Intracellular uptake of recombinant superoxide dismutase after intratracheal administration

SANTANU DAS, STUART HOROWITZ, CAROLYN G. ROBBINS, MARWAN EID EL-SABBAN, NAMITA SAHGAL, AND JONATHAN M. DAVIS
Departments of Pediatrics (Neonatology) and Medicine (Nephrology) and The CardioPulmonary Research Institute, Winthrop University Hospital, State University of New York Stony Brook School of Medicine, Mineola, New York 11501

Das, Santanu, Stuart Horowitz, Carolyn G. Robbins, Marwan Eid El-Sabban, Namita Sahgal, and Jonathan M. Davis. Intracellular uptake of recombinant superoxide dismutase after intratracheal administration. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L673–L677, 1998.—We have previously demonstrated that recombinant human copper-zinc superoxide dismutase (rhCu,ZnSOD) is rapidly incorporated into cells of airways, respiratory bronchioles, and alveoli after intratracheal administration. The present study examines whether this cellular uptake is specific for rhCu,ZnSOD or whether other proteins are similarly incorporated into lung cells. Twenty-two newborn piglets (2–3 days old, 1.2–2.0 kg) were intubated and mechanically ventilated. Eight piglets received fluorescently labeled recombinant human manganese superoxide dismutase (rhMnSOD), six received fluorescently labeled albumin, two received free (unbound) fluorescent label intratracheally, and two piglets served as untreated controls. To determine whether endogenous surfactant was important in the process of intracellular uptake, four additional piglets were made surfactant deficient by repeated bronchoalveolar lavage and then given rhCu,ZnSOD intratracheally. All animals were killed after 30–60 min. Lung sections were examined blindly by laser confocal microscopy. Similar to our previous observations with rhCu,ZnSOD, intracellular uptake of rhMnSOD and albumin was noted throughout the lung. The free label did not localize intracellularly. The uptake of proteins did not appear to be affected by surfactant deficiency. rhMnSOD administration was associated with a greater than twofold increase in lung MnSOD activity. Data suggest that the cellular uptake of antioxidants and other proteins in the lung may reflect a nonspecific host defense system for clearing proteins from the lumen of airways and alveoli.

recombinant human superoxide dismutase; antioxidants; lung injury; surfactant

BRONCHOPULMONARY DYSPLASIA (BPD) is a chronic lung disease seen in neonates treated with O2 and mechanical ventilation for primary lung disorders (7, 21). With the increasing survival of many critically ill neonates, BPD has become one of the most important sequelae of neonatal intensive care. BPD is believed to begin as acute inflammatory changes secondary to cell injury from extremely toxic reactive oxygen intermediates (ROI), which then evolve into chronic lung disease (5–7, 30). Under conditions of hyperoxia and/or inflammation, increased amounts of these ROI are produced (14). ROI can react with cellular macromolecules, including membrane lipids, structural and enzymatic proteins, and DNA, causing significant cell injury and even cell death by necrosis and apoptosis (10, 11, 13).

Under normal physiological conditions, a delicate balance exists between the production of ROI and the antioxidant defenses that protect cells in vivo (10, 11). The balance can be disrupted if there is increased production of ROI or if there are inadequate antioxidant defenses. Premature infants are especially susceptible to free radical injury, since birth can occur before the maturation of the antioxidant system (12). Therefore, even 21% O2 may be considered to be supraphysiologic for a premature infant. Consequently, there is significant interest in therapeutic approaches that supplement lung antioxidants in infants to prevent injury from ROI and the subsequent development of chronic lung disease.

Superoxide dismutases (SODs) are important antioxidant enzymes, the primary function of which is the conversion of the extremely reactive superoxide radical (O2·−) to the potentially less toxic species H2O2 (11). Several animal studies have demonstrated that intravenous, intraperitoneal, or intratracheal (IT) administration of SOD (native or encapsulated in surfactant liposomes) significantly ameliorates lung damage and improves survival from prolonged hyperoxia and mechanical ventilation (6, 20, 22, 26, 28, 29). It has previously been demonstrated that a single IT dose of recombinant human copper-zinc SOD (rhCu,ZnSOD) reduced pulmonary inflammation and significantly increased SOD concentration in serum, tracheal aspirates, bronchoalveolar lavage (BAL), and lung tissue in newborn piglets injured by 48 h of hyperoxia and mechanical ventilation (6). With the use of fluorescently labeled rhCu,ZnSOD and laser confocal microscopy, it has also been demonstrated that rhCu,ZnSOD administered intratracheally is taken up rapidly (by 60 min) into a variety of cell types in the lung and that lung tissue SOD activity was significantly increased (25). This intracellular localization of rhCu,ZnSOD within the lung may partially explain the persistent elevation of SOD concentration and activity observed in the tracheal aspirates, BAL fluids, and lung tissue of treated piglets as well as in the tracheal aspirates of infants in clinical trials (6, 24).

