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Secretion of extracellular superoxide dismutase in neonatal lungs

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Nozik-Grayck, Eva, Christine S. Dieterle, Claude A. Piantadosi, Jan J. Enghild, and Tim D. Oury. Secretion of extracellular superoxide dismutase in neonatal lungs. *Am J Physiol Lung Cell Mol Physiol* 279: L977–L984, 2000.—Extracellular superoxide dismutase (EC-SOD), the only known enzymatic scavenger of extracellular superoxide, may modulate reactions of nitric oxide (NO) in the lungs by preventing reactions between superoxide and NO. The regulation of EC-SOD has not been examined in developing lungs. We hypothesize that EC-SOD plays a pivotal role in the response to increased oxygen tension and NO in the neonatal lung. This study characterizes rabbit EC-SOD and investigates the developmental regulation of EC-SOD activity, protein expression, and localization. Purified rabbit EC-SOD was found to have several unique biochemical attributes distinct from EC-SOD in other species. Rabbit lung EC-SOD contains predominantly uncleaved subunits that do not form disulfide-linked dimers. The lack of intersubunit disulfide bonds may contribute to the decreased heparin affinity and lower EC-SOD content in rabbit lung. EC-SOD activity in rabbit lungs is low before birth and increases soon after gestation. In addition, the enzyme is localized intracellularly in preterm and term rabbit lungs. Secretion of active EC-SOD into the extracellular compartment increases with age. The changes in EC-SOD localization and activity have implications for the neonatal pulmonary response to oxidative stress and the biological activity of NO at birth.

oxidative stress; antioxidant enzyme; nitric oxide; development

SUPEROXIDE DISMUTASES (SODs) are a class of antioxidant enzymes that catalyze the rapid dismutation of superoxide to hydrogen peroxide and oxygen [dissociation constant (K_d) ~ 1 $\times 10^9$] (12). There are three known isoforms of SOD in mammals. The intracellular Cu,Zn-containing SOD is located primarily in the cytoplasm and nucleus of cells (7), and Mn-containing SOD is predominantly in mitochondria (25, 28, 31, 32). The third isoform of SOD is the extracellular superoxide dismutase (EC-SOD), which also contains Cu,Zn in its active site (17, 18). EC-SOD is localized predominantly in the extracellular matrix of tissues as well as in extracellular fluids depending on the presence of a carboxy-terminal heparin-binding domain (19). The heparin-binding domain is formed by a cluster of positively charged amino acids that enable EC-SOD to bind to heparin sulfate in extracellular matrices and cell surfaces (11, 21, 24, 30). The heparin-binding domain is sensitive to proteolytic removal. Proteolysis of this domain does not decrease enzyme activity but determines the affinity of the protein to the extracellular matrix and the distribution of EC-SOD activity (9).

EC-SOD is highly expressed in several tissues including vascular tissue, lung, and uterus. EC-SOD constitutes as much as 70% of total SOD activity in human and baboon aorta (24). In the mature lung, EC-SOD is produced by alveolar type II cells, airway epithelial cells, and vascular endothelial cells and is immunolocalized to the extracellular space around vessels and airway spaces (11, 21, 24, 29, 30).

One putative function of EC-SOD is the modulation of nitric oxide (NO) in the pulmonary vasculature and airways by preventing reactions between superoxide and NO. Superoxide reacts rapidly with NO (K_d ~ 6.7×10^9) to inactivate the vasodilator activity of NO and form a strong oxidant, peroxynitrite ($OONO^-$) (14). The fate of extracellular superoxide depends on the local concentrations of both EC-SOD and NO. Scavenging superoxide by EC-SOD would enhance smooth muscle relaxation in pulmonary vessels or airways by NO.

Changes in antioxidant defenses and NO production are important in the adaptation of the fetal lung to postnatal conditions. Adequate antioxidant defenses are necessary as the neonatal lung adapts to a relatively oxygen-rich environment. During late gestation in rabbit lungs, antioxidant enzyme activity increases for Cu,Zn SOD, catalase, and glutathione peroxidase (13). Cu,Zn SOD and catalase mRNA expression also have been shown to increase with development in rat lungs (4, 5). In human lung, catalase mRNA and activity, as well as Cu,Zn SOD and Mn SOD mRNAs,

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increase from the prenatal period into adulthood (2). It is not known how EC-SOD expression or activity change in the developing lung. NO synthesis increases in the developing lung and mediates the decrease in pulmonary vascular resistance that must occur at birth. Since EC-SOD can limit the reaction between superoxide and NO, EC-SOD may have an important physiological role in the transition to adult pulmonary circulation.

