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Comparison of inhibitory effects of oxygen radicals and calf serum protein on surfactant activity

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Abstract

The effects of the reactive oxygen species (ROS) superoxide anion ($O_2^{\cdot}$-) and hydroxyl radical (•OH) on the surface tension lowering properties of bovine lipid extract surfactant (BLES) were compared to the effects of calf serum protein (CSP) in a captive bubble surfactometer (CBS). $O_2^{\cdot}$- was generated from xanthine/xanthine oxidase (X/XO), and •OH was generated by the Fenton reaction. ROS were demonstrated by electron spin resonance (ESR) using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as the spin trap. Lipid peroxidation was measured using the thiobarbituric acid method. •OH had broad inhibitory effects on surface tension parameters, including adsorption, minimum surface tension, percentage film area change and film compressibility. $O_2^{\cdot}$- showed inhibitory effects on adsorption, film area change and film compressibility but had no significant effect on minimum surface tension. Both $O_2^{\cdot}$- and •OH treatment were associated with a large 'squeezeout' plateau around 20–25 mN/m in the surface tension–area relation, indicating poor film organization during the compression phase. At the concentrations used, ROS were associated with lipid peroxidation of BLES, which also demonstrated radical scavenging properties. Calf serum protein produced inhibitory effects on adsorption, minimum surface tension and percentage film area change that were quantitatively similar to those produced by •OH. The effects on film compression were significantly greater and qualitatively different from those seen with either $O_2^{\cdot}$- or •OH. We conclude that the inhibition of BLES surface activity by ROS and inhibitory proteins can be distinguished in the captive bubble surfactometer and, particularly, by changes in the film compressibility modulus. (Mol Cell Biochem **259**: 15–22, 2004)

Key words: oxygen radicals, surfactant, bovine lipid extract surfactant, lipid peroxidation, surface tension

Introduction

Pulmonary surfactant is composed of phospholipids, neutral lipids and four distinct surfactant proteins [1–3]. It is critical for the maintenance of low surface tension in the alveoli, preventing collapse [4]. During acute lung injury reactive oxygen species (ROS) and serum proteins are released into the alveolar space and the composition and function of pulmonary surfactant are altered. Patients with adult respiratory distress syndrome (ARDS) have significantly less phosphatidylcholine (PC) and phosphatidylglycerol (PG) in bronchoalveolar lavage (BAL) fluid than control patients, surfactant protein concentrations are decreased, there is increased conversion of large to small surfactant aggregates and function is impaired [5–7].

ROS such as superoxide anion ($O_2^{\cdot}$-), and hydroxyl radical (•OH) are generated in the lung following exposure to toxic levels of oxygen, oxidizing gases such as ozone [8] and NO_2 [9], drugs such as paraquat [10, 11] and ionizing radiation. Low levels of ROS are constantly generated in the lung

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from xanthine oxidase during normal metabolic processes [12]. Activated leukocytes also produce oxygen species such as H_2O_2, $O_2^{\cdot}$-, peroxynitrite, nitric oxide and •OH [13–15]. Most are beneficially utilized and the remainder scavenged by antioxidant defenses. These defenses include superoxide dismutase (SOD), catalase and glutathione peroxidase, many of which are found in the alveolar lining fluid [16, 17].

There are many potential mechanisms for surfactant impairment in acute lung injury [18, 19]. These include lipid peroxidation of unsaturated fatty acids [20], denaturation of surfactant apoproteins [21, 22], protein inhibition [23] and alterations in surfactant recycling [24]. Several proteins have been shown to inhibit the activity of surfactant, particularly serum proteins such as fibrin and its associated degradation products which are found in high concentration in the lungs of patients with ARDS [5–7, 25]. These proteins impair the absorption of DPPC at the air liquid interface by competition between protein and surfactant molecules [23]. ROS by contrast produce biochemical alterations in surfactant lipids and proteins [25]. Both lead to abnormalities in the biophysical properties of surfactant both *in vitro* [13, 20, 25, 26] and *in vivo* [9, 14].

Intuitively, it would be expected that the ROS and inhibitory proteins would enhance each other's deleterious effects on surfactant function. However, recent *in vitro* studies have demonstrated that addition of serum proteins to surfactant protects against, rather than promotes, ROS mediated chemical changes in surfactant lipid and protein constituents [25].

The purpose of this study was to determine whether two clinically important ROS ($O_2^{\cdot}$- and •OH) produce similar or different abnormalities in the biophysical properties of surfactant compared to protein inhibitors. As a working hypothesis, we postulated that the effects of ROS and inhibitory proteins on surfactant would have distinct biophysical signatures, thus potentially offering a means to distinguish between the two types of injury in bronchoalveolar fluid from patients with ARDS. The Fenton reaction was used to generate •OH, and xanthine (X) and xanthine oxidase (XO) system used for $O_2^{\cdot}$- generation. Calf serum protein (CSP) was the source of inhibitory proteins. We report that the inhibition of surfactant activity by ROS in the captive bubble surfactometer (CBS) is qualitatively and quantitatively different from the inhibition caused by CSP.

