Synthetic surfactant scavenges oxidants and protects against hyperoxic lung injury

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Ghio, Andrew J., Philip J. Fracica, Stephen L. Young, and Claude A. Piantadosi. Synthetic surfactant scavenges oxidants and protects against hyperoxic lung injury. *J. Appl. Physiol.* 77(3): 1217–1223, 1994.—Injury and mortality after exposure to 100% oxygen can be diminished by surfactants that may operate by mechanisms other than those responsible for surface tension effects. We tested the hypotheses that *1*) synthetic surfactant and its components function as antioxidants in vitro and *2*) decrements in hyperoxic injury after treatment with a surfactant and its components are associated with decreases in oxidative stress to the lung. A synthetic surfactant (Exosurf) and its non-surface-active components tyloxapol and cetyl alcohol were incubated in an iron-containing hydroxyl radical-generating system to determine their abilities to prevent oxidation of deoxyribose. Doses of tyloxapol, cetyl alcohol, and artificial surfactant diminished the absorbance of thiobarbituric acid-reactive products of deoxyribose. Similarly, tyloxapol, cetyl alcohol, and the surfactant decreased hydroxylated products of salicylate in the same system. Rats were instilled intratracheally with saline, tyloxapol, tyloxapol plus cetyl alcohol, or artificial surfactant and immediately exposed to air or 100% oxygen. After 61 h of oxygen exposure, pleural fluid volume and wet-to-dry lung weight ratios were decreased in animals treated with surfactant and/or its components. There were also decrements in thiobarbituric acid-reactive products of lung tissue. In separate experiments, mean survival of saline-treated rats exposed to 100% oxygen was 67.3 ± 8.1 h and >96 h for rats given the surfactant or its components. We conclude that tyloxapol, cetyl alcohol, and Exosurf can function as antioxidants in vitro and their in vivo instillation is associated with reduction in measures of hyperoxic injury, oxidized tissue products, and mortality.

oxygen toxicity; free radicals; antioxidants

PULMONARY SURFACTANT is a combination of lipids, proteins, and carbohydrates that provides alveolar stability at low lung volumes. The principal component (80%) of natural surfactant is phospholipid, most (55–60%) of which is dipalmitoylphosphatidylcholine (DPPC), which is essential for surfactant activity (5). Surfactant lowers surface tension at the air-liquid interface, thus minimizing or reducing alveolar collapse at end exhalation. Surfactant deficiency or dysfunction leads to increased respiratory work, atelectasis, hypoxemia, and pulmonary edema. Lack of lung surfactant in newborns causes the infant respiratory distress syndrome (IRDS), which results in the deaths of many untreated patients (1). Natural surfactant and a commercial synthetic surfactant (Exosurf, Burroughs-Welcome) significantly decrease the mortality of IRDS (22, 24). Exosurf is a wholly synthetic surfactant containing DPPC, cetyl alcohol to solubilize the phospholipid, and the nonionic detergent tyloxapol to disperse it.

Supplemental oxygen is the therapy of choice to reverse arterial hypoxemia resulting from inadequate pulmonary exchange of oxygen. Prolonged exposures to high partial pressures of oxygen, however, induce a diffuse lung injury that includes abnormalities in the composition, quantity, and function of surfactant (13, 18). Similar to IRDS, treatment of hyperoxic lung injury with exogenous natural surfactant or Exosurf can improve lung mechanics and gas exchange (15, 17, 23). The major action of surfactants has been assumed to be replacement of depleted or dysfunctional phospholipids, thus restoring surface tension toward normal (16). After exposures to hyperoxia, however, exogenous surfactant ameliorates defective epithelial permeability (8, 23) and decreases mortality without improving lung compliance (17). These observations suggest a mechanism of action in addition to any effects of the drug on surface tension. Oxygen toxicity is mediated by an incomplete reduction of bimolecular oxygen, which generates reactive species including O_2, H_2O_2, and $\cdot OH$ (11, 21). These partially reduced species of oxygen react with and damage biomolecules including enzymes, membrane lipids, and nucleic acids. In addition to improving mechanical and gas exchange functions by lowering surface tension, therapy with surfactants after 100% oxygen may diminish oxidation of critical constituents of the lung.

