Prenatal hypoxia decreases lung extracellular superoxide dismutase expression and activity

BRENDA-LOUISE GILES,1 HAGIR SULIMAN,2 LISA B. MAMO,3 CLAUDE A. PIANTADOSI,4 TIM D. OURY,4 AND EVA NOZIK-GRAYCK3
1Department of Pediatrics, University of Manitoba, Winnipeg, Manitoba R3A 1S1; Departments of 2Medicine and 3Pediatrics, Duke University Medical Center, Durham, North Carolina 27710; and 4Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

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Giles, Brenda-Louise, Hagir Suliman, Lisa B. Mamo, Claude A. Piantadosi, Tim D. Oury, and Eva Nozik-Grayck. Prenatal hypoxia decreases lung extracellular superoxide dismutase expression and activity. Am J Physiol Lung Cell Mol Physiol 283: L549–L554, 2002. First published April 19, 2002; 10.1152/ajplung.00018.2002.—Extracellular superoxide dismutase (EC-SOD), which scavenges extracellular superoxide (O2•−), is highly regulated in the developing lung. In the prenatal rabbit, EC-SOD is predominantly intracellular and inactive, and postnatally, active EC-SOD is secreted. We hypothesized that prenatal hypoxia would delay the normal postnatal secretion of active EC-SOD in the lung. Pregnant New Zealand White rabbits were exposed to hypobaric hypoxia (15,000 ft × 36 h) to alter fetal O2 tension or were maintained in room air. Lungs were harvested from preterm (28 days), term (30 ± 1 day), and 1-wk-old kits. After prenatal hypobaric hypoxia, EC-SOD mRNA expression was significantly decreased in lungs of full-term kits, whereas EC-SOD protein decreased at all ages. Immunohistochemical staining for EC-SOD showed that hypoxia delayed secretion of the isoenzyme in the airways and pulmonary vasculature. Furthermore, pulmonary EC-SOD enzyme activity was significantly decreased in the 1-wk-old kits exposed to prenatal hypoxia. We conclude that prenatal hypoxia downregulates EC-SOD expression at both the transcriptional and posttranslational levels. Furthermore, prenatal hypoxia delays secretion of active EC-SOD enzyme. These findings have important implications for the effects of prenatal asphyxia on postnatal response to oxidant stress.

THE NEONATAL LUNG ADAPTS to postnatal conditions by decreasing the pulmonary vascular resistance to facilitate pulmonary blood flow and increasing antioxidant defenses to protect against the rise in alveolar oxygen tension associated with room air breathing. Multiple antioxidant enzymes are developmentally regulated in the neonatal lung in preparation for birth, including catalase, glutathione peroxidase, Cu,Zn superoxide dismutase (SOD), and extracellular SOD (EC-SOD) (2, 4, 9, 13, 14, 25). The isoforms of the SODs, which include mitochondrial Mn SOD, cytosolic Cu,Zn SOD, and EC-SOD, each have a specific cellular distribution and catalyze the dismutation of O2•− (Kd = 1 × 109) (21, 23, 35). EC-SOD, the only known enzymatic scavenger of extracellular O2•−, is also involved in the preservation of nitric oxide (NO) activity in the vasculature and airways due to its ability to compete with the rapid reaction between O2•− and NO (1, 28). Therefore, the regulation of EC-SOD in the neonatal lung is potentially important both to protect from extracellular oxidative stress with the change in oxygen tension and to preserve NO-dependent transition from fetal to adult circulation.

EC-SOD is highly expressed in the lung and is produced by alveolar type II cells, airway epithelial cells, and vascular endothelial cells (12, 26, 27, 32). We have previously shown that EC-SOD is located inside the cell in the prenatal rabbit lung and that active EC-SOD is secreted after birth (25). Although EC-SOD has been shown to protect against hyperoxic lung injury in animal models (3, 6, 11), the factors that regulate the secretion of active EC-SOD or the consequence of alterations in secretion of EC-SOD in the immature lung are not known.