Materials and methods

Reagents

Bovine lipid extract surfactant (BLES) was donated by BLES Biochemicals, London, Ontario, Canada. Briefly, BLES is a chloroform:methanol extract of bronchoalveolar lavage which is treated with acetone to remove neutral lipids. BLES contains surfactant phospholipids plus surfactant proteins B and C (SP-B, SP-C) but lacks antioxidant enzymes and surfactant proteins A and D [3, 27]. It is dispersed in saline with 1.5 mM calcium chloride [27]. Xanthine (X-0626), XO (X-4500), calf serum protein (CSP) and H_2O_2 were obtained from Sigma Chemicals (St. Louis, MO, USA). 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was obtained from Aldrich Chemicals (Milwaukee, WI, USA).

Superoxide anion and hydroxyl radical generation

$O_2^{\cdot}$- radicals were generated through the reaction of xanthine (X) and xanthine oxidase (XO) [28]. The standard reaction mixture in a 1-ml vol. contained 300 μg BLES, 0.35 mM X and 0.0024 units/ml of XO in 0.05M buffered EDTA at pH 7.4. At these concentrations of X and XO we expected the $O_2^{\cdot}$- radicals to have effects on both proteins and lipids [29]. Control experiments used SOD (0.04 mg/ml) to inhibit generation of $O_2^{\cdot}$- and catalase and deferoxamine to inhibit •OH.

•OH was generated from H_2O_2 and $Fe(NH_4)SO_4$. The reaction mixture in 1 ml final volume contained 50 mM H_2O_2 and 50 mM $Fe(NH_4)SO_4$. Control experiments were conducted with and without H_2O_2 and in the presence of oxygen radical scavengers, catalase and deferoxamine.

Calf serum protein

Calf serum protein at a concentration of 1 mg/ml was used to inhibit the surface activity of 300 μg/ml of BLES [30].

Measurement of surface tension

Surface tension measurements were made using a captive bubble surfactometer [31–33]. Briefly, the method consisted of introducing an aqueous suspension of 300 μg/ml BLES into a cylindrical glass chamber beneath an agarose gel. A bubble is introduced into the aqueous phase and the bubble shape monitored with a video camera. The surface area of the bubble is changed by varying the pressure in the liquid surrounding the bubble. Surfactant adsorption at the air/liquid interface, film surface area during quasi-static conditions, minimum surface tension and film compressibility were calculated according to the methods described previously [30]. At the conclusion of the surface tension measurements, aliquots of the bubble chamber fluid were analyzed for phospholipid phosphorus to confirm surfactant content [34]. The surface tension experiments were conducted 1 h after mixing the BLES with the ROS reagents or CSP. This incubation period was judged optimal based on previous studies using protein inhibitors [30] and on kinetic experiments for $O_2^{\cdot}$- and •OH using incubation periods ranging from 5 min

to 3 h. A 1-h incubation time corresponded to ~85% decay in the ESR signal, resulted in plateauing of lipid peroxidation and was short enough to avoid effects of auto-oxidation that have been demonstrated at 4 h [21]. Control experiments using radical scavengers were not used in the CBS experiments due to the fact that the scavengers (being mostly proteins) independently impaired the surface activity of BLES.

Electron spin resonance

Electron spin resonance (ESR) measurements were performed with a Varian E 109 ESR spectrometer operating at X-band (9.7 GHz) frequency [35]. Short-lived free radical intermediates were spin trapped with (DMPO) present in the reaction medium [36]. Hyperfine couplings were measured (0.1 G), directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydroxyl. All the measurements were carried out at room temperature at 50 mW microwave power, 2G modulation amplitude and a magnetic field of 3370 ± 50 G. Reactants were mixed in a 3 ml syringe for 10 sec and transferred directly to a flat cell for ESR measurements. All the measurements were started immediately after mixing the reactants and ESR spectra were read at room temperature. The receiver gain, time constant, sweep time, sweep width, modulation frequency/amplitude, and microwave power was kept constant to allow relative peak intensity comparisons.

Lipid peroxidation

Lipid peroxidation was assayed by measuring thiobarbituric acid reactive substance (TBARS) according to a modified method described earlier [35]. The reaction mixture in a total volume of 1 ml contained 75 μg BLES, 50 μM X and 0.0024 units/ml of XO in EDTA buffered medium containing 140 mM NaCl, 5 mM KCl, 10 mM of (N-[2-hydroxyethyl]piperazine-N′[2-ethanesulfonic acid]) (HEPES) at pH 7.4. The reaction was terminated by the addition of 625 μl 40% of trichloroacetic acid and 300 μl of 5 N HCl. Tubes were vortexed and heated for 20 min at 90°C. The TBARS was measured at 540 nm and compared with malondialdehyde standards prepared from 1,1,3,3-tetramethoxypropane. Control experiments were carried out with X and XO alone, H_2O_2 without iron and with the addition of antioxidant butyl hydroxy toluene to inhibit lipid peroxidation. Linoleic acid was used as a positive control.