EC-SOD is the only known extracellular antioxidant enzyme that scavenges superoxide in the lung and therefore may be a critical component in both the responses to increased oxidant stress and preservation of NO-dependent processes in the neonatal lung. Therefore, we hypothesized that lung EC-SOD is regulated developmentally. This study illustrates that EC-SOD activity in rabbit lungs increases soon after gestation. We report that EC-SOD is intracellular in preterm and term lungs and that secretion of active EC-SOD into the extracellular compartment increases with age, beginning in the first week of life. The changes in EC-SOD localization and activity have implications for the neonatal pulmonary response to oxidative stress and biological activity of NO at birth.

MATERIALS AND METHODS:

Purification of rabbit aorta EC-SOD. Forty-five grams of rabbit aortas were obtained from Pel Freeze (Rogers, AR) and homogenized with an Osterizer blender at 4°C in 450 ml of 50 mM potassium phosphate, pH 7.4 and 0.3 M potassium bromide with the proteinase inhibitors diethylenetriaminepentaacetic acid (3 mM) and phenylmethylsulfonyl flouride (0.5 mM). The homogenate was centrifuged at 20,000 *g* for 20 min at 4°C. The supernatant was collected and treated with 20,000 units of DNase (Boehringer Mannheim, Indianapolis) and 0.5 mg/ml of RNase (Worthington Biochemical, Lakewood, NJ) at 4°C overnight. The supernatant was then filtered through a 0.45-μm filter (Millipore, Bedford, MA).

Concanavalin A Sepharose chromatography. The aorta homogenate was applied to a 10-ml concanavalin A Sepharose column (Sigma, St. Louis) connected to a fast-performance liquid chromatography (FPLC) system (Amersham Pharmacia Biotech UK) at 4 ml/min. The column was then washed with 50 mM HEPES and 0.25 M NaCl, pH 7.5, until the change in absorbance at 280 nm (A_{280}) of the flowthrough was zero. Binding proteins were eluted with a 0–200 mM gradient of methyl-α-mannopyranoside at 0.5%/min, with a flow rate of 2 ml/min. Fractions containing EC-SOD were determined by Western blot analysis as described below and pooled.

Heparin Sepharose chromatography. The pooled sample was dialyzed into 50 mM Tris-Cl, pH 7.5, and 50 mM NaCl. After dialysis the sample was applied to 50 ml of heparin Sepharose (Sigma) connected to a FPLC system (Amersham Pharmacia Biotech UK). The heparin Sepharose was washed with buffer (50 mM Tris-Cl, pH 7.5, and 50 mM NaCl) until the A_{280} of the flowthrough was zero. Heparin binding proteins were then eluted with a 50 mM to 1 M gradient of NaCl at a rate of 0.6%/min, with a flow rate of 5 ml/min, and collected in 5-ml fractions. Fractions containing EC-SOD were determined by Western blot analysis as described below and pooled.

Mono Q chromatography. The heparin Sepharose pool was dialyzed into 50 mM Tris-Cl, pH 7.5, and 50 mM NaCl. The sample was applied to a Mono Q Sepharose column connected to an FPLC system (Amersham Pharmacia Biotech UK). Binding protein was eluted with a 0–1 M gradient of NaCl at a rate of 0.5%/min. Fractions containing EC-SOD were determined by Western blot analysis and pooled. The purity of the protein was determined by Coomassie blue staining of the pooled sample after reducing SDS-PAGE.

PAGE. SDS-PAGE was performed with 5–15% gradient gels using a glycine-2-amino-2-methyl-1,3-propanediol-HCl system described by Bury (29). For reducing SDS-PAGE, samples of purified rabbit EC-SOD were boiled for 10 min in the presence of 30 mM dithiothreitol and 1% SDS before electrophoresis. For nonreducing SDS-PAGE, the dithiothreitol was omitted from the samples. Nondenaturing PAGE was performed in the same system by removing SDS from all buffers and not boiling the samples before electrophoresis.

