

Neutrophil-generated Free Radicals: Possible Mechanisms of Injury in Adult Respiratory Distress Syndrome

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The acute lung injury resulting from adult respiratory distress syndrome (ARDS) is thought to be largely mediated by activated neutrophils. Because activated neutrophils produce the superoxide radical, which is both bactericidal and cytotoxic to host cells, this oxygen-derived free radical is likely responsible for at least part of the neutrophil-mediated lung injury. In a rat model of ARDS resulting from intratracheal instillation of interleukin-1, recombinant human manganous superoxide dismutase significantly decreased lung leak. One detrimental action of proteases released by adherent neutrophils may be the degradation of extracellular superoxide dismutase (ECSOD), which normally binds to the heparan sulfate on the surface of the endothelium. We found that rabbit ECSOD incubated with either trypsin or activated neutrophils loses affinity for heparin. Furthermore, soluble ECSOD is elevated in the serum of patients with ARDS, consistent with this hypothesis. — *Environ Health Perspect* 102(Suppl 10):57–60 (1994)

Key words: neutrophils, adult respiratory distress syndrome, superoxide dismutase, free radicals, ferritin, iron

Introduction

Adult respiratory distress syndrome (ARDS) is a common and frequently lethal complication of a number of conditions, especially sepsis and trauma. The mortality remains at around 50%, resulting from leakage of fluid into the pulmonary interstitium and alveoli (1). Considerable evidence implicates the neutrophil as a primary source of injury to the lungs (2). In addition, there are numerous indications that patients with ARDS are in oxidative stress, including elevated levels of hydrogen peroxide in their expired breath (3), oxidatively inactivated antiprotease in their blood and lavage fluid (4), lipid peroxidation products in their blood (5), and decreased levels of reduced glutathione in blood and alveolar lining fluids (6).

In many laboratory models and in a few clinical trials, superoxide dismutase (SOD) has proven therapeutically useful in protecting injured tissues from superoxide radical, whether generated intracellularly or by infiltrating activated neutrophils (7). Its ability to protect tissues against any particular insult (ischemia, inflammation, hyper-

oxia, etc.) depends on several parameters, such as the rate of plasma clearance (8), the ability to equilibrate between extracellular fluid compartments (9), and the ability to closely approach negatively charged cell surfaces by virtue of its own net charge (10).

In humans, three types of SOD have been extensively characterized: the cytosolic Cu,Zn-SOD, a 32-kDa dimer (11), the mitochondrial Mn-SOD, an 89-kDa tetramer (12), and an extracellular SOD (ECSOD) (13). This ECSOD (a 135,000-mw tetrameric glycoprotein) is also a cuprozinic enzyme. ECSOD is genetically related to the smaller cytosolic enzyme, and is found in a number of tissues but at a much lower concentration than either of the other two enzymes (14,15). However, the major SOD in extracellular fluids is the ECSOD. It is found as three post translationally generated isozymes: ECSOD-A with no heparin affinity; ECSOD-B with low heparin affinity; and ECSOD-C with high heparin affinity, causing it to be largely bound to heparan sulfate on endothelial surfaces. The affinity for polyanions is due to a highly hydrophilic, positively charged carboxy-terminal "tail" on each subunit of ECSOD-C (the native form) (16).

Most studies published to date showing the ability of SOD to protect injured tissues have employed the cytosolic Cu,Zn-SOD (bovine, yeast, or human). This molecule has the advantage of being commercially available and relatively inexpensive, coupled with the enormous disadvantage of having

very undesirable pharmacologic properties. Its half-life in the plasma following iv injection is from 6 to 15 min, depending on species. Its net charge at physiologic pH is negative, like that of cell surfaces; hence, it is repelled from those surfaces. Recently, the human ECSOD has become available in sufficient quantity to compare its pharmacologic properties with those of the other SODs, and it appears that ECSOD may have a substantial advantage over cytosolic Cu,Zn-SOD because of its ability to bind to the endothelium (17,18).