Data analysis

For the calculation of the bubble surface tension, area, and volume we used the method of Schoel *et al.* [31]. For the quasi-static cycling and film compressibility calculations we used the methods of Lee *et al.* [30]. Film compressibility at a particular surface tension, γ_1, was calculated from the formula:

$$C = \frac{1}{A_1}\,\frac{dA_1}{d\gamma}$$

A_1 is the film area at a particular surface tension γ_1 and $dA_1/d\gamma$ is the inverse value of the derivative of the γ vs. area curve at point (A_1, γ_1). The surface tension properties and film compressibility values were determined from the 4th quasi-static compression curves. A 4th degree polynomial was fitted to the surface tension vs. area data. At the point of inflection, the slope of the 4th degree polynomial assumes its minimum value, which was used to determine the maximum compressibility (C plateau). If there was no obvious plateau, the compressibility was calculated at 25 mN/m (C 25). Other parameters, including the surface tension obtained after a 5 min adsorption period ($\gamma_{ads}5'$), the relative film area change during compression from 25 mN/m to the minimum surface tension (ΔA%) and minimum surface tension (γ_{min}), were computed as described in Lee *et al.* [30].

Statistical analyses

Data presented are the means and standard errors. Analysis of variance was used to determine the significance in the differences of the means between treated and control experiments. A probability value of less than $p = 0.05$ was considered significant.

Results

Surface tension measurements

Adsorption

In control experiments using BLES (300 μg/ml), the surface tension after 5 min of adsorption ($\gamma_{ads}5'$) was 23.5 mN/m ± 0.5 mN/m (S.E., n = 6). This value fell well within the accepted equilibrium values of 22–25 mN/m for pulmonary surfactant films, which indicated that BLES adsorption was complete in 5 min. Statistically significant ($p < 0.05$) increases in surface tension were seen for $O_2^{\cdot}$-, •OH and CSP compared to BLES alone (Fig. 1).

Minimum surface tension

During quasi-static conditions, there were significant ($p < 0.05$) increases in minimum surface tension (γ_{min}) compared to the BLES control following the addition of CSP and •OH. The γ_{min} was not significantly affected by the presence of $O_2^{\cdot}$- (Fig. 2).

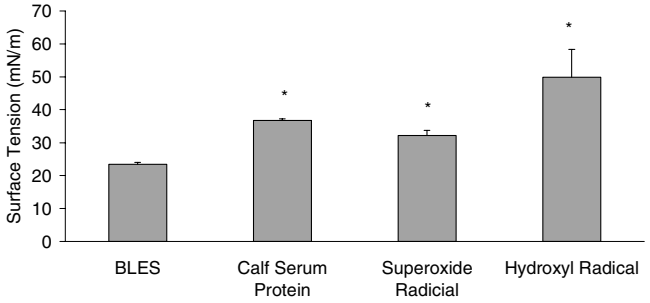

Fig. 1. Graph of the equilibrium surface tension after 5 min adsorption for BLES alone and BLES incubated with one of the following: calf serum protein, superoxide radical or hydroxyl radical. The equilibrium surface tensions are increased for all treatments, with greatest effect seen for hydroxyl radical. *$p < 0.05$.

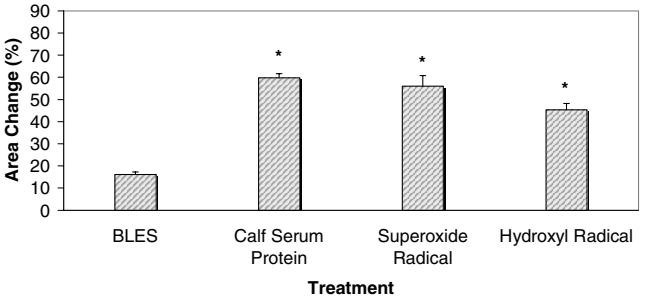

Fig. 3. Graph of the percent area change to achieve minimum surface tension on the 4th quasi-static compression for BLES alone and BLES incubated with one of the following: calf serum protein, superoxide radical or hydroxyl radical. All treatments had significant ($p < 0.05$) effects on area change, with the greatest effect seen for hydrogen peroxide.