The secretion of active EC-SOD in the neonatal lung over the first week of life correlates with the adaptation to room air breathing. On the basis of this observation, we speculated that changes in oxygen tension in the prenatal period regulate the postnatal secretion of EC-SOD in the lung. It is well known that maternal factors, including maternal infection or placental insufficiency, which limit oxygen delivery to the fetus, disrupt alveolarization and lung vascular growth and alter the health outcome of the newborn infant (33). In this study, we hypothesized that maternal exposure to hypoxia in late gestation would delay the secretion of active EC-SOD in the lung. We tested this hypothesis by treating pregnant rabbits in late gestation to 36 h of hypobaric hypoxia (15,000 ft) and measuring EC-SOD expression and activity in the lungs of preterm, term, and 1-wk-old kits.

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MATERIALS AND METHODS

Animal model and environmental exposures. Animal studies were performed in the Center for Hyperbaric Medicine and Environmental Physiology at Duke University Medical Center. Protocols were developed and approved by the Institutional Animal Care and Use Committee. Timed pregnant New Zealand White rabbits were obtained at 24 days of gestation (Robinson’s Animal Farm) and housed in the vivarium. All animals were given food and water ad libitum and were maintained on a regular 12-h light/dark cycle.

Pregnant animals were maintained in room air throughout pregnancy (control) or exposed to hypobaric hypoxia for 36 h in late gestation to produce transient prenatal hypoxia. Pregnant rabbits at day 26 of gestation were exposed to 15,000 ft in an altitude chamber [0.5 atmospheres (atm)] for 36 h. Pregnant rabbits consumed food and drank well during the exposures. Use of hypobaric hypoxia at this altitude produces a reproducible decrease in oxygen tension with no measurable changes in lung function resulting from the change in atmospheric pressure (7).

Lung tissue from the kits of control or hypoxia-exposed pregnant rabbits was harvested immediately after environmental exposure, at term, and at 1 wk of age. We delivered preterm rabbits by cesarean section after euthanizing the mental exposure, at term, and at 1 wk of age. We delivered atmospheric pressure (7).

RNA isolation and RT-PCR. Total RNA was isolated from rabbit lung by TRizol reagent (Life Technologies, Gaithersburg, MD) based on the Chomczynski and Sacchi (8) method. Briefly, tissue was homogenized in TRizol reagent containing phenol and guanidine isothiocyanate, followed by chloroform extraction and isopropanol precipitation. Samples were quantified by absorption spectrophotometry, and RNA integrity was confirmed using nondenaturing agarose gel electrophoresis. RNA samples were treated with RNase-free DNase (Promega, Madison, WI) at 37°C for 30 min to remove contaminating DNA, followed by 20 mM EGTA for 10 min at 65°C to inactivate the enzyme.

Total RNA (1 μg) extracted from the lungs was reverse transcribed in a final volume of 25 μl containing 0.4 units of avian myeloblastosis virus (AMV) RT (Promega), 1× AMV RT buffer (50 mM Tris-HCl, pH 8.3), 50 mM KCl, 10 mM MgCl2, 0.5 mM spermidine, and 10 mM dithiothreitol, 0.01 μg oligo(dT), 0.8 mM 2′-deoxynucleoside 5′-triphosphate (dNTP) (Life Technologies), and 1.6 units RNasin ribonuclease inhibitor. The samples were incubated at 42°C for 90 min and enzyme inactivated at 95°C for 10 min. The synthesized single-stranded cDNA was stored at -20°C until use.

EC-SOD was amplified by PCR using 20 pmol rabbit EC-SOD sense and antisense primers, 0.2 mM dNTP, 0.075 units Taq DNA polymerase (Qiagen, Valencia, CA), and 1× PCR buffer (25 mM KCl, 25 mM Tris-HCl (pH 8.3), 0.75 mM MgCl2). Primers were designed by MacVector to span the intron-exon 3 coding region of rabbit EC-SOD (sense: 5′ TGG ATG TTT CAA GTG ACC AG 3′; anti-sense: 5′ GAC TAC CAA GCC GCT GAG TC 3′) (Life Technologies). The cDNA was amplified in Hot Start reaction tubes (Molecular Bio-Products, San Diego, CA) with the Hybrid OmniGene thermocycler (Hybaid, Teddington, United Kingdom) using the following conditions: 1 min at 95°C × 1 cycle; 3 min at 95°C × 1 cycle; 30 s at 95°C, 40 s at 59.4°C, and 30 s at 72°C × 35 cycles followed by a terminal extension of 7 min at 72°C × 1 cycle. EC-SOD amplicons were normalized to 18S ribosomal RNA (rRNA) as an external standard.