To address whether intracellular uptake was specific for rhCu,ZnSOD, the present studies examined whether other antioxidants such as recombinant human manganese SOD (rhMnSOD) or larger proteins such as albumin also enter lung cells after IT administration. In addition, we examined whether normal concentrations of endogenous surfactant are necessary for intracellular localization.
METHODS

Twenty-two newborn piglets 2–3 days old and weighing 1.2–2.0 kg were studied. At the start of each experiment, the piglets were anesthetized with intraperitoneal pentobarbital sodium (25 mg/kg) and placed on a heating blanket. They were intubated (3.5–4.0 endotracheal tube) and mechanically ventilated with a Bournes BP 200 infant ventilator. A 3.5-Fr umbilical arterial catheter (UAC) was inserted. Arterial Po2 was maintained at 60–80 Torr, and arterial Pco2 was maintained between 35 and 45 Torr. Five percent dextrose in 0.33 M NaCl was administered via the UAC at 80 ml·kg−1·day−1. Additional sedation was used as required. Four animals had repeated BAL performed with 30-ml aliquots of warmed normal saline to induce surfactant deficiency. BAL was performed until the arterial Po2 was <100 Torr while each animal was receiving 100% inspired O2. It has previously been demonstrated that this is associated with significant surfactant deficiency (8).

Study material was instilled intratracheally to all piglets with the right side down to enhance delivery to the right lower lobe (RLL). The piglets were then ventilated for the next 30–60 min. At the end of this period, they were killed with lethal doses of pentobarbital sodium. The chest was opened (with the lungs inflated with 20 cmH2O of continuous pressure), the pulmonary artery was cannulated, and the lungs were perfused with normal saline (3 × 60 ml) to minimize red blood cell contamination.

Proteins and labeling. Eighteen animals received one of the following proteins by IT instillation: rhCu,ZnSOD (mol mass 31 kDa, n = 4), rhMnSOD (mol mass 45 kDa, n = 8), and albumin (mol mass 66 kDa, n = 6). The rhSOD used in this study was supplied by Bio-Technology General (Iselin, NJ). The rhSOD is produced in Escherichia coli by recombinant DNA technology. The amino acid sequence is identical to human SOD, although the NH2 terminus is not acetylated.

The proteins were fluorescently labeled with fluorescein-X succinimidy l ester [SFX; 6-(fluorescein-5-carboxamido)hexanoic acid succinimidyl ester; Molecular Probes, Eugene, OR]. One milligram of SFX was dissolved in 10 µl of DMSO. Ten milligrams of protein (in 250 µl of buffered saline) were then added. The final solution was incubated at room temperature for 1 h and then incubated at 4°C for 12–24 h. The reaction mixture was subjected to exclusion chromatography using a Sephadex G25 spin column (Isolab QS-2B) to remove unbound fluorophore before use. The protein was then diluted with normal saline to a concentration of 2.5 mg/ml and administered intratracheally at 2 ml/kg (total dose of 5 mg/kg). Nine piglets received only free (not bound to any protein) fluorescent label as an additional control group. One milligram of SFX was added to 250 µl of saline. This mixture was further diluted so that the piglets received 2 ml/kg intratracheally. Finally, two piglets acted as untreated control animals and did not receive any protein or fluorescent label.

Tissue preparation. The lungs were perfused with 2% paraformaldehyde (2 × 60 ml). The RLL was then isolated and immersed in 2% paraformaldehyde for at least 48 h to fix the tissue, followed by immersion in 20% sucrose for 24 h. Parallel slices of the RLL were cut from the periphery to the main airway, and each piece was embedded in optimum cutting temperature compound (OCT compound, Tissue-Tek) and then flash-frozen in liquid nitrogen. Cryosections of 10-µm thickness were obtained with a Jung cryomicrotome. The sections were mounted on glass slides using Antifade compound (Molecular Probes).