Film surface area reduction

During quasi-static conditions, minimal surface tension was achieved in control samples of BLES with approximately 16% compression of the bubble surface area (the percent area change between the values of $\gamma = 25$ mN/m and $\gamma = \text{min}$) on the 4th compression cycle. Significant and similar adverse effects on film surface area ($p < 0.05$) were seen for all treatment groups compared to BLES alone (Fig. 3).

An interesting finding is that the Student–Newman–Keuls test showed that the treatment of the dilute superoxide radicals for minimum surface tension is different from the that of the effects produced by hydroxyl radicals and hydrogen peroxide.

Film compressibility

Film compressibility is an indication of film behaviour during compression. Pure DPPC films are highly incompressible, while mixed films of DPPC and unsaturated phospholipids show high compressibility due to squeeze-out. Thus compressibility gives an indication of film quality in terms of DPPC content at the surface tensions studied. Compressibility was computed from compression curves during the 4th quasi-static compression cycle. Figure 4 shows a representative first order least square fit curve for values obtained with 300 μg/ml of BLES suspended in buffer at 37°C. The compression curves for 300 μg/ml BLES incubated with 1 mg/ml of CSP showed an elongated plateau region in the surface tension vs. area relation between 40–45 mN/m (Fig. 4). This likely represents squeeze-out of serum protein until a relatively pure surfactant film is attained. In contrast, in the presence of $O_2^{\cdot}$- and •OH (Fig. 4), compression profiles were very different from those seen with BLES alone or BLES with CSP. During the compression phase for these treatments there was an initial drop in surface tension followed by a plateau region characterized by a decrease in surface area but little decrease in surface tension. This region was observed between surface

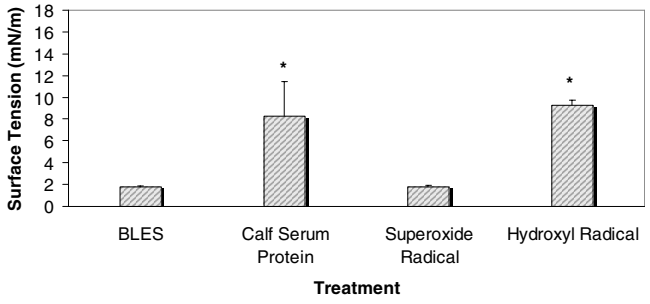

Fig. 2. Graph of minimum surface tension achieved on the 4th quasi-static compression of BLES alone and BLES incubated with one of the following: calf serum protein, superoxide radical or hydroxyl radical. Significant ($p < 0.05$) effects are seen for hydroxyl radical and calf serum protein, but not for superoxide radical. The value of superoxide radical was not significantly different from control.

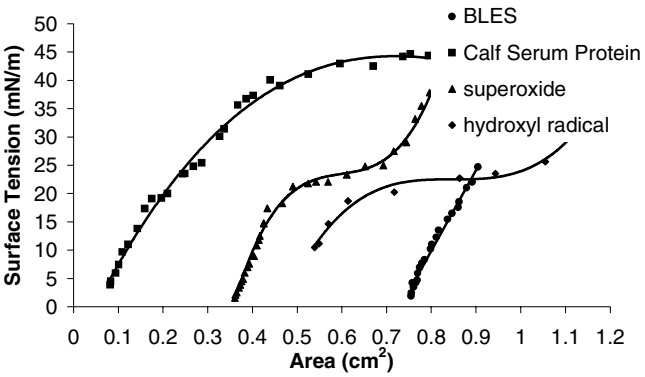

Fig. 4. Representative compression plots of surface tension vs. area of 300 μg/ml of BLES alone compared to 3 treatment groups: (1) BLES incubated with 1 mg/ml of calf serum protein (CSP); (2) BLES incubated with xanthine/xanthine oxidase to produce $O_2^{\cdot}$-, and (3) BLES incubated with H_2O_2/ferrous sulphate mixture to produce hydroxyl radicals. All three treatments resulted in distinctive surface tension–area relationships. Note the plateau region between 20 and 25 mN/m for ROS treatments and between 40 and 45 mN/m for protein treatment.

Table 1. Effect of various agents on BLES film compressibility, C (m/mN); mean ± S.E. (n)

Experiment	$C_{plateau}$ [1]	C_{15}
BLES alone	0.008 ± 0.0005	0.008 ± 0.0005
•OH + BLES	0.089 ± 0.0219†	0.020 ± 0.0025*
$O_2^{\cdot}$- + BLES	0.053 ± 0.0032†	0.015 ± 0.0020*
CSP + BLES	0.118 ± 0.0644†	0.032 ± 0.0126*

[1]The compressibility at $\gamma = 25$ mN/m (C_{25}) was used as there was no plateau on the compression curve. †Compressibility values significantly different from BLES control values using the Scheffé test (ANOVA). *Compressibility values significantly different from the corresponding $C_{plateau}$ or C_{25} values using the paired *t*-test.

tensions of 20–25 mN/m, which was characteristic for ROS inhibition in these experiments and was clearly different from the protein-induced inhibition. Film compressibility (m/mN; mean ± S.E.), calculated at C plateau or C_{25} and at 15 mN/m, is presented in Table 1. BLES alone had a compressibility value comparable to the reported value of 0.005 m/mN at 15 mN/m for pure DPPC. Compressibility values for BLES with $O_2^{\cdot}$- and •OH or CSP were all significantly different ($p < 0.05$) from BLES alone.

