Inhibition of surfactant function by copper-zinc superoxide dismutase (CuZn-SOD)

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Haddad, Imad Y., Bedford Nieves-Cruz, and Sadis Matalon. Inhibition of surfactant function by copper-zinc superoxide dismutase (CuZn-SOD). J. Appl. Physiol. 83(5): 1545-1550, 1997.—The efficacy of antioxidant enzymes to limit oxidant lung injury by instillation with surfactant mixtures in preterm infants with hyaline membrane disease is under investigation. However, there is concern that instillation of proteins in the alveolar space may inactivate pulmonary surfactant. We studied the effects of bovine copper-zinc superoxide dismutase (CuZn-SOD) on the biophysical properties of two distinct surfactant preparations. Incubation of calf lung surfactant extract (CLSE, 1 mg phospholipid/ml) and Exosurf (0.1 mg phospholipid/ml) with CuZn-SOD (1-10 mg/ml) prevented the fall of surface tension at minimal bubble radius (Tmin) to low values with dynamic compression in a pulsating bubble surfactometer. CuZn-SOD also enhanced the sensitivity to inactivation by albumin, normal human serum, and after treatment with peroxynitrite. The inhibitory effects of CuZn-SOD on CLSE, but not Exosurf, were abolished at high lipid concentrations (3 mg/ml) and after the addition of human surfactant protein A (by weight). We conclude that CuZn-SOD may interfere with the surface activity of surfactant mixtures, leading to decreased effectiveness of surfactant replacement therapy.

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determined synergistic effects of serum protein inactivation and the protective effects of SP-A.

**MATERIALS AND METHODS**

Chemicals. Normal human serum was obtained by pooling from several healthy volunteers, and its protein concentration was determined by the bicinchoninic acid (BCA) method. Bovine CuZn-SOD was obtained from DDI Pharmaceuticals (Mountain View, CA). Bovine serum albumin (BSA) and all other chemicals unless specified were from Sigma Chemical (St. Louis, MO).

Surfactants preparations. CLSE (Infrasurf) was a generous gift of ONY (Buffalo, NY). The organic solvent was removed by vacuum distillation and nitrogen evaporation, and the dried CLSE was resuspended in 0.15 M NaCl by mechanical vortexing. Exosurf was provided by Glaxo-Wellcome (Research Triangle Park, NC).

SP-A was purified from bronchoalveolar lavage of patients with alveolar proteinosis as previously described (10, 11). SP-A was dissolved in 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.4), and protein concentration was determined by the BCA method and stored in small aliquots at −20°C until use.

Surface tension measurements. Stock solutions of CLSE and Exosurf were diluted to the desired lipid concentrations [0.1–3 mg phospholipid (PL)/ml] in a 10 mM HEPES buffer (150 mM NaCl, 2 mM CaCl2, 5 mM KCl, and 1.2 mM MgCl2; pH 7.4). The surfactant suspension was incubated with the indicated concentration of CuZn-SOD, BSA, SP-A, and serum (see below) for 15 min at 37°C. The surface tension of the surfactant mixtures was measured during dynamic compression using a pulsating bubble surfactometer (PBS, Electronetics, Amherst, NY), as described in detail by Enhorning et al. (8).

Briefly, this apparatus allows the continuous determination of the pressure drop across the interface of a small air bubble formed in a surfactant-containing subphase. The bubble was pulsed at a rate of 20 cycles/min between maximal and minimal radii of 0.55 and 0.4 mm (surface compression of 50%). Surface pressure and bubble radius were recorded every 100 ms, and surface tension was calculated from the law of Young and Laplace for a sphere. Oscillation of the bubble continued for 10 min or until the surface tension at minimal radius (Tmin) was <3 mN/m.

Exposure of surfactant to ONOO−. ONOO− was synthesized in a quenched-flow reactor (25). Solutions of 0.6 M sodium nitrite and 0.6 M HCl-0.7 M hydrogen peroxide were pumped at 10 ml/s into a T junction and mixed in a 3-mm-diameter 10-cm glass tube. The acid-catalyzed reaction of nitrous acid with hydrogen peroxide to form peroxynitrous acid was quenched by pumping 1.5 M sodium hydroxide at the same rate into a second T junction at the end of the glass tubing. The solution was treated with manganese dioxide to convert hydrogen peroxide to water, and the mixture was quenched by manganese dioxide to form peroxynitrous acid. Solutions of 0.6 M HCl-0.7 M hydrogen peroxide were added to 0.5 M FeSO4·EDTA at pH 7.4. The solution was oxygenated with a stream of nitrogen gas for 10 min to prepare ONOO− solution.

Our previous studies indicate that exposure of CLSE to ONOO− plus 100 µM FeSO4·EDTA inhibits its ability to achieve low Tmin on dynamic compression (12). In this study, we exposed CLSE or Exosurf (1 mg PL/ml) to ONOO− (0.5–1 mM) plus 100 µM FeSO4·EDTA for 15 min at 23°C and evaluated the effects of CuZn-SOD on modification of the ONOO−-mediated injury to surface activity of CLSE and Exosurf by using the PBS.