Protein sequencing and amino acid analysis. Purified rabbit EC-SOD was subjected to SDS-PAGE in the presence of 10 mM dithiothreitol and then transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA) for amino-terminal sequence analysis. Samples were then analyzed by automated Edman degradation using an Applied Biosystems model 477A sequencer with on-line phenylthiohydantoin analysis employing an Applied Biosystems model 120A HPLC. Samples were applied to Porton peptide or protein sample support disks and were sequenced utilizing the modified cycles PI-BGN and PI-1 recommended by Porton Instruments.

Animal model. Lungs were obtained from preterm, term, 1-wk-old, 1-mo-old, and adult rabbits for analysis of EC-SOD protein activity, expression, and localization. Preterm rabbits were delivered by cesarean section on *day 28* (normal gestation 30 ± 1 days). Lungs from term rabbits were obtained within 12 h of birth. Tissue was either flash-frozen in

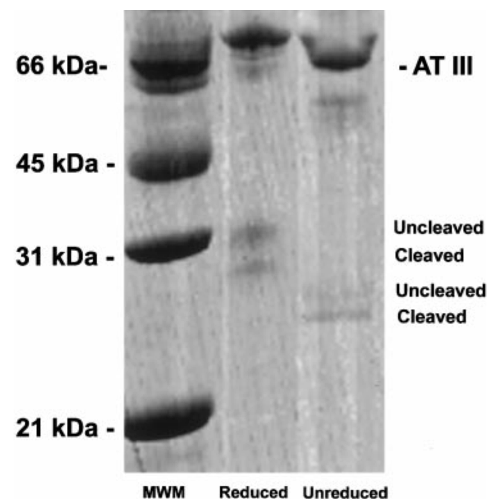

Fig. 1. Analysis of rabbit extracellular superoxide dismutase (EC-SOD) by SDS-PAGE. Purified rabbit aorta EC-SOD migrates as an ~30-kDa doublet on SDS-PAGE gel. The difference in mobility of the upper and lower bands in the EC-SOD doublet is presumed to be due to proteolytic removal of the heparin-binding domain in the lower subunit as has previously been shown for both human and mouse EC-SODs. Purified EC-SOD was analyzed under reduced and unreduced conditions (*lanes 2* and *3*, respectively). *Lane 1*, molecular mass markers (MWM; nos. on *left*). This analysis revealed that rabbit EC-SOD does not contain an intersubunit disulfide bond as shown by the lack of 60-kDa dimer and presence of monomers in both reduced and unreduced samples. Purified rabbit aorta EC-SOD was contaminated by antithrombin III (AT III).

Fig. 2. Western blot analysis of purified rabbit aorta EC-SOD. SDS-PAGE electrophoresis was performed with purified EC-SOD under reducing conditions for Western blot analysis. The polyclonal antibody against rabbit EC-SOD peptide recognized only EC-SOD monomers at 24 and 28 kDa.

liquid nitrogen for activity assays and Western blot analysis or inflation-fixed in 4% paraformaldehyde for immunohistochemistry.

Separation of SOD isoenzymes. Lung tissue was homogenized in 10 volumes of ice-cold 50 mM potassium phosphate, pH 7.4, with 0.3 M KBr, 0.05 mM phenylmethylsulfonyl flouride, and 3 mM diethylenetriaminepentaacetic acid. EC-SOD was separated from intracellular SOD (Cu,Zn SOD and Mn SOD) by passing the lung homogenates over a concanavalin A Sepharose column as previously described (16).

Measurement of SOD activity. EC-SOD activity and total SOD activity (Cu,Zn SOD and Mn SOD) remaining in the lung homogenate after EC-SOD extraction were measured by inhibition of cytochrome *c* reduction at pH 10.0 as previously described (6). Total SOD activity predominantly reflects Cu,Zn SOD since Cu,Zn SOD activity increases by 10-fold at pH 10.0 compared with that at pH 7.8, without a concomitant increase in Mn SOD activity. Total protein was determined by the Coomassie Plus protein assay (Pierce, Rockford, IL).

EC-SOD antibody production. A polyclonal antibody against a 20-amino acid peptide from the carboxy-terminal region of rabbit EC-SOD (CVVGASGPAPWARQAQEHAE) was developed in mouse ascites. The peptide was synthesized and conjugated to keyhole limpet hemocyanin at Biosynthesis (Lewisville, TX). Antibody was produced by injecting a 9:1 emulsion of complete Freund's adjuvant and peptide intraperitoneally into 8-wk-old A/J mice (Jackson Laboratories, Bar Harbor, ME). Mice were boosted four times using incomplete Freund's adjuvant, and once ascites developed, fluid was removed every 3–5 days. Dot blots were performed to confirm antibody production.