PCR products were analyzed by agarose gel electrophoresis (0.8%). A 315-bp and a 489-bp fragment of EC-SOD and 18S, respectively, were stained with gelStar nucleic acid gel stain (BioWhittaker Molecular Applications, Rockland, MD) and visualized under ultraviolet transillumination. Band intensity was determined by densitometry analysis via the GS-710 calibrated imaging densitometer and Quantity One software (Bio-Rad, Hercules, CA). Results were expressed as the ratio between optical density (OD) of EC-SOD and 18S amplicons to determine relative expression values.

Western blot analysis. Lungs from kits of different ages were used without exposure to prenatal hypoxia were examined by Western blot analysis for expression of EC-SOD protein. Tissue was homogenized in lysis buffer (50 mM Tris, pH 7.6, 3% Igepal, 150 mM NaCl, 1 mM MgCl2, 5 mM EDTA, pH 7.6) with 1:20 protease inhibitor (2 mM 1,10-phenanthroline, 2 mM 3,4-diisocoumarin, 0.4 mM trans-epoxyucci

DNA-l-Leucylamido(4-guanidino)butane] on ice. Homogenates were centrifuged at 10,000 g for 20 min, and supernatants were used for protein assay (BCA protein assay kit, Sigma). Twenty micrograms of protein were loaded onto a 12% polyacrylamide gel for electrophoresis and electrophotographically transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Each membrane was blocked in 3% milk-Tris-buffered saline-Tween 20 (TBST) overnight at 4°C. The membranes were incubated with a mouse IgG antibody against a 20-amino acid EC-SOD peptide (1:1,000 in 3% milk-TBST) (25) for 1 h at room temperature, followed by a secondary goat anti-mouse IgG antibody conjugated to horseradish peroxidase (1:20,000 in 3% milk-TBST; BD Transduction Laboratories, San Diego, CA) for 1 h at room temperature. The blots were developed with enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ). The blots were stripped with 0.1 M glycine (pH 2.9) and reprobed using a monoclonal mouse IgG antibody against β-actin (1:5,000 in 3% milk-TBST), followed by a secondary goat anti-mouse IgG antibody conjugated to horseradish peroxidase (1:20,000 in 3% milk-TBST; BD Transduction Laboratories) to confirm equal protein loading.

Immunohistochemistry. Paraffin-embedded tissue sections (4 μm) were immunostained to assess localization of EC-SOD protein in airways and vessels. Sections were deparaffinized, and endogenous peroxidase activity was inhibited using graded ethanol and 3% hydrogen peroxide. Sections were subsequently blocked with 5% normal goat serum, 1% BSA, and 3% milk in PBS for 1 h at room temperature. Slides were incubated with primary mouse IgG against EC-SOD (25) overnight at 4°C. The secondary antibody incubation was performed with a biotinylated goat anti-mouse IgG antibody 1:20 in 1% BSA-PBS for 1 h at room temperature, followed by labeling with peroxidase-conjugated streptavidin in 1% BSA-PBS for 1 h (Biogenex Link and Label Kit; Biogenex, San Ramon, CA). Negative controls were performed with normal mouse serum. Slides were washed with PBS and Tris-Cl and developed with 3,3-diaminobenzidine. Slides were counterstained with hematoxylin, rinsed, and dehydrated with graded alcohol. Sections were examined by light microscopy and photographed at ×180.
EC-SOD activity measurement. EC-SOD activity was measured after separation from intracellular SOD (Cu,Zn SOD and Mn SOD). Lung tissue was homogenized in 10 vol of ice-cold buffer (50 mM potassium phosphate, pH 7.4, with 0.3 M KBr, 0.05 mM phenylmethylsulfonyl fluoride, and 3 mM diethylenetriaminepentaacetic acid) and then passed over a concavalin A sepharose column as described (22). EC-SOD activity was measured by inhibition of cytochrome c reduction at pH 10.0 as described by Crapo et al. (10).