In this study, we tested three hypotheses: *1*) synthetic surfactant and its components function as antioxidants in vitro; *2*) decrements in hyperoxic injury after treatment with surfactant and its components are associated with evidence of decreased oxidative stress to the lung; and *3*) injury and mortality after exposure to 100% oxygen can be diminished by components of a synthetic surfactant other than those responsible for effects on surface tension.

METHODS

Materials. The artificial surfactant Exosurf was donated by Burroughs-Wellcome (Research Triangle Park, NC). Tyloxapol, cetyl alcohol, and all other reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise specified.

In vitro assays for oxidant scavenging. The in vitro system employed to generate reactive oxygen species was a reaction mixture containing 10.0 μM $FeCl_3$, 1.0 mM ascorbate, and 1.0 mM H_2O_2 in Hanks' balanced salt solution (GIBCO, Grand Island, NY). The molecular target used in the first assay was the pentose sugar deoxyribose (1.0 mM), which reacts with oxidants to yield a mixture of products. On heating with thiobarbituric acid (TBA) at low pH, these reaction products form a pink chromophore that can be quantified by the change in absorbance at 532 nm (A_{532}).

Normal saline (0.1 ml), tyloxapol (final concn 0.0–10.0 mg/ml), cetyl alcohol (final concn 0.0–10.0 mM), or Exosurf (final concn 0.0–10.0 mg/ml) was added to the reaction mixture. DPPC is not easily solubilized in aqueous buffer and therefore

was not separately employed in these studies. The reaction mixtures, which included tyloxapol or Exosurf, were incubated at 45°C for 30 min. To promote solubilization of cetyl alcohol, mixtures containing cetyl alcohol were incubated at 55°C. After incubation, the samples were centrifuged at 1,200 *g* for 10 min and 1.0 ml of both 1.0% (wt/vol) TBA and 2.8% (wt/vol) trichloroacetic acid was added to 1.0 ml of supernatant. The samples were heated at 100°C for 10 min and cooled in ice, and the chromophore was determined in triplicate by its A_{532}.

Oxidant scavenging by tyloxapol, cetyl alcohol, and Exosurf was also measured by assay of hydroxylation products of salicylate in vitro. Salicylic acid (2-hydroxybenzoic acid) reacts with hydroxyl radical to produce 2,3- and 2,5-dihydroxybenzoic acids (9). The detection of 2,3- and 2,5-dihydroxybenzoic acid was performed using high-performance liquid chromatography (HPLC) with electrochemical detection (9). Suspensions of 10 μM $FeCl_3$, 1.0 mM ascorbate, and 1.0 mM H_2O_2 were employed to generate oxidants in the presence of 10.0 μM salicylate. Normal saline (0.1 ml), tyloxapol (final concn 0.0–10.0 mg/ml), cetyl alcohol (final concn 0.0–10.0 mM), or Exosurf (final concn 0.0–10.0 mg/ml) was added. The reaction mixtures were incubated at 45°C (except those with cetyl alcohol, which were incubated at 55°C) for 30 min and centrifuged at 1,200 *g* for 10 min. Supernatant was centrifuged (Beckman Microfuge E) through a 0.22-μm microfuge tube filter (no. 352–118, PGC Scientific) at 15,000 *g*. A 100-μl sample of the eluate was injected onto a C_{18} reverse-phase HPLC column (250 × 4.6 mm, Beckman no. 235329). Hydroxylated products of salicylate were quantified with a Coulochem electrochemical detector (ESA model 5100A), with the detector set at a reducing potential of −0.40 V direct current. The guard cell was set at an oxidizing potential of +0.40 V direct current (29). Measurements were done in duplicate.

The in vitro capacity of tyloxapol to scavenge ·OH also was measured relative to that of salicylate. The reaction mixture included 50 μM $FeCl_2$, 50 μM EDTA, and 5.0 mM H_2O_2 in phosphate-buffered saline (pH 7.0). Salicylate was present at 5 mM (680 μg/ml). Tyloxapol was added in concentrations of 1.0, 10.0, 100, 500, and 1,000 μg/ml. The solutions were irradiated using deuterium broad-band ultraviolet light for 2 min, and the reaction was then stopped with catalase (200 U). After precipitation of the protein with use of trichloroacetic acid and centrifugation, hydroxylated products of salicylate in the supernatant were quantified in duplicate by use of HPLC coupled with electrochemical detection.