Statistical analysis. All results are expressed as means ± SD. Statistical differences among group means at 0, 5, and 10 min of pulsation were determined by using one-way analysis of variance and the Bonferroni modification of the t-test.

**RESULTS**

Inhibition of surface activity by CuZn-SOD. Samples of CLSE (1 mg PL/ml), suspended in HEPES buffer (pH 7.4) displayed a surface tension of 27 ± 0.9 mN/m just before pulsation and a Tmin of <3 mN/m within 1 min of pulsation in a PBS. Addition of CuZn-SOD (5 or 10 mg/ml) impaired its adsorption to the interface, as indicated by the increased Tmin at time 0 min, and prevented the rapid drop to low Tmin with dynamic compression in a dose-dependent manner (Fig. 1). The inhibitory effects of CuZn-SOD were ameliorated by increasing CLSE concentration to 3 mg PL/ml (data not shown). In contrast, BSA in concentrations up to 100 mg/ml did not inhibit the rapid drop of Tmin with pulsation, although Tmin just before pulsation was significantly higher than control (Fig. 1). After pulsation in a PBS, the Tmin of Exosurf samples at low PL concentrations (0.1 mg/ml) decreased to <3 mN/m within 3 min. The presence of CuZn-SOD (1 or 2 mg/ml) delayed the drop of Tmin of Exosurf (Fig. 2). At higher PL concentrations (>0.1 mg/ml), Tmin of Exosurf remained >28 mN/m, as previously reported (3, 9).

Effects of BSA, serum, and SP-A on CuZn-SOD-mediated inhibition. The addition of CuZn-SOD (1–10 mg/ml) to CLSE (1 mg PL/ml) or Exosurf (0.1 mg PL/ml) samples in the presence of BSA (3–50% by weight) at low concentrations (0.1 mg/ml) decreased to <3 mN/m within 3 min. The presence of CuZn-SOD (1 or 2 mg/ml) delayed the drop of Tmin of Exosurf (Fig. 2). At higher PL concentrations (>0.1 mg/ml), Tmin of Exosurf remained >28 mN/m, as previously reported (3, 9).

Normal human serum (2–5% by weight) significantly increased Tmin of CLSE (1 mg/ml) and Exosurf (0.1 mg/ml) at 0 min and prevented the drop of Tmin below 6 mN/m. The presence of CuZn-SOD (2 mg/ml) also potentiated the inhibitory effects of serum and prevented the drop of Tmin of CLSE below 10 mN/m even after 10 min of pulsation were determined by using one-way analysis of variance and the Bonferroni modification of the t-test.
pulsation (Figs. 5 and 6). On the other hand, addition of human SP-A (3% by weight) restored the ability of CLSE plus CuZn-SOD samples, but not Exosurf plus CuZn-SOD samples (not shown), to rapidly achieve low Tmin with dynamic compression (Fig. 3).

Effects of SOD on ONOO\textsuperscript{-}injured CLSE. Exposure of CLSE (1 mg PL/ml) to 0.5 mM ONOO\textsuperscript{-} plus 100 µM Fe\textsuperscript{3+}-EDTA, capable of initiation and propagation of lipid peroxidation and oxidation and nitration of surfactant proteins (12), significantly impaired the ability of CLSE to adsorb to the interface, as indicated by the increased Tmin immediately before the initiation of pulsations, and inhibited its ability to rapidly reach a low Tmin with dynamic compression. This oxidant-mediated injury to CLSE was more severe in the presence of CuZn-SOD (5 mg/ml). SP-A (3% by weight) restored the ability of ONOO\textsuperscript{-}-exposed CLSE to baseline (control) values (Fig. 7). In contrast, exposure of Exosurf samples (0.1 mg PL/ml) to ONOO\textsuperscript{-} (0.5–4 mM) plus 100 µM Fe\textsuperscript{3+}-EDTA did not alter their surface properties (Tmin <3 mN/m within 3 min of pulsation).

This finding is consistent with the absence of unsaturated lipids and surfactant proteins in Exosurf.