Western blot analysis. Lung tissue from rabbits at different ages were examined by Western blot analysis for expression of the EC-SOD protein. All tissue was homogenized in 150 mM NaCl, 1 mM $MgCl_2$, 5 mM EDTA, 50 mM Tris, pH 7.6, and 3% (nonylphenoxy)polyethoxyethanol (NP-40). Lysis buffer included 1:20 protease inhibitor 2 mM 1,10 phenanthroline, 2 mM 3,4 diisocoumarin, 0.4 mM *trans*-epoxysuccinyl-L-leucylamideo(4-guanidino)butane (E-64). Tissue was homogenized on ice and centrifuged at 10,000 *g* for 20 min, and the supernatant was used for protein assay and Western blot analysis. Twenty micrograms of protein were loaded onto a 12% polyacrylamide gel and electrophoretically transferred to an Immobilon-P membrane. The membrane was blocked in 3% milk-Tris-buffered saline-Tween 20 (TBS-T) overnight at 4°C. The membrane was then incubated with the antibody against rabbit EC-SOD peptide (1:1,000 in 3% milk-TBS-T) for 1 h at room temperature followed by a secondary goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY) (1:20,000) for 1 h at room temperature. The blot was developed with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech UK).

Immunohistochemical analysis. Paraffin-embedded tissue sections were examined by immunohistochemistry to determine the localization of the EC-SOD protein in developing rabbit lungs. Sections were deparaffinized, and endogenous peroxidase activity was inhibited with ethanol and 3% hydrogen peroxide and blocked with 5% normal goat serum, 1% BSA, and 3% milk in PBS for 1 h at room temperature. The slides were incubated with the primary antibody (1:200 in 1% BSA-PBS) overnight at 4°C. The secondary antibody incubation was performed with Biogenex Link and Label Kit (Biogenex, San Ramon, CA) containing biotinylated goat anti-mouse IgG antibody 1:20 in 1% BSA-PBS for 1 h at room temperature. Negative controls were performed with normal mouse serum and with antibody preabsorbed with purified EC-SOD. Sections were then labeled with peroxidase-conjugated streptavidin (Biogenex) in 1% BSA-PBS for 1 h at room temperature, washed with PBS and Tris-Cl, and developed with 3,3′-diaminobenzidine. Sections were counterstained with hematoxylin, rinsed, and dehydrated with graded alcohol. Sections were examined by light microscopy and photographed at ×160 and ×400.

Reagents. Reagents, unless specified, were from Sigma (St. Louis, MO).

RESULTS

Characterization of rabbit EC-SOD. Purified rabbit aorta EC-SOD migrates as an ~30-kDa doublet on SDS-PAGE (Fig. 1). The amino terminus of both bands in the doublet were sequenced and resulted in the same sequence: WSGPAAVELGSDTVE. This sequence is identical to the sequence of rabbit EC-SOD as predicted from the sequence of rabbit cDNA (NIH GenBank accession no. Z67878). The difference in mobility of the upper and lower bands in the EC-SOD doublet is presumed to be due to proteolytic removal of the heparin-binding domain in the lower subunit, as has previously been shown for both human and mouse EC-SODs (9). The major contaminant was a band of ~100 kDa. Amino-terminal sequence analysis revealed a sequence of DEFPVEDICTAKPRD. This sequence is identical to pig antithrombin III except for the first two amino acids. This band therefore is likely to represent rabbit antithrombin III, which has not been sequenced to date. During heparin Sepharose chromatography, EC-SOD eluted early in the NaCl gradient at 18–42% of 1 M NaCl compared with 50–75% of 1 M NaCl for human EC-SOD, indicating that rabbit EC-SOD has lower affinity to heparin than does human EC-SOD.