In vivo lung injury with 100% oxygen. Sixty-day-old (273 ± 16 g) specific pathogen-free male Sprague-Dawley rats (Charles River, Wilmington, MA) were instilled intratracheally with normal saline, tyloxapol (6.0 mg), tyloxapol (6.0 mg) plus cetyl alcohol (11.0 mg), or Exosurf (equivalent to 7.5 mg tyloxapol, 11.3 mg cetyl alcohol, and 101.3 mg DPPC). All treatments were administered using an instillation volume of 0.5 ml. As a result of insolubility in aqueous buffers at body temperature, cetyl alcohol alone was not tested, but rather a combination of tyloxapol and cetyl alcohol was instilled in a ratio approximately equal to that present in Exosurf. Rats were then exposed to air (n = 40) or 100% oxygen (n = 40) in Plexiglas chambers that were flushed with gas at a flow rate of 10 l/min. Oxygen percentage was continuously monitored in the chambers by a polarographic analyzer (Servomex, Sybron, Norwood, MA) and maintained at >98%. CO_2 levels were <0.5%. Temperature was kept between 20 and 22°C. Food (Ralston Purina, St. Louis, MO) and water were available ad libitum. After 61 h of exposure, animals were killed with pentobarbital sodium (100 mg/kg ip; Abbott Laboratories, North Chicago, IL). Lung wet-to-dry weight ratios were calculated after tissue samples were dried for 96 h at 60°C. Pleural fluid volume was measured by aspirating fluid from the chest cavity through a small incision in the diaphragm. In some animals the trachea was cannulated, and 2% glutaraldehyde in 84 mM sodium cacodylate buffer at pH 7.4 was instilled at a constant pressure of 20 cmH_2O. Tissue was examined by light microscopy after it was sectioned and then stained with hematoxylin and eosin.

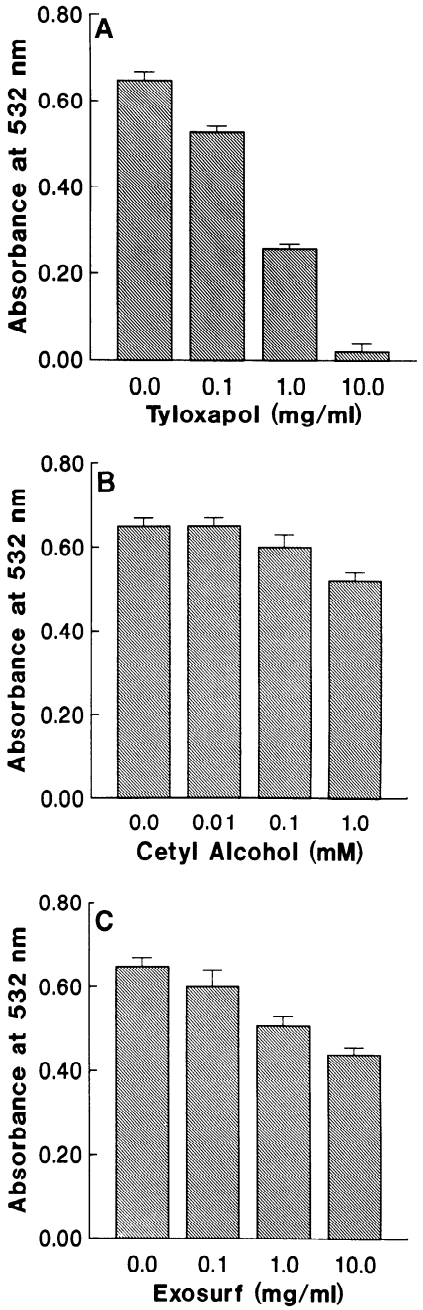

FIG. 1. Absorbance of thiobarbituric acid-reactive products of deoxyribose after in vitro incubation of deoxyribose in an oxidant-generating system with tyloxapol, cetyl alcohol, and Exosurf. Dose-dependent decreases in oxidized products of deoxyribose are evident with all 3 compounds.