DISCUSSION

Exogenous surfactant improves the lung distribution of intratracheally instilled substances and enhances their uptake into the alveolar epithelium (19, 24). Jobe et al. (18) recently reported that the administration of recombinant adenoviral vectors used for gene therapy mixed with a surfactant preparation (Survanta), enhanced the efficiency of gene transfer into murine lungs. Similarly, Survanta delayed the clearance of instilled rhCuZn-SOD protein and prolonged its antioxidant activity measured at 48 h (6). This enhanced antioxidant activity in preterm animal and human lungs protects against oxidant stress and thus may prevent the development of BPD (7). However, before the widespread use of this approach, the effects of CuZn-SOD on the biophysical properties of surfactant need to be examined.
Our data show that CuZn-SOD (0.5–10 mg/ml) adversely affected the ability of two distinct surfactant preparations at low PL concentrations to achieve a low Tmin with dynamic compression in a PBS. We used the smallest SOD concentration necessary to inhibit the Tmin of the surfactant under study. At first glance, surfactant and SOD concentrations used in these studies may seem outside the doses used clinically. However, it should be kept in mind that alveolar concentrations may differ dramatically from instilled ones because surfactant and SOD are cleared from the alveolar space at different rates. Matalon et al. (22) instilled two 125-mg (162 µmol) boluses of CLSE into the lungs of oxygen-treated rabbits 12 h apart. Matalon et al. (22) instilled two 125-mg (162 µmol) boluses of CLSE into the lungs of oxygen-treated rabbits 12 h apart. Values are means ± SD (in some cases SD is less than width of symbols) for n ≥ 3. *P < 0.05 from corresponding Tmin of control Exosurf (○).

![Figure 6. Effects of CuZn-SOD on normal human serum-mediated inhibition of Tmin of Exosurf. Samples of Exosurf (0.1 mg PL/ml) were incubated with serum (2% by weight, ○) or serum (2% by weight) in presence of CuZn-SOD (1 mg/ml, ●) for 15 min at 37°C before Tmin measurement in a PBS. Values are means ± SD (in some cases SD is less than width of symbols) for n ≥ 3. *P < 0.05 from corresponding Tmin of control Exosurf (○).](image)

Fig. 6. Effects of bovine CuZn-SOD on normal human serum-mediated inhibition of Tmin of Exosurf. Samples of Exosurf (0.1 mg PL/ml) were incubated with serum (2% by weight, ○) or serum (2% by weight) in presence of CuZn-SOD (1 mg/ml, ●) for 15 min at 37°C before Tmin measurement in a PBS. Values are means ± SD (in some cases SD is less than width of symbols) for n ≥ 3. *P < 0.05 from corresponding Tmin of control Exosurf (○).

Proteins inhibit surfactant function by competing with the phospholipids for space at the air-liquid interface (15). This is consistent with the observation that the albumin-mediated effect is abolished by an increase in surfactant concentration (16). CuZn-SOD may also delay adsorption and increase Tmin of CLSE and Exosurf by interfering with the formation of a lipid monolayer at the air-liquid interface. However, the marked sensitivity to inhibition by CuZn-SOD compared with BSA suggests additional mechanisms may be responsible. One potential mechanism is a direct molecular interaction between CuZn-SOD and the lipid or protein components of the surfactant mixtures. For example, Seeger et al. (28) reported that fibrin monomers are very potent inhibitors of surfactant and suggested they formed a complex with one or more of the surfactant components. Differential sensitivity of the various surfactant to the inhibitory effects of serum proteins has been observed (29, 30). Plausible reasons include differences in lipid composition, surfactant protein (SP-A, SP-B, SP-C) content, and presence of contaminating material during extraction. We were unable to directly compare the sensitivity of Exosurf and CLSE to inhibition by CuZn-SOD because we measured the Tmin of these two preparations at different PL concentrations. The Tmin of Exosurf at PL concentration of 1 mg/ml does not drop <28 mN/m with pulsation in a bubble surfactometer (50% area compression) (12). However, we observed, as previously noted (3, 9), that at lower PL concentration (0.1 mg/ml) low Tmin was achieved within few minutes of pulsation. It is important to note that this concentration of Exosurf may be lower than the clinically relevant range after exogenous replacement. On the other hand, the Tmin of CLSE (0.1 mg/ml) remained high during dynamic pulsation. Despite these limitations, two important differences between CLSE and Exosurf emerged. First, Exosurf was resistant to inactivation by ONOO· plus Fe2+-EDTA. This finding is consistent with the fact that Exosurf lacks oxidizable unsaturated lipids and surfactant proteins. Second, SP-A protected CLSE, but not Exosurf (data not shown), against inactivation by CuZn-
SOD, consistent with the knowledge that SP-A requires the presence of SP-B to enhance surface activity of surfactant (12, 15).

Because bovine CuZn-SOD and rhCuZn-SOD are structurally and functionally very similar and the experiments were performed in vitro, the bovine source of CuZn-SOD was used in these studies. However, it should be noted this form of SOD may contain contaminants that may have contributed to the inhibition of surface activity.

The clinical use of CuZn-SOD may have important protective effects against oxidant-mediated tissue and surfactant injury. Therefore, despite our observation that at low phospholipid concentrations CuZn-SOD inhibited the surface activity of surfactant mixtures, the net effect may still be beneficial. Fortunately, this surfactant inactivation can be overcome either by increasing surfactant PL concentration or by adding SP-A to currently available hydrophobic protein-based surfactant preparations. Further in vivo studies are needed to determine the value and efficacy of each of these modifications.

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