factors other than surfactant deficiency contribute to lung injury in this setting. Because the immature lung lacks sufficient antioxidant host defenses to

combat the oxidant stress induced by exposure to high inspired oxygen concentrations after premature delivery (7–9), therapies designed to augment endogenous antioxidant activity have engendered considerable interest (10).

Inhaled nitric oxide (iNO) holds promise as an adjunctive treatment for RDS because of its effects on pulmonary vasodilation, $\dot{V}/\dot{Q}$ matching, lung inflammation, and oxidant stress (1, 11). Intratracheal administration of recombinant human superoxide dismutase (rhSOD) has also been shown to reduce the severity of lung injury caused by mechanical ventilation with hyperoxia in newborn piglets (12, 13). Moreover, via its effects on reducing the activity of toxic free radicals, intratracheal treatment with rhSOD may enhance the bioavailability of endogenously produced NO and augment the pulmonary vasodilator response to iNO (14). However, the mechanisms responsible for the beneficial effects of intratracheal treatment with rhSOD and the interactive effects of rhSOD and iNO have not been studied in a premature animal model of RDS.

The role of oxidant stress in the pathophysiology of RDS and mechanisms by which antioxidant therapy may improve gas exchange in premature animals with RDS are uncertain. We hypothesized that administration of intratracheal rhSOD in premature lambs with RDS would improve oxygenation and ameliorate the pulmonary hypertension that is typical of this animal model after prolonged mechanical ventilation with high inspired oxygen concentrations. We tested this hypothesis in 46 premature lambs with RDS in four separate protocols to determine optimal dosing and the timing of rhSOD administration, whether rhSOD treatment augmented the response to iNO, and the effects of rhSOD administration on pulmonary hemodynamics.

METHODS

Mixed-breed (Columbia-Rambouillet) pregnant ewes were used in this study. All procedures and protocols were reviewed and approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center. This study includes four treatment protocols that examine the independent and combined effects of rhSOD and iNO on gas exchange, lung injury, and pulmonary hemodynamics in this premature (122–125 days' gestation; term, 147 days) ovine model of severe RDS (1).

Study Design—General

An abbreviated description of the methods used is provided here. Additional details on the methods and experimental design are provided in an online supplement.

A uterine incision was made under sterile conditions, the fetal head was exteriorized, and a right paramedian skin incision was made in the neck after local infiltration with lidocaine (1% solution, 2–3 ml). Polyvinyl catheters (20-gauge; Martech Medical Products, Lansdale, PA) were advanced into the ascending aorta through the carotid artery and into the superior vena cava through the jugular vein. All animals were treated with exogenous surfactant (Infasurf; provided by E. A. Egan, M.D.) at an estimated dose of 3 ml/kg (105 mg phospholipid/kg) before the first breath.

For Protocol 4, pulmonary hemodynamics were measured as previously described (1, 11). In brief, before delivery, a left thoracotomy

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was performed to expose the heart and great vessels. Catheters were placed in the ascending aorta, left atrium, and main pulmonary artery, and a flow probe was placed around the left pulmonary artery to measure lung blood flow. On delivery, the animals were treated with exogenous surfactant and 10 mg/kg of rhSOD or an equivalent volume of saline for control animals.

Experimental Design

Four experimental protocols were conducted. The treatment group for each animal was assigned before delivery. In Protocol 1, we tested the effects of treatment with three different doses of rhSOD on delivery (control, $n = 5$; 2.5 mg/kg rhSOD, $n = 5$; 5.0 mg/kg rhSOD, $n = 5$; 10.0 mg/kg rhSOD, $n = 5$). In Protocol 2, we compared treatment with iNO (5 ppm, $n = 5$) versus iNO (5 ppm) + rhSOD (2.5 mg/kg, $n = 5$) on delivery. In Protocol 3, we provided late treatment with rhSOD (10 mg/kg, $n = 6$), after 2 hours of mechanical ventilation. In Protocol 4, we measured the effects of treatment with rhSOD (10 mg/kg, $n = 5$) on pulmonary vascular resistance compared with control animals ($n = 5$). For Protocol 4, animals were instrumented before delivery to measure left lung blood flow, pulmonary artery pressure, and left atrial pressure, as previously described, and left lung vascular resistance was calculated (11).