Table 1. *EC-SOD and IC-SOD activity in rabbit lungs*

	Preterm (n = 5)	Term (n = 6)	8 Day Old (n = 5)	1 Month Old (n = 6)	Adult (n = 5)
EC-SOD, U/mg protein	0.20 ± 0.07*†	0.28 ± 0.39*	0.37 ± 0.07*	0.64 ± 0.09*	1.34 ± 0.24
IC-SOD, U/mg protein	30.0 ± 7.5*†	31.4 ± 4.0*†	26.0 ± 4.1*†	90.5 ± 19.0	86.5 ± 12.2

Values are means ± SE; n, no. of lungs. Both extracellular (EC) and intracellular (IC) superoxide dismutase (SOD) activity significantly increases with age in rabbit lungs. *P < 0.05 vs. adult rabbit lungs. †P < 0.05 vs. 1-mo-old rabbit lungs by ANOVA.

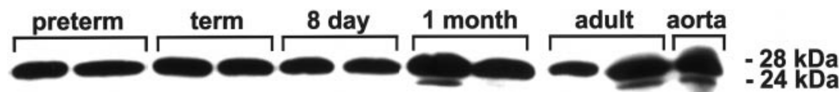

Fig. 3. Developmental expression of rabbit lung EC-SOD by Western blot. The expression of EC-SOD protein was examined in preterm, term, 8-day-old, 1-mo-old, and adult rabbit lungs, which showed a similar amount of EC-SOD protein at each age. Rabbit lung at all ages contained predominantly uncleaved monomers. Aorta was used as a positive control.

Analysis of EC-SOD by nondenaturing PAGE revealed that rabbit EC-SOD also exists as large multimers similar to human EC-SOD (data not shown) (22). The polyclonal antibody developed against rabbit EC-SOD peptide recognized purified rabbit aorta EC-SOD on Western blot (Fig. 2).

Rabbit EC-SOD lacks an interchain disulfide bond. Analysis of rabbit EC-SOD under reducing and nonreducing conditions reveals that it does not contain an intersubunit disulfide bond as shown by the lack of 60-kDa dimer and presence of monomers in both reduced and unreduced samples. (Fig. 1). A band present below antithrombin III in both the reduced and unreduced samples was not identified due to insufficient protein for amino-terminal sequence analysis. This is unlikely to represent EC-SOD dimer, since it persists after reduction with DTT and it is unrecognized by the antibody to rabbit EC-SOD on Western blots. Furthermore, full-length uncleaved monomer is also present in the unreduced lane, indicating that no interchain disulfide bond is present in the majority of the unproteolyzed protein. Thus rabbit EC-SOD differs from both mouse and human EC-SOD, which have been shown to contain an intersubunit disulfide bond that links the heparin-binding domains of two subunits together (9a, 22). Notably, rabbit EC-SOD does contain an intrasubunit disulfide bond as illustrated by the slower migration of both subunits in the ~30-kDa dimer on

Fig. 4. Immunohistochemical localization of rabbit lung EC-SOD with age: alveoli and airways. In preterm (*A*) and term lungs (*B*), EC-SOD was primarily localized to the intracellular compartment of airway epithelial and alveolar epithelial cells. There was minimal staining in the extracellular matrix, characteristic of mature lungs. At 8 days of life (*C*), extracellular staining was apparent, although there was still dark staining for EC-SOD within epithelial cells. At 1 mo of age (*D*), there was significant intracellular staining and an increase in extracellular matrix surrounding airways. In adult lungs (*E*), the extracellular matrix of airways stained, without appreciable staining within cells. Adult negative control (*F*) shows absence of staining with normal mouse serum. Tissue sections were photographed at ×400.

reduction (Fig. 1). An intrasubunit disulfide is also present in human and mouse EC-SODs (9a, 22).

Changes in EC-SOD activity in developing rabbit lungs. EC-SOD and intracellular SOD (IC-SOD) activity levels were measured in rabbit lung tissue at different ages. EC-SOD activity in rabbit lungs increased from preterm to adult. There was also an increase in IC-SOD activity in developing rabbit lung tissue (Table 1).

EC-SOD protein levels in developing rabbit lungs. The expression of EC-SOD protein was examined in lungs during development and showed a similar amount of EC-SOD protein in immature lungs, which tends to increase with age (Fig. 3). Notably, in contrast to human and mouse lungs that have a mixture of full-length and cleaved EC-SOD subunits, rabbit lungs at all ages contained predominantly uncleaved monomers. This finding was consistent for Western blot data using antibody isolated from three different mice.