Microscopy. Slides were examined by laser confocal microscopy. Images were obtained with a Nikon RCM 8000 laser confocal imaging system using a Nikon Planapo ×40 objective lens. Images were captured and analyzed using Nikon RCM 8000 software. All images were captured under identical conditions. To further demonstrate that the signal was from within (and not on the surface of) the cell, Z-series images (optical sections) were obtained at 0.1-µm intervals.

Lung tissue MnSOD activity measurements. Two piglets that received rhMnSOD and the two untreated control animals had lung tissue from the RLL homogenized and assayed for MnSOD activity as previously described (25). Data are presented as units of MnSOD activity per milligram of protein. The difference in MnSOD activity between the two experimental groups was analyzed using an unpaired Student’s t-test.

RESULTS

Previous work showed that rhCu,ZnSOD, which is a relatively small protein (31 kDa), readily entered a variety of lung cells shortly after IT instillation (25). To determine whether a much larger antioxidant enzyme could also localize intracellularly after IT delivery, rhMnSOD (45 kDa) was fluorescently labeled and instilled in the trachea of newborn piglets. Figure 1A shows representative laser confocal micrographs of lung tissue 1 h after rhMnSOD instillation. At this time point, ~90% of the fluorescent label remains bound to the protein within the lung (25). rhMnSOD fluorescence was evident within a variety of airway and alveolar cells, similar to what was previously described for rhCu,ZnSOD. Figure 2 shows a Z series of optical

Fig. 1. Uptake of proteins in lung. A: a representative laser confocal microscopic image 1 h after intratracheal (IT) administration of labeled recombinant human manganese superoxide dismutase (rhMnSOD). B: 0.5 hour after IT administration of labeled albumin. C: 1 h after IT administration of labeled albumin. D: 1 h after administration of labeled recombinant human copper-zinc SOD (rhCu,ZnSOD) to a surfactant-deficient piglet. See RESULTS for details.
sections of 0.1 µm each taken through the fluorescent regions of airway and alveoli. This series shows that the fluorescence was intracellular because it persists in each section, is limited to the cytoplasm, and does not include the nucleus. This yields an annular fluorescence pattern. rhMnSOD administration was associated with a 116% increase in lung tissue MnSOD activity compared with the untreated control animals ($P < 0.005$).

Although rhMnSOD is $\sim$150% of the molecular mass of rhCu,ZnSOD, both are antioxidant enzymes. It was important to determine whether this process was specific for antioxidant enzymes or whether nonantioxidant proteins are also taken up by lung cells. To address this issue, fluorescently labeled BSA was also administered to piglets. Figure 1, B and C, shows that BSA also localized within cells of the lung after IT administration. A Z series of optical sections further indicated that some BSA was localized within the cells, similar to rhMnSOD and rhCu,ZnSOD.

In the experiments described in METHODS, all piglets were born at term and were thus surfactant sufficient. Other studies have reported that SOD was found to protect cells from hyperoxia only when first encapsulated in surfactant liposomes, suggesting that the protein could not enter cells directly unless it was part of a larger macromolecular complex that might be endocytosed (22, 28). In vivo, pulmonary surfactant could, in principle, combine with instilled proteins to create complexes that might facilitate cellular uptake. To test this notion, piglets were rendered surfactant deficient by repeated BAL with buffered saline. Under these conditions, at least 80–90% of the surfactant phospholipids are removed from the lung (8). One hour after repeated lavage, piglets were given labeled rhCu,ZnSOD intratracheally. Again, fluorescence was observed within a variety of cell types in the lung (Fig. 1D), suggesting that intracellular uptake is not dependent on normal concentrations of pulmonary surfactant. Optical sections through the cells (Fig. 3) confirmed that the rhCu,ZnSOD had indeed localized intracellularly in the surfactant-deficient animals.

Although the vast majority (>90%) of the fluorescent label remains covalently attached to protein over this time period, the possibility exists that the observed cellular fluorescence was an artifact of the fluorophore that had become dissociated after IT administration. It became important to eliminate this possibility by administering unbound fluorophore directly into the lungs. If free fluorescent dye were responsible for the observed intracellular fluorescence, it might increase if 100% of the dye were unbound. Figure 4 demonstrates that the intracellular labeling pattern was not observed when free fluorescent dye was used, suggesting that uptake was dependent on the association of the fluorophore with a macromolecule.