Serum and lung tissue concentrations of rhSOD were measured using an ELISA system, as developed by M. Zvilich at Biotechnology General Laboratories (Iselin, NJ). In brief, this assay uses rabbit-anti-CuZnSOD antibodies to capture the CuZnSOD within the samples. After removal of nonbound proteins, the CuZnSOD-anti-CuZnSOD antibody complex is then exposed to anti-CuZnSOD biotin-conjugated antibodies. The biotinylated antibody is activated by a streptavidin-horseradish peroxidase conjugate, and measurement of the specific absorbance of the activated complexes is compared with a standard curve of absorbance (15).

Statistical Analysis

For Protocols 1 and 2, two-way repeated-measures analysis of variance (ANOVA) was used to analyze the interaction of time and treatment group. Statistical comparisons of within-group continuous variables were performed using one-way repeated-measures ANOVA. Where significant differences were identified, *post hoc* analysis was performed using Student-Newman-Keuls test. Comparisons of responses to each intervention (among treatment groups) at the hourly time points were performed using one-way ANOVA and the Student-Newman-Keuls test. The level of statistical significance was set at $p < 0.05$; results are reported as mean $\pm$ SE.

RESULTS

There were no differences in bodyweight among the study groups (mean for all animals, 2.302 ± 0.33 kg). Before initiation of mechanical ventilation, baseline PaO_2 , PaCO_2 , arterial pH, heart rate, and mean systemic arterial blood pressure were similar among groups.

There were no differences in arterial pH, PaCO_2 , peak inspiratory pressure, or mean airway pressure among the treatment groups at the 4-hour time point (Table 1). In Protocol 1, PaO_2 in the control animals declined over the 4-hour treatment period

(PaO_2 , 25 ± 8 mm Hg at 4 hours; Table 1 and Figure 1). Treatment with rhSOD at 5 and 10 mg/kg improved PaO_2 at 4 hours compared with control animals (167 ± 44 mm Hg, $p = 0.013$, 95% confidence interval [CI], 39, 245; and 269 ± 33 mm Hg, $p < 0.0001$, 95% CI, 167, 323, respectively; Figure 1 and Table 1) in a dose-dependent fashion (posttest for linear trend, $p = 0.016$). Two-way repeated-measures ANOVA yielded differences for PaO_2 over time ($p < 0.001$), by dose of rhSOD ($p = 0.0093$), and by interaction ($p = 0.0272$). Two-way repeated-measures ANOVA for Protocol 2 showed no difference in the interaction of time and treatment group ($p = 0.64$) on PaO_2 .

Analysis of lung compliance measurements with pressure-volume curves showed no differences among the groups (Figure 2A). Lung edema, as reflected by the ratio of wet-to-dry lung weights, was also not different among the groups (Figure 2B).

Serum concentrations of rhSOD increased over time in all early-treatment groups (Figure 3A). Within-group analyses showed significantly higher serum levels at each time point for all dosages tested. However, increasing the dosage of intratracheally administered rhSOD did not cause a dose-dependent increase among all the treatment groups. Only rhSOD at the 10 mg/kg dose caused a significantly higher serum concentration than either of the lower doses at each time point.

Lung concentrations of rhSOD paralleled the serum measurements (Figure 3B); that is, there was no difference between the 2.5- and 5.0-mg/kg dose in lung rhSOD levels at the end of the 4-hour study period. However, the 10-mg/kg rhSOD group had significantly higher lung rhSOD concentrations than either of the lower dose groups.

Treatment with iNO improved PaO_2 (196 ± 22 mm Hg) compared with control animals ($p = 0.0023$; 95% CI, 102, 328), and was not different from combined therapy (SOD + iNO, 240 ± 48 mm Hg; $p < 0.0001$; 95% CI, 117, 226, compared with control animals; Figure 4 and Table 1). For Protocol 3, delaying treatment with high-dose SOD until 2 hours after delivery caused no improvement in oxygenation compared with control animals ($p = 0.56$; 95% CI, -105, 184; Table 1). Neither lung compliance nor edema (wet/dry weight, 9.4 ± 0.36) was different from the other groups.

Treatment with rhSOD prevented the increase in pulmonary vascular resistance over 4 hours of mechanical ventilation (Figure 5; $p = 0.028$; 95% CI, -0.329, -0.025). Left pulmonary artery blood flow was higher in the SOD-treated group compared with control animals (121 ± 13 vs. 82 ± 9 ml/minute, $p < 0.05$). Mean pulmonary artery pressure was not significantly lower in the SOD-treated group (30 ± 13 vs. 35 ± 13 mm Hg, $p = 0.08$).