ESR and lipid peroxidation measurements

Kinetic studies at 3, 15, 30 and 60 min showed that the ESR signal for ROS increased linearly up to 1 h thereafter plateauing. The results of ESR experiments for the radical generating treatments with BLES or linoleic acid as a positive substrate are presented in Fig. 5. In the presence of BLES there were significant ($p < 0.05$) effects with both ROS treatments. The relative effects on ESR peak height were •OH > $O_2^{\cdot}$-. Significantly greater ESR peak heights were seen with linoleic acid than with BLES under the same experimental conditions. The differences in ESR peak height for linoleic acid and BLES in the presence of ROS is consistent with a greater radical scavenging capacity of the BLES. Figure 6 shows the results of the lipid peroxidation experiments for $O_2^{\cdot}$- and •OH. Significant increases in TBARS were seen for BLES incubated with •OH, and $O_2^{\cdot}$- compared to controls. However, these values were significantly less than the values for TBARS formed when linoleic acid was the substrate (Fig. 6). Control experiments using the appropriate radical scavenger inhibited these reactions by 70–80%. There was no effect of X or XO alone on ESR signal or lipid peroxidation.

Discussion

Surface tension in alveolar spaces on full inspiration plateaus at ~30 mN. During expiration alveolar surface tension falls to less than 2 mN/m at residual lung capacity [4, 32]. These *in situ* properties of pulmonary surfactant can be reproduced *in vitro* with the captive bubble surfactometer [31–33]. Specifically, leakage artifacts are eliminated as no artificial walls interrupt the continuous surfactant film. The biophysical properties of adsorption, compression, equilibrium surface tension and surface tension/area relationships are all measured in the CBS. Furthermore, dynamic behavior of the surface film can be detected visually such as in the case when the bubble jumps from one metastable conformation to another metastable conformation, what we termed as 'clicks' [33].

In control experiments using BLES alone there was rapid adsorption to the air/liquid interface to reach a stable equilibrium surface tension of ~25 mN/m (Fig. 1). Following four quasi static compressions a γ min of approximately 2 mN/m was achieved on mild (10–20%) area compression (Figs 2 and 3). These desirable attributes are attributable to the presence of the low molecular weight hydrophobic proteins SP-B and SP-C in the preparation [3, 37].

The addition of ROS or proteins to BLES in the CBS resulted in distinct patterns of abnormality. CSP, $O_2^{\cdot}$- and •OH

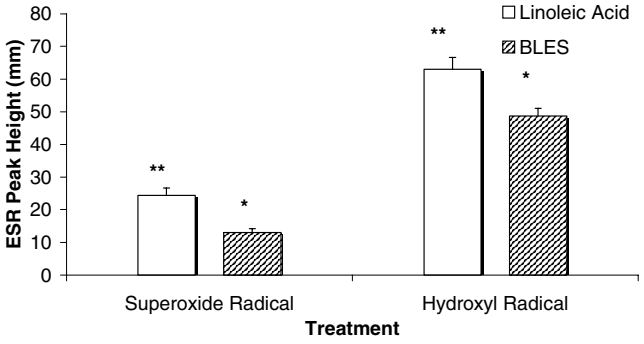

Fig. 5. Graph of ESR peak height (mm) for $O_2^{\cdot}$- and •OH incubation with either BLES or linoleic acid. Both ROS treatments resulted in significant ($p < 0.05$) trapping of ROS. However, the effect was significantly attenuated in the presence of BLES compared to linoleic acid, suggesting radical scavenging by BLES.

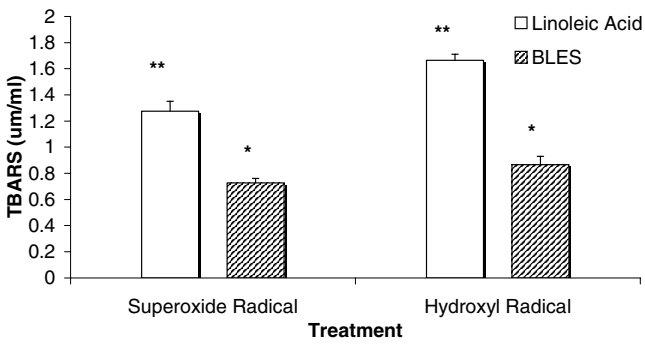

Fig. 6. Graph of lipid peroxidation products (TBARS) formed from various mixtures: $O_2^{\cdot}$- and •OH, with either BLES or linoleic acid. Both ROS treatments resulted in significant ($p < 0.05$) increases in lipid peroxidation products. However, the effect was significantly ($p < 0.05$) attenuated in the presence of BLES compared to linoleic acid.

all significantly increased surface tension following adsoption (γ_{ads} 5 min) compared to BLES alone, and the effects of ROS and CSP on this parameter were similar (Fig. 1).