The contribution made by ECSOD to the protection of the endothelium during pathologic conditions, and especially when the endothelium may be covered with adherent, activated neutrophils, is largely unexplored. Activated neutrophils release a variety of proteolytic activities. The likelihood that the hydrophilic tail of ECSOD, rich in arginine and lysine, would be a target for these proteases prompted us to examine the role of ECSOD in ARDS, and its vulnerability to degradation by neutrophil proteases.

Materials and Methods

Enzymes, Assays, and Purifications

Trypsin (Type III, bovine pancreas) was from Sigma Chemical Co. (St. Louis, MO). Human recombinant Cu,Zn- and Mn-SODs were generously provided by Biotechnology General, Inc. (New York, NY). SOD was assayed by the method of McCord and Fridovich (11) modified as

This paper was presented at the Conference on Oxygen Radicals and Lung Injury held 30 August–2 September 1993 in Morgantown, West Virginia.

This work was supported by a Cardiovascular Discovery Grant from Glaxo, Inc., and by National Institutes of Health grant 5P50 HL40784.

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necessary for increased sensitivity (19). Rabbit ECSOD-C was purified by administering a lethal dose of sodium pentobarbital (60 mg/kg) to a rabbit also injected iv with 200 U heparin/kg bw. The rabbit was exsanguinated and the blood centrifuged to remove the cells. The plasma was fractionated by heparin affinity chromatography as described below. After elution by the sodium chloride gradient, the peak fractions were dialyzed against HBSS and concentrated by ultrafiltration (Amicon, PM-10 membrane).

Heparin Affinity Chromatography

A sample of SOD (500–2000 U) was applied to a small (2–3 ml bed volume) column of heparin-agarose (Sigma) in 10 mM Tris-Cl buffer, pH 7.4. The column was washed with this buffer to remove unbound enzyme, then eluted with a gradient of NaCl, 0 to 2.0 M. Fractions were assayed for SOD activity and for conductivity. Native Cu,Zn- or Mn-SODs showed no affinity for this column; rabbit ECSOD-C was retained by the column, eluting at 0.4 M NaCl.

Neutrophil Isolation and Activation

Human peripheral neutrophils were isolated from freshly drawn blood obtained from healthy donors as previously described (20) and resuspended in HBSS at 1×10^7 cells/ml. The neutrophils were activated by 1 μ M phorbol myristate acetate.

Interleukin-1-induced Model of Pulmonary Injury

Administration of IL-1 to Intact Rats. Rats were anesthetized with ketamine (90 mg/kg) and xylazine (7 mg/kg) ip and supplemented as needed with ketamine (68 mg/kg) and xylazine (5 mg/kg). Subsequently, the trachea was cannulated with an indwelling 16-g stub adaptor tube. After five ventilated breaths (Harvard Apparatus Co., South Natick, MA), 0.5 ml containing 50 ng IL-1 in sterile saline was rapidly instilled intratracheally and then followed by another five ventilated breaths. Sham-treated rats received identical anesthesia and surgery but were injected with sterile saline or heat-inactivated IL-1 intratracheally. Control rats received no surgery or treatment and were anesthetized only immediately before sacrifice. Rats were kept on a warming blanket, and temperatures were monitored throughout the experiment.

Assessment of Lung Leak. Rats were injected iv with 1.0 μ Ci (0.5 ml) of 125 I-labeled bovine serum albumin 4.5 hr after IL-1 administration. Rats were ventilated

25 min later with a Harvard small animal respirator and then subjected to laparotomy, thoracotomy, and then right ventricular injection of heparin (200 U, 0.2 ml). Subsequently, 30 min after 125 I injection, blood samples were obtained, lungs were perfused blood-free with phosphate-buffered saline and excised. Right lungs and blood samples were counted in a gamma counter (Beckman, Fullerton, CA). Lung leak index (lung leak) is defined as cpm of 125 I in the right lung per cpm in 1.0 ml of blood.