DISCUSSION

We found that treatment with intratracheal rhSOD at delivery increases oxygenation in a dose-dependent fashion during mechanical ventilation of premature lambs with RDS. The improvement

TABLE 1. VENTILATOR SETTINGS AND BLOOD GAS TENSIONS AT 4 HOURS

	Control ($n = 5$)	SOD, 2.5 mg/kg ($n = 5$)	SOD, 5 mg/kg ($n = 5$)	SOD, 10 mg/kg ($n = 5$)	SOD, Late Tx ($n = 6$)	iNO, 5 ppm ($n = 5$)	iNO + SOD ($n = 5$)
Arterial pH	7.00 ± 0.13	7.15 ± 0.10	7.22 ± 0.08	7.18 ± 0.06	7.17 ± 0.03	7.21 ± 0.04	7.20 ± 0.03
PaCO_2	74 ± 8	63 ± 13	50 ± 10	55 ± 4	53 ± 2	44 ± 4	50 ± 3
PaO_2	25 ± 8	129 ± 63	$167 \pm 44^*$	$269 \pm 33^*$	90 ± 27	$196 \pm 22^*$	$240 \pm 48^*$
PIP	28 ± 2	24 ± 1	25 ± 1	24 ± 1	24 ± 1	24 ± 1	25 ± 1
Mean Paw	10.6 ± 0.7	10.1 ± 0.5	10.3 ± 0.7	9.2 ± 0.5	8.7 ± 0.3	10.2 ± 0.4	9.8 ± 0.3

Definition of abbreviations: iNO = inhaled nitric oxide; Paw = mean airway pressure; PIP = peak inspiratory pressure; SOD = superoxide dismutase; Tx = treatment.
* $p < 0.05$ versus control.

Figure 1. Effects of intratracheal administration of recombinant human superoxide dismutase (rhSOD) on oxygenation in premature lambs with respiratory distress syndrome (RDS). Early treatment with 5 and 10 mg/kg rhSOD improves oxygenation after 4 hours of mechanical ventilation. * $p = 0.013$, 95% confidence interval [CI], 39, 245, for 5 mg/kg group versus control; and $p < 0.0001$, 95% CI, 167, 323, for 10 mg/kg versus control. CTRL = control.

in oxygenation was similar for animals treated with either high-dose rhSOD or iNO. However, delaying treatment with high-dose rhSOD until 2 hours after delivery abrogated the improvement observed with early treatment. Early rhSOD treatment did not improve lung edema or compliance, despite the improvement in PaO_2 . However, rhSOD treatment prevented the predictable rise in pulmonary vascular resistance associated with mechanical ventilation in premature lambs with RDS, suggesting that the improvement in oxygenation was likely caused by reductions in both intrapulmonary and extrapulmonary right-to-left shunting. Previous studies suggest that both mechanisms likely contribute to the improvement in oxygenation in this animal model with pulmonary hypertension and severe parenchymal lung disease (1, 10, 16). Intratracheal administration of rhSOD

Figure 2. Effects of intratracheal treatment with rhSOD at delivery in premature lambs with RDS. Intratracheal rhSOD does not change lung compliance (A) or pulmonary edema (wet/dry lung weights; B). A: solid squares = control; solid triangles = SOD 2.5 mg/kg; inverted triangles = SOD 5 mg/kg; solid diamonds = SOD 10 mg/kg; solid dots = 2.5 SOD + 5 ppm inhaled nitric oxide (iNO); open squares = iNO 5 ppm.

Figure 3. Effects of increasing doses of intratracheal rhSOD on serum (A) and lung tissue (B) rhSOD concentrations. A: Squares = 2.5 mg/kg; triangles = 5.0 mg/kg; inverted triangles = 10 mg/kg. A, B: * $p < 0.01$ versus 2.5 and 5 mg/kg.

has been shown to reduce the severity of lung injury caused by mechanical ventilation with hyperoxia in newborn piglets, potentially improving intrapulmonary shunt (12, 13). Moreover, via its effects on reducing the activity of toxic free radicals, intratracheal treatment with rhSOD may enhance the bioavailability of endogenously produced NO and augment the pulmonary vasodilator response to endogenously produced and exogenously delivered NO (14).