Change in localization of EC-SOD in developing rabbit lungs. EC-SOD localization changed in developing rabbit lungs. In preterm and term lungs, EC-SOD was primarily localized to the intracellular compartment of airway epithelial and alveolar epithelial cells. There was minimal staining in the extracellular matrix, characteristic of mature lungs. At 1 wk of life, extracellular staining was apparent, although there was still dark staining for EC-SOD within epithelial cells. At 1 mo of age, significantly less intracellular staining and an increase in extracellular matrix staining was seen around vessels and on the surface of airways and (Figs. 4 and 5). In adult lungs, the extracellular matrix of vessels and airways stained without appreciable staining within cells. A negative control performed with purified protein-absorbed antibody inhibited the binding of the antibody to tissue sections from rabbit lung (Fig. 6).

DISCUSSION

This study is the first to examine EC-SOD activity and expression in developing lungs. EC-SOD is an important extracellular antioxidant enzyme in the lung and has been characterized in the adult human, baboon, rat, and mouse. We chose the rabbit lung for studies of developmental expression of EC-SOD because the neonatal rabbit lung is relatively large, which facilitates biological and physiological studies. Notably, rabbit EC-SOD was found to have several features that differ from EC-SOD of other species.

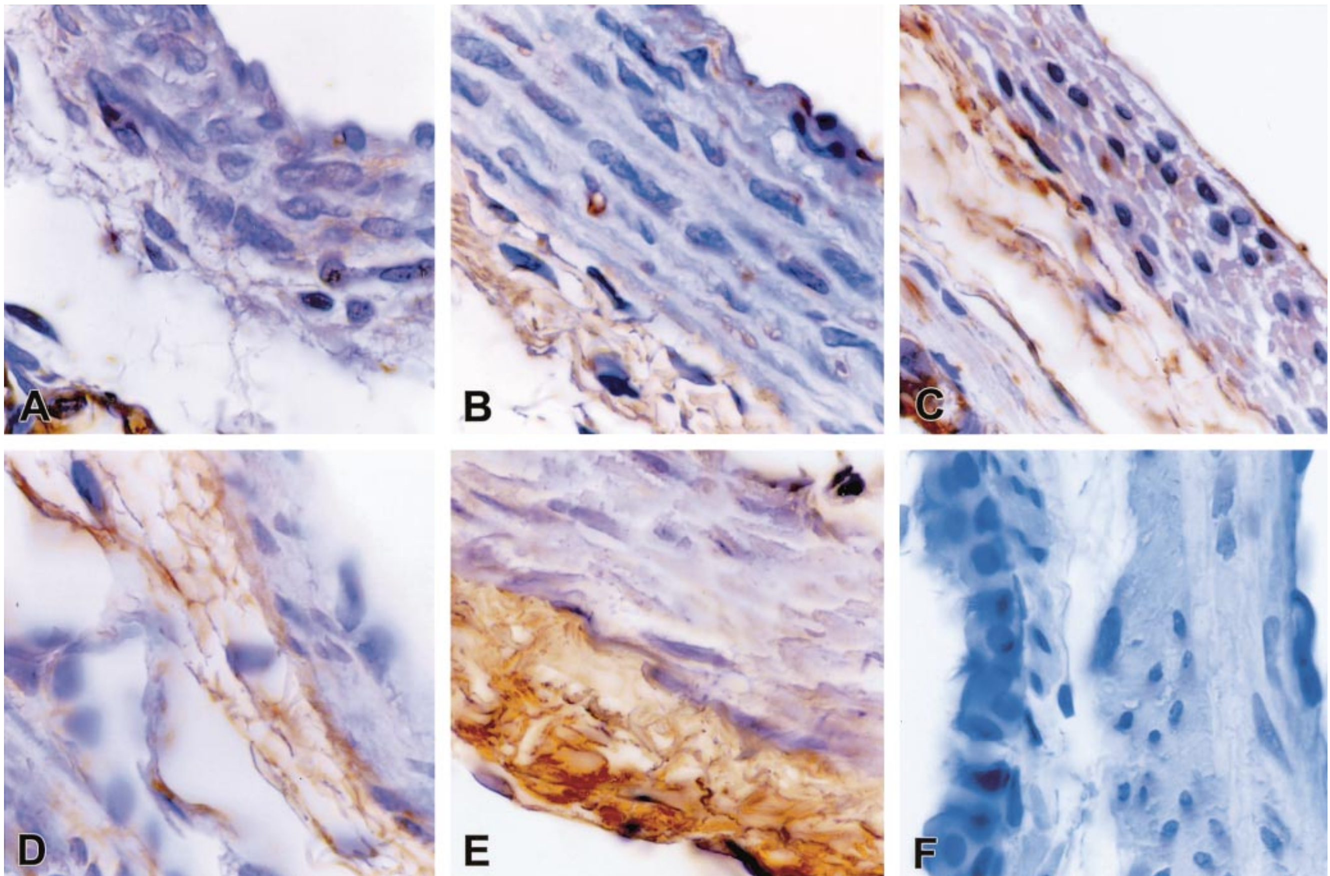

Fig. 5. Immunohistochemical localization of rabbit lung EC-SOD with age: vessels. Staining of the extracellular matrix surrounding small pulmonary arteries increased with age. Representative vessels are shown from lungs of preterm (*A*), term (*B*), 8-day-old (*C*), 1-mo-old (*D*), and adult (*E*) rabbits. Adult negative control (*F*) shows absence of staining with normal mouse serum. Tissue sections were photographed at ×400.