Results

Protection by MnSOD against IL-1-induced Lung Leak

Four groups of rats were treated as described above, and lung leak was quantified: group 1, sham-treated animals (no IL-1); group 2, sham-treated animals pretreated by iv injection of 2 mg MnSOD; group 3, IL-1-treated animals; and group 4, IL-1-treated animals pretreated by iv injection of 2 mg MnSOD. The results are shown in Figure 1. The IL-1-induced increase in lung leak was suppressed by approximately 50% in the MnSOD-treated animals. This group was significantly different from both control groups and from the untreated IL-1 group.

Elimination by Trypsin of the Ability of ECSOD to Bind to Heparin

Trypsin (5 μ g in 0.5 μ l of 1 mM HCl) was added to 120 U of rabbit ECSOD-C in 0.5 ml of HBSS and incubated for 1 hr at 37°C. The solution was then subjected to heparin-agarose affinity chromatography as described above. The results (Figure 2) show that while the native ECSOD-C binds to the heparin column and is eluted at a salt concentration of about 0.4 M, the trypsinized ECSOD-C lost all affinity for the heparin column.

Activated Neutrophils Decrease the Heparin Affinity of ECSOD

Isolated human neutrophils from a healthy donor were resuspended at a concentration of 1.4×10^8 cells/ml in HBSS containing rabbit ECSOD-C (240 U/ml). The cells in one aliquot (0.5 ml) were activated by the addition of 1 μ M phorbol myristate acetate. The mixtures were then incubated for 1 hr at 37°C and each was subjected to heparin-agarose affinity chromatography. The results (Figure 3) show that while resting neutrophils did not affect the affinity of the enzyme for heparin, incubation with activated neutrophils decreased the interaction

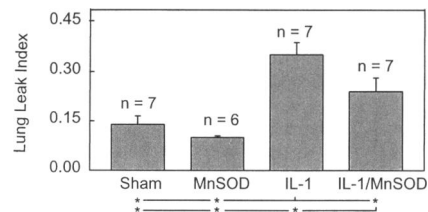


Figure 1. Mn-SOD significantly decreases lung leak in rats administered IL-1 intratracheally. Differences among groups are significant at $p < 0.05$ where indicated by an asterisk.

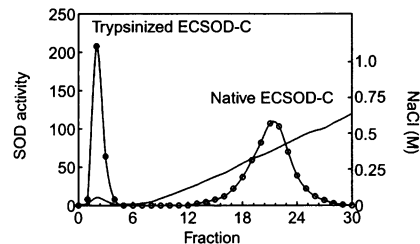


Figure 2. Native rabbit ECSOD-C binds to the heparin affinity column and is released when the sodium chloride gradient reaches about 0.4 M. Incubation with trypsin eliminates all affinity for heparin, although the enzyme activity remains.

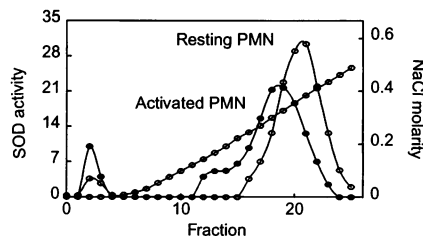


Figure 3. Incubation of rabbit ECSOD-C with resting human neutrophils does not affect heparin affinity, whereas incubation with neutrophils stimulated by phorbol myristate acetate results in decreased binding to heparin.

significantly. The major peak from this incubation eluted at a NaCl concentration of about 0.3 M, compared to 0.4 M for the native ECSOD-C, and two smaller peaks eluted at about 0.15 and 0 M salt.

SOD Content of Plasma from ARDS

To determine whether the plasma concentrations of SODs are perturbed in ARDS patients, we fractionated plasma from a patient with ARDS and from a control by gel exclusion chromatography on Sephacryl S-200 (Pharmacia, Uppsala, Sweden). Plasma samples (1.2 ml) were applied to a column (1 \times 50 cm) and eluted with 10 mM potassium phosphate, pH 7.4, containing 0.15 M NaCl. The column had

been standardized with samples of purified rabbit ECSOD-C and human recombinant Cu,Zn- and Mn-SODs. The elution profiles (Figure 4) reveal only trace amounts of SOD activity in the healthy plasma, but significant amounts of all three SODs in the ARDS plasma. The appearance of the soluble ECSOD-A peak suggests that degradation of ECSOD-C is taking place in this patient. The appearance of the intracellular Cu,Zn- and Mn-SODs suggests that cell lysis is also occurring in the patient.