TBA-reactive products in lung tissue are measured easily as

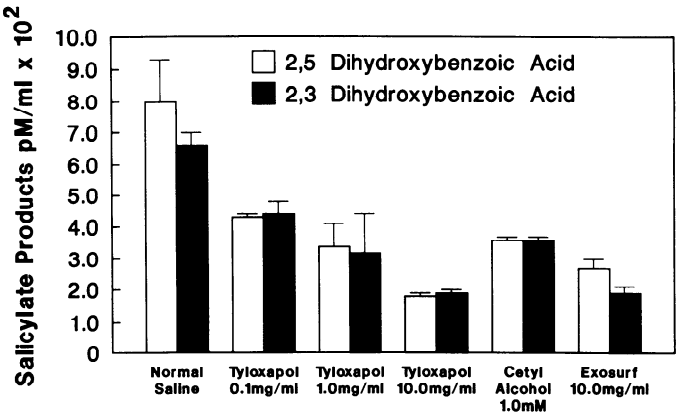

FIG. 2. Hydroxylated products of salicylate after in vitro incubation of salicylate in an oxidant-generating system along with tyloxapol, cetyl alcohol, and Exosurf. All 3 compounds decreased hydroxylation of salicylate. Decrements with tyloxapol were dose dependent. Significant differences in hydroxylated products were found between reaction mixtures with normal saline and those with 10.0 mg/ml tyloxapol, 1.0 mM cetyl alcohol, and 10.0 mg/ml Exosurf.

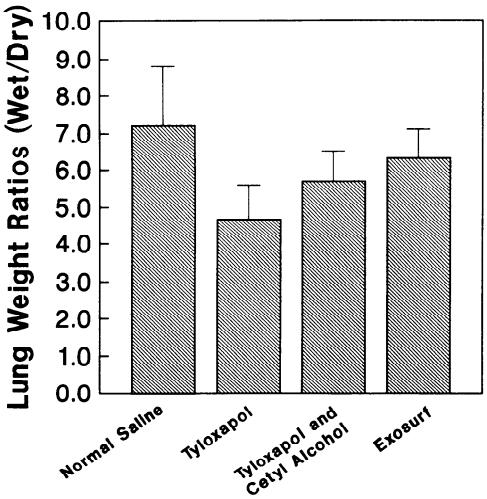

FIG. 3. Wet-to-dry lung weight ratios after 61 h of continuous exposure of rats to 100% oxygen. Significant differences were demonstrated only between saline- and tyloxapol-treated rats.

a sensitive index of oxidant stress. Peroxidation of lipids occurs during exposure of tissue to oxygen in vitro (10) and in vivo (34), although other oxidized tissue components also react with TBA and lead to an overestimate of fatty peroxide formation. At the end of the experiments, ~0.5 g of lung tissue was excised and TBA-reactive products were measured as previously described (28).

Mortality studies. Sprague-Dawley rats were instilled intratracheally with 0.5 ml of normal saline, tyloxapol (6.0 mg), tyloxapol (6.0 mg) plus cetyl alcohol (11.0 mg), or Exosurf (equivalent to 7.5 mg tyloxapol, 11.3 mg cetyl alcohol, and 101.3 mg DPPC). These rats were then exposed to air (n = 48) or 100% oxygen (n = 48). Cages were checked every 3 h after 60 h for dead animals, and the data were recorded as percent survival.

Statistics. The salicylate hydroxylation experiments in a Fenton system were performed twice. All other experiments were performed three times. Data are expressed as means ± SD. An analysis of variance was used to determine differences

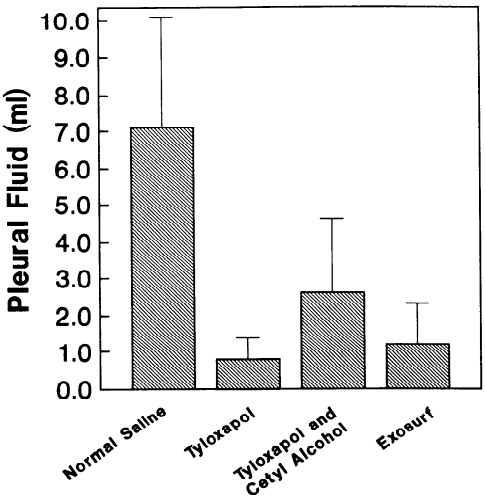

FIG. 4. Pleural fluid accumulation after 61 h of continuous exposure to 100% oxygen. There were significant differences between saline and all other therapies.

between multiple groups (6). When F ratios were significant, means were compared post hoc using Scheffé's test. For mortality studies, χ^2 values were calculated and used to analyze differences in total survival (6). Significance was assumed at $P < 0.05$.