BLES alone attained low surface tensions (< 2 mN/m) after four compression cycles consistent with a film highly enriched in DPPC (Fig. 2). Significant increases in γ min were seen when BLES was incubated with either CSP or •OH. By contrast $O_2^{\cdot}$- had no effect on minimum surface tension.

Area reduction correlates with DPPC concentration and its organization within the film [2]. All treatments resulted in significant effects on area change (Fig. 3); however, at the concentrations used, there were no significant differences between CSP, $O_2^{\cdot}$- and •OH.

These data indicate that the treatments either reduced selective DPPC adsorption and/or reduced preferential squeeze out of non-DPPC components from the monolayer. For ROS experiments it is likely that ROS modified the surfactant proteins SP-B and SP-C [21] thereby resulting in impaired adsorption [3]. The observations on CSP are consistent with non-surfactant proteins competing with surfactant phospholipids for the air-water interface [23]. The presence of serum proteins in the interface would explain the initial high surface tension observed in the presence of serum proteins and the need for high compression ratios to achieve low surface tension (Figs. 2 and 4).

Both •OH and $O_2^{\cdot}$- formed lipid peroxidation products when incubated with BLES. At the concentrations used in these experiments the formation of lipid peroxides were approximately 30% greater following •OH treatment compared to $O_2^{\cdot}$- (Fig. 6). Thus these data indicate the presence of fluid oxidation products which could not be squeezed out of the DPPC film during maximal compression in the •OH treated group. Seeger *et al.* [26] examined the effects of ROS on crude rabbit lavage surfactant with the Langmuir-Wilhelmy surface balance. Oxygen exposure resulted in delayed surface tension reduction during compression of dilute films (50–60 mN/m), consistent with the area reduction studies in Fig. 3. These investigators also observed elevated surface tensions during film expansion, implying hampered adsorption and/or respreading. In their studies, minimum surface tension was approximately 20 mN/m, a high value, possibly due to the leakage problems associated with the Langmuir-Wilhelmy balance. Monolayer instability at minimum surface tension was also increased. These results, although obtained at higher surface tensions, are consistent with the presence of fluid film components such as oxidized phospholipids. The crude lavage surfactant studied by Seeger *et al.* [26] would also contain SP-A, which is susceptible to oxidation reactions [8, 9, 21, 22].

The lack of an effect of $O_2^{\cdot}$- on γ min is less easily explained. It might have resulted from small quantitative differences in lipid peroxides (Fig. 6), the formation of lipid breakdown products, other than TBARS, such as free acyl alcohols and lysophopholipid [20, 38] or to the greater reactivity of •OH radical proteins compared to $O_2^{\cdot}$- [28]. Haddad *et al.* [13] observed that peroxynitrite, which decomposes to release products with •OH like reactivity [39], markedly depressed the surface activity of CLSE assayed with the pulsating bubble surfactometer, but found little effect with X/XO-based oxidation. The latter observation is in keeping with our observation that $O_2^{\cdot}$- generated from X/XO had no significant effect on minimum surface tension (Fig. 2). Gilliard *et al.* [20] demonstrated that oxidation of porcine surfactant lipid extracts with either Fe/H_2O_2 or $CuCl_2/H_2O_2$ led to severe impairment of adsorption and film compression in the pulsating bubble surfactometer. These investigators also found that oxidation markedly reduced the biological effectiveness of their surfactant extract in premature rabbit pups. In similar experiments, Mark and Ingenito [21] studied the effects of •OH radicals on purified CLSE. At 1 mg/ml CLSE they found evidence of lipid peroxidation and modification of SP-A, but only minimal effects on surface tension lowering properties compared to controls. The greater effects noted on surfactant function reported in this study may reflect our use of a lower concentration of surfactant (300 μg/ml) or the absence of SP-A in BLES.