Premature lambs delivered at 122 to 125 days' gestation develop severe RDS manifested by hypercarbia and hypoxemia despite mechanical ventilation with high inspired oxygen concentrations (17, 18). Treatment with exogenous surfactant at delivery improves gas exchange initially; however, mechanical ventilation with hyperoxia causes lung injury and inflammation with progressive deterioration in gas exchange over 4 hours (1). As is typical of this model, the hypercarbia noted after 4 hours of mechanical ventilation is exacerbated by right-to-left shunting of blood across the ductus arteriosus and foramen ovale. Because of its similarity to severe clinical RDS and its particular susceptibility to acute lung injury during conventional gas ventilation, we used extremely premature lambs to study the effects of both dose and timing of intratracheal treatment with rhSOD, and the interactive effects of rhSOD and iNO. Both of these agents hold promise as potentially beneficial therapies in the premature infant. iNO has effects on pulmonary vasodilation, $\dot{V}/\dot{Q}$ matching, lung inflammation, and antioxidant activity (1, 19). In addition, a recent clinical study suggested that exogenous rhSOD therapy during the first days after birth did not reduce the

Figure 4. Low-dose iNO treatment improves oxygenation in RDS. * $p = 0.0023$ for iNO versus control; 95% CI, 102, 328; and $p < 0.0001$ for iNO + SOD versus control; 95% CI, 117, 226.

Figure 5. Effects of rhSOD treatment on pulmonary vascular resistance (PVR) in premature lambs with RDS. Treatment with rhSOD (10 mg/kg) prevents the development of pulmonary hypertension in mechanically ventilated premature lambs with RDS. * $p = 0.0246$, 95% CI, -0.33 , -0.031 , versus SOD group.

incidence of bronchopulmonary dysplasia, but reduced late pulmonary morbidity, such as respiratory hospitalizations and the need for asthma medications (20). Our results provide support for the potential benefit of rhSOD therapy via improvement in intrapulmonary and extrapulmonary shunting in RDS, and emphasize the importance of early administration for optimal effect.

The improvement in oxygenation in animals treated with iNO was similar to the high-dose rhSOD group. There may be several intriguing explanations for this observation. This premature lamb model is exposed to 100% O_2 over the 4-hour study period, and we have previously shown that marked lung neutrophil accumulation rapidly occurs (an important source of oxidant radicals) (1). Hyperoxia causes increased production of reactive oxygen species (e.g., superoxide radical), which are particularly injurious to the immature lung with diminished antioxidant defenses. SOD, in combination with catalase and glutathione peroxidase, catalyzes the reduction of superoxide to hydrogen and water, thus diminishing its potential for oxidant injury. Ilizarov and colleagues (21) have demonstrated that overexpression of SOD protects lung epithelial cells against oxidant injury. It is possible that exogenous treatment with rhSOD on delivery also has a protective effect on the endothelial cell, potentially preserving the ability of the endothelial cell to produce vasoactive substances such as NO and prostacyclin. Moreover, high concentrations of SOD are also required for NO synthase to effectively catalyze the production of biologically active NO, suggesting an interactive effect of exogenously administered rhSOD on the endogenous production and activity of NO (22, 23). Finally, because the intratracheal administration of rhSOD would deliver the enzyme to the best-ventilated lung units, its regional distribution within the lung would enhance $\dot{V}/\dot{Q}$ matching via its effects on preservation of endogenous NO activity in those well-ventilated areas.

We found a dose-dependent improvement in oxygenation associated with rhSOD treatment, and a dose-dependent increase in lung and serum concentrations of rhSOD. The duration of the experiment was not sufficient to characterize drug elimination or the half-life of rhSOD in this model, but the marked increase in serum and lung concentrations after administration of 10 mg/kg rhSOD suggests zero-order kinetics at this dose. These data demonstrate that administration of rhSOD to the lung causes rapid absorption, with a plateau occurring by 4 hours at the lowest dose (2.5 mg/kg). The elimination of rhSOD appears to become saturated at doses between 5 and 10 mg/kg.

We conclude that treatment with rhSOD on delivery improves oxygenation by reducing shunt in a dose-dependent fashion in premature lambs with RDS. The improvement in oxygenation

was similar for animals treated with either high-dose rhSOD or iNO. Because the improvement in Pa_{O_2} was not associated with changes in lung edema or compliance but was associated with marked improvement in pulmonary vascular resistance in SOD-treated animals, we speculate that rhSOD improves Pa_{O_2} by reducing both intrapulmonary and extrapulmonary shunt in severe RDS. The lack of improvement in oxygenation with late rhSOD treatment may reflect the unique susceptibility of the extremely premature lung to even brief exposure to oxidant injury, and these findings may have important implications for clinical trial design of antioxidant therapy interventions.

Conflict of Interest Statement: J.P.K. serves on the Scientific Advisory Board for INO Therapeutics. T.A.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.M.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.H.A. serves on the Scientific Advisory Board for INO Therapeutics.

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