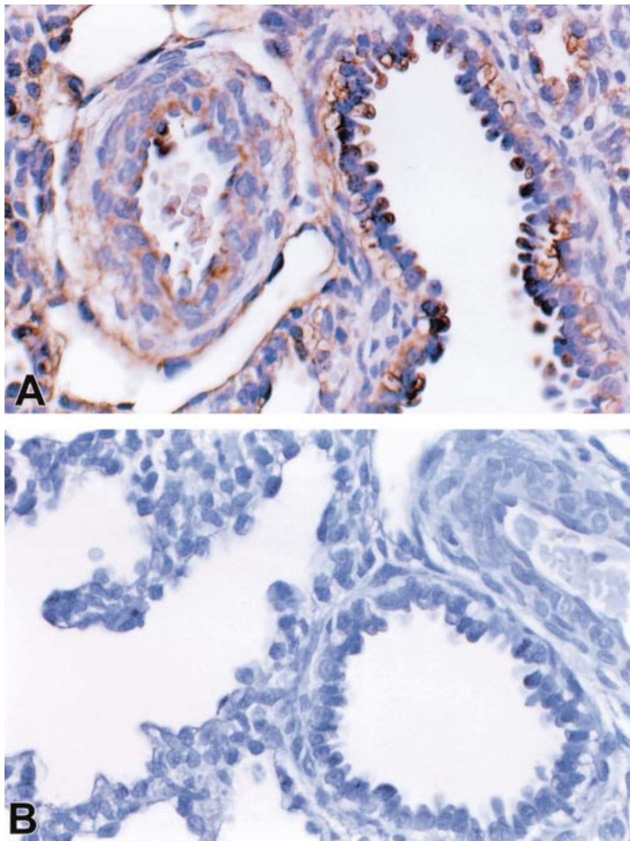

Fig. 6. Inhibition of staining for EC-SOD with protein-absorbed antibody. Control experiments were performed in preterm rabbit lungs treated with EC-SOD antibody (*A*) or with EC-SOD antibody preabsorbed with purified EC-SOD (*B*). Staining was not present in lung tissue treated with protein-absorbed antibody. Tissue sections were photographed at ×160.

Rabbit EC-SOD has a lower affinity to heparin than human EC-SOD, although it has not been previously known how the rabbit protein differs from other species (15). These new data indicate that rabbit EC-SOD lacks an interchain disulfide bond to link the heparin-binding domains of two subunits together. This interchain disulfide bond is present in both human and mouse EC-SODs (9a, 22) and may increase the affinity of the protein to heparin by linking two heparin-binding domains together (22). Thus the absence of an interchain disulfide bond in rabbit EC-SOD may be partly responsible for its decrease in affinity for heparin.

Low heparin affinity of rabbit EC-SOD likely accounts for the relatively lower activity of EC-SOD in adult rabbit lung and pulmonary artery compared with that in human and baboon. The ratio of EC-SOD activity in rabbit lung to pulmonary artery, however, is similar to that in human and baboon, where lung contains 10–20% of EC-SOD activity measured in pulmonary artery (24). The lower heparin affinity may account for the relatively higher activity levels of EC-SOD in rabbit serum compared with that in other species (24); however, EC-SOD is still localized predominantly in tissue (27).