RESULTS

The synthetic surfactant and two of its components, tyloxapol and cetyl alcohol, diminished in vitro oxidation of deoxyribose, as reflected by the absorbance of TBA-reactive products (Fig. 1). These decrements in oxidant generation were dependent on the concentration of the compounds or the mixture. Post hoc tests demonstrated significant differences in absorbances between reaction mixtures with normal saline and those with 0.1, 1.0, and 10.0 mg/ml tyloxapol. Also, 1.0 mM cetyl alcohol and 1.0 and 10.0 mg/ml Exosurf were significantly different from control. The same three compounds diminished in vitro hydroxylation of salicylate in a Fenton system (Fig. 2). The decreases in hydroxylation of salicylate associated with tyloxapol also were concentration dependent. Tyloxapol competed successfully with salicylate for ·OH generated using $FeCl_2$, H_2O_2, and ultraviolet light. At 1,000 μg/ml of the detergent, hydroxylated products of salicylate were diminished by >99%. This indicates that tyloxapol, on a weight basis, is a more efficient ·OH scavenger than salicylate.

After prolonged exposure to 100% oxygen, the lungs of rats develop a defect in the barrier function of the alveolar-capillary membrane characterized by pulmonary edema and large pleural effusions. Wet-to-dry weight ratios of lung tissue from rats exposed to air and treated with saline, tyloxapol, tyloxapol plus cetyl alcohol, or Exosurf demonstrated no differences (control = 4.78 ± 0.31). Exposure to 100% oxygen elevated the wet-to-dry weight ratio (Fig. 3). Whereas wet-to-dry lung weight ratios in rats exposed to oxygen and treated with tyloxapol, tyloxapol plus cetyl alcohol, and Exosurf were lower, post hoc tests indicated that only tyloxapol decreased