Discussion

In terms of protecting the external surfaces of cells from attack by superoxide and secondarily derived species, ECSOD-C may play a vital role. The endothelium, which is subjected to the oxidants produced by adherent neutrophils during times of inflammation, is especially vulnerable. It is normally covered by a protective coating of ECSOD-C, an enzyme that appears to be specifically designed for this purpose. It adheres tightly to the polyanionic heparan sulfate on the endothelial surface by virtue of the positively charged hydrophilic carboxy-terminal tail, rich in lysine and arginine residues (16,21). When neutrophils become activated as in ARDS, several significant changes occur. The exposure of the endothelium to superoxide goes up dramatically as the adherent neutrophils activate their NADPH oxidase. As the cells

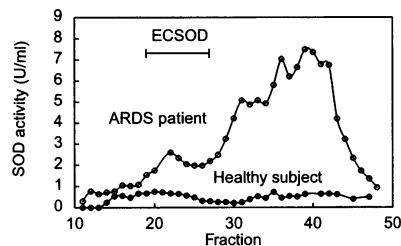


Figure 4. Fractionation of sera from a normal individual and an ARDS patient on Sephacryl S-200 gel filtration medium. Only trace amounts of SOD activity are present in the normal serum, but the ARDS serum shows significant amounts of all three SODs, separated here by their size differences. Purified rabbit ECSOD-C elutes from this column in the bracketed range indicated.

degranulate, a variety of proteolytic enzymes is released. We have found that activated neutrophils are capable of causing partial proteolysis of ECSOD-C, resulting in a diminished affinity for the endothelial surface. These hypothetical changes are illustrated in Figure 5.

Karlsson et al. (22) have also noted that plasmin as well as trypsin can abolish the heparin affinity of ECSOD-C. This vulnerability of ECSOD-C to proteolysis, we believe, raises the possibility of its involvement in a wide range of diseases that produce increased levels of proteolytic activity in the plasma (whether locally or systemically), as well as increased levels of oxida-

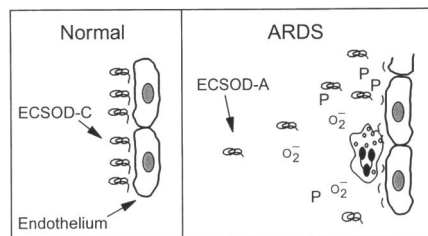


Figure 5. While the normal endothelium is protected by a coating of ECSOD-C, this coating may be stripped away by the proteases (P) released from adherent activated neutrophils, leaving the endothelium vulnerable to oxidative attack.

tive stress. The phenomenon also casts new light on the therapeutic use of superoxide dismutases. A relatively high concentration of soluble SOD in the extracellular fluid may not be able to provide the necessary protection to the endothelium that could be provided by a much smaller amount of SOD if it were bound to the cell surfaces. Note that in Figure 1 the exogenously administered Mn-SOD provided significant protection to the IL-1-treated lung, but not maximal protection. This might mean, of course, that the injury is not totally superoxide-mediated, or it might mean the effective concentration of SOD at the endothelial surface was simply inadequate.