The mechanism of pulmonary surfactant dysfunction resulting from free radical damage or lipid peroxidation is complex and only partially understood. ROS-induced oxidation of surfactant results in the generation of unstable peroxides which form cleaved fatty acid chain products and lysophospholipids [20]. These products with high fluidity could fuse with other lipids or with the hydrophobic SP-B and SP-C [3]. In our studies, incubation of BLES with $O_2^{\cdot}$-, or •OH, resulted in free radical generation (Fig. 5), and the appearance of TBARS (Fig. 6) indicating that the free radicals reacted with polyunsaturated fatty acids to form lipid peroxides [20]. Films derived from BLES exposed to ROS showed broad elongated plateaus around 20–30 mN/m during compression (Fig. 4), which can be explained most directly by phospholipid structural alterations, but could also be explained by increased fluidity arising from oxidized fatty acids. The compression curves of films from BLES incubated with $O_2^{\cdot}$- or •OH demonstrate a large flat plateau in the zone near the equilibrium adsorption tension (i.e. 20–25 mN/m) of BLES. The changes in surfactant compressibility induced by ROS were different from the compression curves in the presence of inhibitory protein which showed a plateau at much higher surface tensions (40–45 mN/m). The interaction(s) between ROS and surfactant are complex and may involve factors in addition to lipid peroxidation. One possibility is that ROS affected the hydrophobic proteins SP-B and SP-C resulting in inactivation of these proteins as has been observed with SP-A [13]. Native SP-B and SP-C are highly surface active compared to other surfactant proteins and when present at low values are considered squeezed out of DPPC, DPPG, or DPPC:DPPG monolayers below 5 mN/m [40, 41]. Studies with SP-A and other soluble proteins suggest methionine and tryptophan oxidation can

occur [42], and that proteins are preferred targets in protein surfactant mixtures [43]. Thus, although it appears likely that ROS could inhibit surfactant adsorption by affecting SP-B and/or SP-C, the mechanism must be determined.

Free radical scavenging is an important property in natural surfactant preparations [39, 43] but is seen to a lesser degree in therapeutic surfactant preparations [39, 44]. The ESR data in our studies indicated that ROS were scavenged more effectively in the presence of BLES than with linoleic acid (Fig. 5). These findings might indicate a role for the surfactant proteins B and C in this process. SP-A and SP-D have been shown to have potent direct antioxidant properties [45]; however, no data exist for a similar role for SP-B and SP-C. Crude natural surfactant is rich in antioxidant enzymes, such as catalase and SOD. However, the enhanced scavenging efficacy of BLES is not likely due to classic protein antioxidants as the manufacture of BLES results in the removal of all proteins except SP-B and SP-C, as well as the neutral lipid cholesterol and vitamin E [3]. Hence, it is unlikely that the observed phenomenon is due to one of the known antioxidant lung defense systems.

In summary, using the captive bubble surfactometer we were able to demonstrate marked quantitative and qualitative differences in BLES surfactant function following treatment with an inhibitory protein, CSP or ROS. Differences in effect were also observed between the two ROS, $O_2^{\cdot}$- and •OH. The abnormalities in the biophysical properties of BLES surfactant exposed to ROS are consistent with a mechanism of action that involves lipid peroxidation and protein oxidation; whereas, the abnormalities associated with CSP are consistent with physical interference with the surfactant film. The most discriminating test for distinguishing the effects of ROS from proteins was the compressibility modulus. The results presented here were based on a single set of concentrations, thus our findings may not necessarily generalize to other situations. The concentrations were, however, carefully selected to produce equivalent effects on overall surfactant function. The results of clinical trials of ARDS indicate that some types of lung injury respond better to surfactant replacement therapy than others [7]. The data presented in this paper suggest that it might be possible to differentiate protein-induced surfactant impairment from that induced by ROS in BAL samples from patients with acute lung injury. Such a test would be valuable in selecting the most appropriate therapy for individual patients.