Reagents. Reagents, unless specified, were obtained from Sigma.

Data analysis. Data are expressed as means ± SE as indicated. Comparisons were made by an unpaired t-test for RT-PCR or Western blot data or two-way analysis of variance for activity data using Statview software (SAS Institute, Cary, NC).

RESULTS

Effects of prenatal hypoxia on EC-SOD mRNA expression. To evaluate the effects of prenatal oxygen tension on EC-SOD gene expression, EC-SOD mRNA transcription was examined by semiquantitative RT-PCR in control preterm, term, and 1-wk-old rabbit lungs and compared with EC-SOD mRNA transcripts after prenatal exposure to hypoxia. Experiments were performed in triplicate. Figure 1 shows a representative EC-SOD mRNA signal along with corresponding 18S rRNA extracted from lung samples from preterm, term, and 1-wk-old rabbits with and without prenatal exposure to hypoxia. OD of the signals expressed as a ratio of EC-SOD to 18S rRNA: 1.19 ± 0.12 in term control lungs vs. 0.36 ± 0.15 in 1-wk control lungs, *P < 0.05 by unpaired t-test). Prenatal hypoxia exposure to hypoxia significantly decreased EC-SOD mRNA transcripts in term animal lung (ratio of EC-SOD/18s rRNA 0.52 ± 0.15, *P < 0.05 vs. term control lung by unpaired t-test). Prenatal hypoxia induced subtle but not significant decreases in EC-SOD mRNA signals in the lungs from preterm and 1-wk-old kits.

Effects of prenatal hypoxia on EC-SOD protein expression. EC-SOD protein was constitutively expressed in preterm and term rabbits. Interestingly, protein expression appeared to be age dependent, because the EC-SOD signal on Western blot analysis tended to decrease in control lung tissue from preterm to wk 1 (P = 0.06 by unpaired t-test, n = 4). Prenatal hypoxia significantly decreased EC-SOD immunoreactive protein in lungs from preterm, term, and 1-wk-old kits. Densitometry showed that with hypoxia, EC-SOD protein levels decreased by 33% in preterm lungs, 45% in term lungs, and 29% in 1-wk-old lungs compared with age-matched control lungs (P < 0.05, n = 4 for each except n = 3 for 1-wk hypoxia; Fig. 2). One week after birth, prenatal hypoxia did not change EC-SOD protein expression in the lung. β-actin levels, used to confirm equal loading of protein samples, were similar in all animals (not illustrated).

By 1 wk of age, EC-SOD was detectable by immunostaining in both the intracellular and extracellular compartments of pulmonary blood vessels in control lungs (Fig. 3, left). We found that prenatal hypoxia strongly decreased the release of EC-SOD protein into the extracellular space of 1-wk-old rabbits lungs, particularly in the extracellular matrix surrounding small pulmonary arteries (Fig. 3, right). Strong intracellular immunostaining was evident in the airway epithelial and alveolar type II epithelial cells (not illustrated) of preterm and term animals with and without exposure to prenatal hypoxia.

Effects of prenatal hypoxia on EC-SOD protein activity. EC-SOD activity increases with age in developing rabbit lung (25). In control animals, EC-SOD activity tended to increase (1.8-fold) 1 wk after birth compared with that of preterm and term kits (n = 5 for each). Prenatal hypoxia sharply lowered EC-SOD activity by one-half in the 1-wk-old rabbit lung, compared with age-matched controls (n = 4, P < 0.05 by ANOVA). EC-SOD activity was not decreased in preterm lungs after prenatal hypoxia (n = 5, P = 0.4; Fig. 4). Low
EC-SOD activity in term lungs (average 0.15 U/mg protein for n/H11005 2) is similar to levels in the preterm (0.14 ± 0.04, n = 5) and 1-wk lungs (0.15 ± 0.07, n = 4), but we are unable to provide statistical analysis with control term lungs due to small sample size.

DISCUSSION

We present new information on the developmental regulation of the important extracellular antioxidant enzyme EC-SOD in the immature rabbit lung and report the interesting finding that prenatal hypoxia disrupts the normal developmental secretion of active EC-SOD in the lung. This study contributes new data to our previous observations that secretion of active EC-SOD into the extracellular compartment is developmentally regulated in the rabbit lung (25).