Purified rabbit aorta EC-SOD contains full-length and cleaved monomers similar to those observed in humans and baboons (22, 24). The rabbit lung, however, contains only the uncleaved full-length subunits of EC-SOD. This differs from mouse, rat, and human lungs, which contain both uncleaved and cleaved EC-SOD subunits (9a, 22). The significant interspecies differences in EC-SOD, e.g., formation of monomers, dimers, or tetramers, and presence as cleaved or uncleaved state influence the distribution of EC-SOD and therefore are important for further investigations of EC-SOD function in the mature and immature lung.

Developmental regulation of antioxidant enzymes is important for the adaptation of the neonatal lung to the relatively high oxygen environment following birth. We found an age-dependent increase in both EC-SOD and IC-SOD activity in rabbit lung. Intracellular Cu,Zn SOD activity has been shown to increase in developing rat lungs (2, 4, 5), but EC-SOD activity had not been reported previously.

Although lung EC-SOD activity increased after parturition, the amount of EC-SOD protein was stable in the lung during late development. An interesting and important change occurred, however, in the immunolocalization of EC-SOD from intracellular to extracellular compartments within the lung. A gradual but clear shift of the protein from the intracellular compartment in airway epithelial cells and endothelial cells to the well-established extracellular distribution in adult lungs took place after birth. The presence of intracellular EC-SOD has also been described in the developing human placenta, with intracellular labeling within cytotrophoblasts early in pregnancy (3). Our findings also suggest the possibility that intracellular EC-SOD is inactive or less active than secreted EC-SOD because the increase in activity correlated with the appearance of EC-SOD outside of the cell, whereas no increase in the amount of apoprotein was detected by Western blots. Neither the size of EC-SOD monomers nor the pattern of intact and cleaved EC-SOD changed in a manner that would account for the low EC-SOD activity in the neonatal lung.

EC-SOD, like cytosolic Cu,Zn SOD, contains copper in its active site. Notably, a specific copper chaperone is essential for the incorporation of copper into the active site of cytosolic Cu,Zn SOD (8, 26). The regulation of copper incorporation into EC-SOD has not been studied to date. However, it is possible that copper is not incorporated into EC-SOD until just before secretion or after it is in the extracellular matrix. Therefore, a lack of copper in the active site of EC-SOD while it is stored intracellularly in the epithelial cells may explain the lower activity for EC-SOD in the neonatal lungs. Further studies are needed to determine the factors that regulate the secretion of active EC-SOD in the lung. Potentially important factors include changes in oxygen tension or maternal hormone adjustment at birth and developmental changes in NO production, the lung extracellular matrix, or copper incorporation.

Other antioxidant enzymes increase in the perinatal period and are felt to protect the lung from the increased oxidative stress during the transition from the hypoxic fetal environment to an oxygen-rich atmosphere. To date, the role of extracellular superoxide and EC-SOD in the lung has been examined only in adult models of oxidative injury. Overexpression of EC-SOD in adult transgenic mice protects from pulmonary oxygen toxicity (10). Extracellular superoxide contributes to pulmonary edema and pulmonary hypertension in an adult rabbit model of hyperoxic lung injury (20). The role of EC-SOD has not been examined in neonatal oxygen toxicity.

Several studies in adult tissues have demonstrated a role for EC-SOD in NO-dependent vasorelaxation. Exogenous EC-SOD can enhance endothelium-dependent vasodilation, and administration of a SOD that binds to heparin sulfate decreases blood pressure in spontaneously hypertensive rats (1). EC-SOD may also have an important role in NO-mediated pulmonary vasodilation in the newborn as it does in adult animals. NO synthase and NO levels increase before birth and mediate the decrease in pulmonary vascular resistance during the transition from fetal to adult circulation. Persistent pulmonary hypertension of the newborn is associated with decreased NO activity and is a significant cause of morbidity and mortality in critically ill full-term infants. The role of EC-SOD in maintaining the biological activity of NO in the neonate under physiological or pathophysiological conditions is an important area for future investigations.

In summary, we have characterized rabbit EC-SOD in the developing lung and shown that the rabbit lung contains primarily uncleaved EC-SOD monomers that do not form intersubunit disulfide bonds. The lack of this interchain disulfide bond in rabbit EC-SOD may explain the low heparin affinity of rabbit EC-SOD observed in this study and previous work. We showed that active EC-SOD is secreted by epithelial cells into the extracellular spaces of the lung shortly following birth. These findings have important implications for the response to oxidative stress and biological activity of NO in the neonatal lung.

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