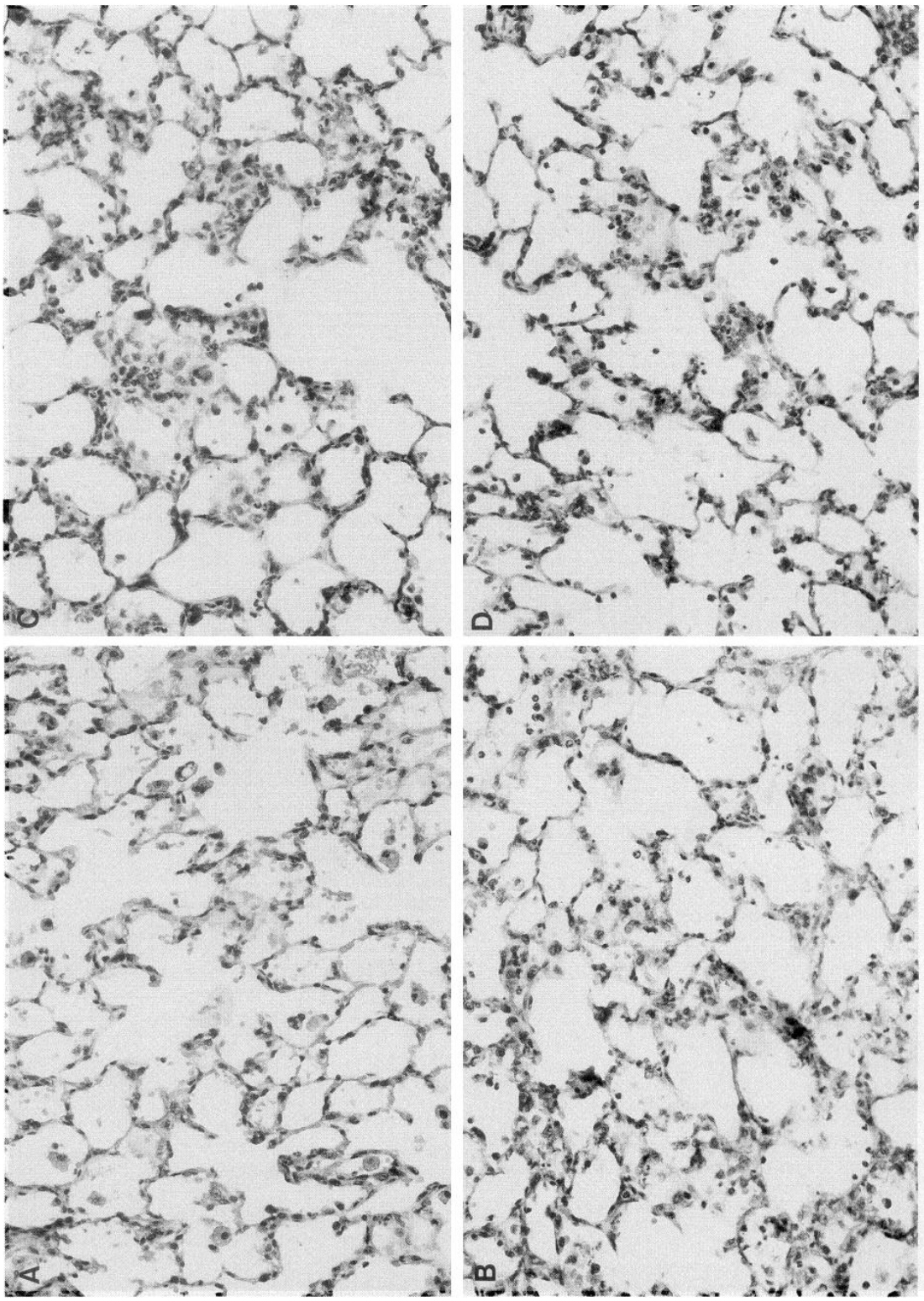

FIG. 5. Histological examination of lung tissue after 61 h of continuous exposure to 100% oxygen. Injury after saline (*A*) appears to be equivalent to that after treatment with tyloxapol (*B*), tyloxapol and cetyl alcohol (*C*), or Exosurf (*D*). Neutrophil aggregation, septal thickening, and epithelial cell sloughing are evident in all micrographs. Hematoxylin-eosin stain; ×200.

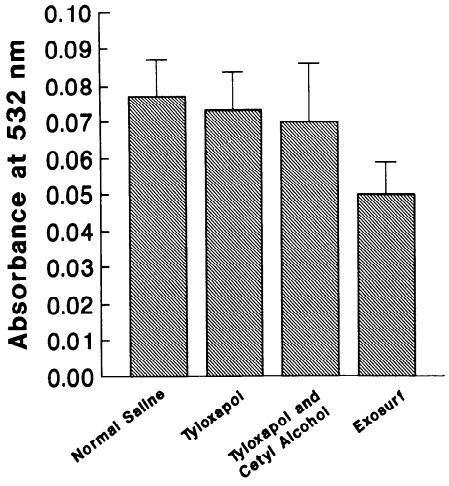

FIG. 6. Absorbance of TBA-reactive products of lung after 61 h of continuous exposure to 100% oxygen. Differences between values with post hoc testing were significant only between saline- and Exosurf-treated rats.

wet-to-dry weight ratios significantly compared with saline treatment.

No pleural fluid was detected in rats exposed to air and treated with saline, tyloxapol, tyloxapol plus cetyl alcohol, or Exosurf. Animals exposed to 100% oxygen accumulated large quantities of pleural fluid (Fig. 4), and post hoc tests demonstrated significantly greater fluid in saline-treated oxygen-exposed animals than in animals given all other treatments. Despite the improvements in several indexes of injury after treatment with Exosurf or its components, histological findings typical of hyperoxic lung injury were observed after all therapies (Fig. 5).