**DISCUSSION**

We have previously reported that rhCu,ZnSOD is rapidly taken up by a variety of cell types in the lung after IT administration in the neonatal piglet (25). The present study was designed to further evaluate the uptake process. Larger proteins including the antioxidant rhMnSOD and nonantioxidant proteins such as albumin also localized intracellularly shortly after IT administration. These observations suggest that intracellular uptake of proteins is probably a nonspecific process, existing perhaps to clear proteins from the lung.

Interestingly, intracellular uptake in the lung does not appear to require the presence of normal quantities of endogenous surfactant, since repeated BAL did not appear to alter intracellular protein uptake. However, up to 20% of surfactant remains in the lung after lavage, which could facilitate intracellular uptake. Moreover, repeated BAL may cause some degree of lung injury with the influx of inflammatory cells and the
development of mild edema. Such injury could also affect the uptake of proteins administered intratra-
cheally.

Airway and alveolar epithelial cells are joined to each other by tight junctions, forming a macromolecular barrier. However, significant amounts of fluid, solutes, and macromolecules (such as protein) can move between cells (16–18). In addition to this paracellular route, protein added to the alveolar space can be cleared by intracellular epithelial cell transport (1–3, 16–19). Cells of the alveolar epithelium and capillary endothelium are actually quite permeable, with estimates of pore radii calculated from experimentally determined reflection coefficients of 6–10 Å in the alveolar epithelium and 40–58 Å for the capillary membrane (27). Although proteins such as albumin are primarily cleared from the lung by paracellular mechanisms, low levels of protein have been shown, using electron microscopy and specific inhibitors of cellular function, to be cleared from the lung by endocytosis, transcytosis, and restricted diffusion through the epithelium (16–18). This pathway may be responsible for our observations. Other investigators have also demonstrated that epithelial cells can directly clear proteins from the alveolar space via epithelial cell transport, although some of the protein might be degraded in lysosomes after uptake (1–3, 9). Ferritin and horseradish peroxidase have been demonstrated to be transported across airway epithelial cells via vesicle formation within 30–60 min of IT instillation in guinea pigs (23). Ito et al. (19) also showed that both endocytosis and transcytosis occurred in Clara cells and type II alveolar cells, whereas no transcytosis was seen in type I alveolar cells. These studies in conjunction with our own observations that rhCu,ZnSOD is detected in both serum and urine of premature infants soon after IT administration suggest that rhCu,ZnSOD is cleared by similar mechanisms, crossing multiple cellular barriers, yet remaining intact and enzymatically active (24).

In conclusion, data indicate that proteins administered into the lung are rapidly taken up into a variety of cell types. Our findings are similar to those reported by several other investigators using a variety of different techniques. We speculate that this nonspecific mechanism exists to clear proteins from airways and alveoli (to minimize oncotic forces that would tend to attract water and inhibit normal lung function). In addition, the fact that rhCu,ZnSOD enters lung cells in surfactant-deficient lungs indicates that uptake does not require normal quantities of pulmonary surfactant. The fluorescent-labeling technique that was used in this study is qualitative, and thus we were not able to quantitate the actual amount of the delivered protein cleared by the intracellular route compared with clear-

Fig. 3. rhCu,ZnSOD localized in alveolar cells of surfactant-deficient piglet. A–F: 0.1-µm optical sections (Z series) through distal lung of a surfactant-deficient piglet 1 h after receiving fluorescently labeled rhCu,ZnSOD as described in METHODS. Threshold was set to minimize autofluorescence.

Fig. 4. Free fluorescent label localizes on cell surface and is not taken up by alveolar cells. Laser confocal microscopic image from lung of a piglet receiving free fluorescent label (unbound to any protein). Label is seen on cell surface and does not localize intracellularly (additional Z-series images not shown here).
ance by other mechanisms. Further studies using electron microscopy and metabolic inhibitors of cellular processes such as endocytosis may better elucidate the exact pathway of rhCu,ZnSOD and other protein entry into the cell. Nonetheless, taken together with the promise of protein-based drug delivery to the lung, the results of this study have important implications for a variety of therapeutic interventions to treat neonatal and other lung disorders.

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Address for reprint requests: J. M. Davis, Dept. of Pediatrics, Winthrop Univ. Hospital, State Univ. of New York Stony Brook School of Medicine, 259 First St., Mineola, NY 11501.

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