In control rabbit lungs, EC-SOD mRNA expression significantly decreased during the first week of life. This pattern is consistent with other antioxidant enzymes, which are upregulated in the immediate prenatal period and subsequently decrease to adult levels (2, 4, 9, 13, 14). Whereas EC-SOD mRNA transcripts were constitutively expressed in preterm and term lungs, the message significantly decreased approximately threefold in the 1-wk-old animals (Fig. 1). The changes

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in mRNA transcription were followed by a similar trend toward less protein expression at 1 wk of age. As noted in our previous report, EC-SOD protein in rabbit lung consists of primarily uncleaved EC-SOD with intact heparin binding domain (25).

The model of maternal exposure to hypobaric hypoxia at 0.5 atm was selected to decrease oxygenation in the fetus by decreasing umbilical venous oxygen saturations. A previous study showed similar levels of hypobaric hypoxia (4, 300 m) in pregnant ewes’ decreased fetal umbilical venous O₂ saturation from 74–61% to 45–28% (20). Human maternal hypoxemia (P AO₂ < 60 mmHg) reduces umbilical venous PvO₂ from 32 to 26 mmHg to reduce fetal oxygen content (36). In our study, although the maternal exposures to hypobaric hypoxia were limited to 36 h, the changes in EC-SOD expression in the lungs of the kits were evident for at least 1 wk.

Intriguingly, transient prenatal exposure to hypoxia decreased EC-SOD mRNA transcription and protein expression for up to 1 wk of life. The significant decrease in both EC-SOD mRNA and protein in term lungs indicates that the regulation of transcription and translation was coordinated in these lungs after prenatal hypoxia. The lack of change in mRNA levels in the 1-wk-old rabbit after hypoxia likely reflects the lower baseline expression of EC-SOD mRNA.

The factors that regulate expression of EC-SOD mRNA or protein are unknown. Hypoxia is known to affect fetal development, matrix production, and secretion of signaling molecules (18, 29). It is possible that hypoxia represses EC-SOD expression in part by decreasing stability of the mRNA transcripts and enhancing the RNA decay (17). On the other hand, hypoxia may induce or repress the expression of a specific factor that would recognize nucleotide consensus sequence in the EC-SOD gene promoter. The formation of complexes with such a factor could regulate EC-SOD gene expression during prenatal hypoxia. Such a mechanism has been implicated in the regulation of Mn SOD in rabbit airway epithelial cells, as well as in mouse, rat, and A549 cells (17, 30). Moreover, EC-SOD regulation may occur via corticosteroid-responsive element in the promoter of EC-SOD gene, which may also suppress the isoenzyme gene expression. Notably, sustained hypoxemia results in an upregulation of adrenocorticotropic hormone expression in association with hypoxia alters free radical reactions and nitrosylation of proteins in this model (19). Future studies will be essential to understand the regulation of EC-SOD and its physiological relevance.

In utero, hypoxia may occur during maternal hypoxia or with placental insufficiency. A reduction in EC-SOD activity in the lung could place an infant at risk for disruption of normal postnatal development of the pulmonary vasculature or pulmonary oxidative damage after birth, especially with supplemental O₂ therapy. Abnormal development of the vasculature is associated with reduced EC-SOD activity (33). In a recent study, administration of NO altered alveolar lung development in fetal lung explants (37), which suggests that changes in EC-SOD expression could influence pulmonary vascular development by a NO-dependent mechanism. In addition, EC-SOD is important in protection from oxygen toxicity, as shown by studies in adult and neonatal transgenic mice overexpressing EC-SOD, which resist pulmonary oxygen toxicity, and EC-SOD knockout mice, which are more sensitive to hypoxia (3, 6, 11).

In summary, we have shown that maternal exposure to hypobaric hypoxia in late gestation decreases EC-SOD expression and activity in the neonatal lung that are sustained over the first week of life. These are the first data that show that EC-SOD expression can be altered by prenatal conditions and provide important rationale for understanding the regulation of EC-SOD.
in the lung and prenatal conditions that influence postnatal susceptibility to lung diseases.

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