No differences were demonstrated among TBA-reactive products of rat lung exposed to air and treated with saline, tyloxapol, tyloxapol plus cetyl alcohol, and Exosurf ($A_{532} = 0.051 \pm 0.004$). Concentrations of TBA-reactive products were significantly elevated in lungs of rats exposed to oxygen (Fig. 6). Whereas all values in rats exposed to oxygen and treated with tyloxapol, tyloxapol plus cetyl alcohol, and Exosurf were lower, only the difference between saline and Exosurf treatments reached significance.

There were no deaths among animals treated with the test compounds and exposed to air. In rats the mean survival during continuous exposure to 100% oxygen at sea level is 60–66 h (7). Mean survival in rats treated with saline and exposed to 100% oxygen was 67.3 ± 8.1 h (Fig. 7). Treatments with tyloxapol, tyloxapol plus cetyl alcohol, and Exosurf significantly increased survival through 96 h. There was no significant difference in survival at 96 h between Exosurf-treated rats and animals instilled with tyloxapol plus cetyl alcohol.

DISCUSSION

Oxygen-derived radicals are produced as normal by-products of metabolism. These oxidants are scavenged by antioxidant enzymes (i.e., superoxide dismutases, catalase, and glutathione peroxidase) and molecular scavengers (i.e., glutathione, α-tocopherol, β-carotene, and ascorbic acid). During exposure to hyperoxia, an increased rate of radical generation is assumed to overwhelm endogenous antioxidant defenses (11, 14). These reactive oxygen species can cause cellular injury and death through lipid peroxidation, protein oxidation, sulfhydryl depletion, and DNA damage. The major source of pulmonary oxidant stress during hyperoxia is likely to be increased cellular production of O_2^- and H_2O_2. Whereas both oxidant species may have deleterious effects on biological materials directly, some portion of their toxicity may be mediated through an iron-catalyzed generation of $\cdot OH$ with O_2^- as the necessary reductant and H_2O_2 as the substrate (2, 14). The hydroxyl radical scavenger dimethylthiourea and the iron chelator deferoxamine decrease hyperoxic injury in an animal model. Consequently, the oxidant-generating system that we selected to test for in vitro antioxidant activity of Exosurf and its components was one that produced hydroxyl radical. Exosurf functioned as an antioxidant in this in vitro system.

Surfactant-enriched material has a capacity to be oxidized (12, 32). The components of Exosurf were tested in vitro, except for DPPC, which is poorly water soluble and chemically the least likely component to react with oxygen-derived radicals. The alcohol and detergent components actively contributed to the antioxidant properties of the mixture. To scavenge radicals, these compounds must be easily oxidized to stable chemical forms. Alcohols are well recognized as antioxidants and are oxidized to aldehydes (4). Some detergents have been observed to stimulate oxidant generation in vitro in acellular (30) and cellular systems (19). The exact mechanisms for the in vitro antioxidant activity of the nonionic detergent and the products of its oxidation are not known. Tyloxapol is an alkylaryl polyether alcohol polymer synthesized from the reaction of 4-(1,1,3,3-tetramethylbutyl)phenol with formaldehyde and oxirane. It retains functional

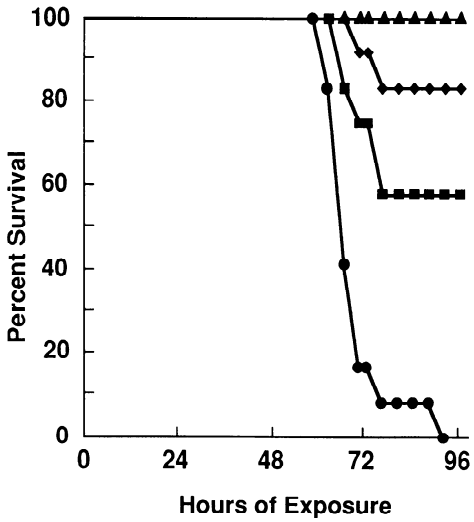

FIG. 7. Survival of rats continuously exposed to 100% oxygen after treatment with saline (●), tyloxapol (■), tyloxapol plus cetyl alcohol (▲), or Exosurf (♦). Relative to saline, therapies with tyloxapol, tyloxapol plus cetyl alcohol, and Exosurf significantly improved survival. Among the 3 therapies, mortality after treatment with tyloxapol plus cetyl alcohol was significantly less than that after tyloxapol alone.

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