REFERENCES

- Repine JE. Scientific perspectives on adult respiratory distress syndrome. *Lancet* 339:466-469 (1992).
- Tate RM, Repine JE. Neutrophils and the adult respiratory distress syndrome. *Am Rev Respir Dis* 128:552-559 (1983).
- Sznajder JI, Fraiman A, Hall JB, Sanders W, Schmidt G, Crawford G, Nahum A, Factor P, Wood LDH. Increased hydrogen peroxide in the expired breath of patients with acute hypoxemic respiratory failure. *Chest* 96:606-612 (1989).
- Cochrane CG, Spragg R, Revak SD. Pathogenesis of the adult respiratory distress syndrome: evidence of oxidant activity in bronchoalveolar lavage fluid. *J Clin Invest* 71:754-758 (1983).
- Bertrand Y. Oxygen-free radicals and lipid peroxidation in adult respiratory distress syndrome. *Intensive Care Med* 11:56-60 (1985).
- Bernard GR. N-acetylcysteine in experimental and clinical acute lung injury. *Am J Med* 91:554-559 (1991).
- McCord JM. Superoxide production and human disease. In: *Molecular Basis of Oxidative Damage by Leukocytes* (Jesaitis A, Dratz E, eds). Boca Raton, FL: CRC Press, 1992;225-239.
- McCord JM, Wong K. Phagocyte-produced free radicals: Roles in cytotoxicity and inflammation. In: *Oxygen Free Radicals and Tissue Damage* (Ciba Foundation Symposium 65 [New Series]). Amsterdam: Excerpta Medica, 1979;343-360.
- Omar BA, McCord JM. Interstitial equilibration of superoxide dismutase correlates with its protective effect in the isolated rabbit heart. *J Mol Cell Cardiol* 23:149-159 (1991).
- McCord JM, Salin ML. Self-directed cytotoxicity of phagocyte-generated superoxide free radical. In: *Movement, Metabolism and Bactericidal Mechanisms of Phagocytes* (Rossi F, Patriarca P, Romeo D, eds). Padova: Piccin Medical Books, 1977;257-264.
- McCord JM, Fridovich I. Superoxide dismutase: an enzymic function for erythrocyte hemocuprein. *J Biol Chem* 244:6049-6055 (1969).
- McCord JM, Boyle JA, Day ED Jr, Rizzolo LJ, Salin ML. A manganese-containing superoxide dismutase from human liver. In: *Superoxide and Superoxide Dismutase* (Michelson AM, McCord JM, Fridovich I, eds). London: Academic Press, 1977;129-138.
- Marklund SL. Human copper-containing superoxide dismutase of high molecular weight. *Proc Natl Acad Sci USA* 79:7634-7638 (1982).
- Marklund SL. Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species. *Biochem J* 222:649-655 (1984).
- Marklund SL. Properties of extracellular superoxide dismutase from human lung. *Biochem J* 220:269-272 (1984).
- Sandstrom J, Carlsson L, Marklund SL, Edlund T. The heparin-binding domain of extracellular superoxide dismutase-c and formation of variants with reduced heparin affinity. *J Biol Chem* 267:18205-18209 (1992).
- Sjöquist P-O, Carlsson L, Jonason G, Marklund SL, Abrahamsson T. Cardioprotective effects of recombinant human extracellular-superoxide dismutase type C in rat isolated heart subjected to ischemia and reperfusion. *J Cardiovasc*

- Pharmacol 17:678–683 (1991).
18. Hatori N, Sjoquist PO, Marklund SL, Ryden L. Effects of recombinant human extracellular-superoxide dismutase type-c on myocardial infarct size in pigs. *Free Radic Biol Med* 13:221–230 (1992).
 19. Crapo JD, McCord JM, Fridovich I. Superoxide dismutases: preparation and assay. *Methods Enzymol* 53:382–393 (1978).
 20. Salin ML, McCord JM. Free radicals and inflammation: Protection of phagocytosing leukocytes by superoxide dismutase. *J Clin Invest* 56:1319–1323 (1975).
 21. Adachi T, Marklund SL. Interactions between human extracellular superoxide dismutase C and sulfated polysaccharides. *J Biol Chem* 264:8537–8541 (1989).
 22. Karlsson K, Edlund A, Sandstrom J, Marklund SL. Proteolytic modification of the heparin-binding affinity of extracellular superoxide dismutase. *Biochem J* 290:623–626 (1993).