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References

1. Wright JR, Clements JA: Metabolism and turnover of lung surfactant. Am Rev Respir Dis 135: 426–444, 1987
2. Veldhuizen RK, Nag K, Orgeig S, Possmayer F: The role of lipids in pulmonary surfactant. Biochim Biophys Acta 1408: 180–202, 1998
3. Possmayer F: Physico-chemical aspects of pulmonary surfactant. In: R.A. Polin, W.W. Fox (eds). Fetal and Neonatal Physiology. W & B Saunders, Philadelphia, PA, 1997, pp 1259–1275
4. Bachofen H, Schürch S, Urbinelli M, Weibel ER: Relations among alveolar surface tension, surface area, volume, and recoil pressure. J Appl Physiol 62: 1878–1887, 1987
5. Spragg RG, Gilliard N, Richman P, Smith RM, Hite D, Papert D, Heldt GP, Merritt TA: The adult respiratory distress syndrome: Clinical aspects relevant to surfactant supplementation. In: B. Robertson, L.M.G. van Golde, J.J. Batenburg (eds). Pulmonary Surfactant: From Molecular Biology to Clinical Practice, vol. 1. Elsevier Science, Amsterdam, 1992, pp 685–703.
6. Ward PA, Johnson KJ, Till GO: Current concepts regarding adult respiratory distress syndrome. Ann Emerg Med 14: 724–728, 1985
7. Lewis JF, Veldhuizen R: The role of exogenous surfactant in the treatment of acute lung injury. Annu Rev Physiol 65: 613–642, 2003
8. Oosting RS, van Iwaarden JF, van Bree L, Verhoef J, van Golde LMG, Haagsman HP: Exposure of surfactant protein A to ozone *in vitro* and *in vivo* impairs its interactions with alveolar cells. Am J Physiol 262: L63–L68, 1992
9. Muller B, Barth P, von Wichert P: Structural and functional impairment of surfactant protein A after exposure to nitrogen dioxide in rats. Am J Physiol 263: L177–L184, 1992
10. Silva MF, Saldiva PH: Paraquat poisoning: An experimental model of dose dependent acute lung injury due to surfactant dysfunction. Braz J Med Biol Res 31: 445–450, 1998
11. Haagsman HP, van Golde LMG: Lung surfactant and pulmonary toxicology. Lung 163: 275–303, 1985
12. Grum CM, Ragsdale RA, Ketai LH, Simon RH: Plasma xanthine oxidase activity in patients with adult respiratory distress syndrome. J Crit Care 2: 22–26, 1987
13. Haddad IY, Ischiropoulos H, Holm BA, Beckman JS, Baker JR, Matalon S: Mechanisms of peroxynitrite-induced injury to pulmonary surfactants. Am J Physiol 265: L555–L564, 1993
14. Matalon S, DeMarco V, Haddad IY, Myles C, Skimming JW, Schürch S, Cheng S, Cassin S: Inhaled nitric oxide injures the pulmonary surfactant system of lambs *in vivo*. Am J Physiol 270: L272–L280, 1996
15. Ryan SF, Ghassibi Y, Liau DF: Effects of activated polymorphonuclear leukocytes upon pulmonary surfactant *in vitro*. Am J Respir Cell Mol Biol 4: 33–41, 1991
16. Cantin AM, North SL, Hubbard RC, Crystal RG: Normal alveolar epithelial lining fluid contains high levels of glutathione. J Appl Physiol 63: 152–157, 1987
17. Davis WB, Pacht ER: Extracellular antioxidant defenses. In: R.G. Crystal, J.B. West (eds). The Lung: Scientific Foundations, 2nd ed. Lippincott-Raven, Philadelphia, PA, 1997, pp 2271–2278
18. Jackson RM: Molecular, pharmacologic, and clinical aspects of oxygen-induced lung injury. Clin Chest Med 11: 73–83, 1990
19. Lewis JF, Veldhuizen RAW: Factors influencing the efficacy of exogenous surfactant in acute lung injury. Biol Neonate 67 (suppl 1): 48–60, 1995
20. Gilliard N, Heldt GP, Gasser H, Redi H, Merritt TA, Spragg RG: Exposure of the hydrophobic components of porcine lung surfactant to oxidant stress alters surface tension properties. J Clin Invest 93: 2608–2615, 1994
21. Mark L, Ingenito EP: Surfactant function and composition after free

radical exposure generated by transition metals. Am J Physiol 276: L491–L500, 1999
22. Haddad IY, Crow JP, Hu P, Yaozu Y, Beckman J, Matalon S: Concurrent generation of nitric oxide and superoxide damages surfactant protein A. Am J Physiol 267: L242–L249, 1994
23. Holm BA, Enhorning G, Notter RH: A biophysical mechanism by which plasma proteins inhibit lung surfactant activity. Chem Phys Lipids 49: 49–55, 1988
24. Holm BA, Hudak BB, Keicher L, Cavanaugh C, Baker RR, Hu P, Matalon S: Mechanisms of H_2O_2-mediated injury to type II cell surfactant metabolism and protection with PEG-catalase. Am J Physiol 261: C751–C757, 1991
25. Marzan Y, Mora R, Butler A, Butler M, Ingenito EP: Effects of simultaneous exposure of surfactant to serum proteins and free radicals. Exp Lung Res 28: 99–121, 2002
26. Seeger W, Lepper H, Hellmut WRD, Neuhof H: Alteration of alveolar surfactant function after exposure to oxidative stress and to oxygenated and native arachidonic acid *in vitro*. Biochim Biophys Acta 835: 58–67, 1985
27. Yu SH, Possmayer F: Comparative studies on the biophysical activities of the low-molecular-weight hydrophobic proteins purified from bovine pulmonary surfactant. Lipids 18: 522–529, 1983
28. Risberg B, Smith L, Örtenwall P: Oxygen radicals and lung injury. Acta Anaesthesiol Scand Suppl 95: 106–116; discussion 116–118, 1991
29. Davies KJA, Goldberg A: Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. J Biol Chem 262: 8220–8226, 1987
30. Lee MM, Green FHY, Roth SH, Karkhanis A, Bjarnason SG, Schürch S: Sulfuric acid aerosol induces changes in alveolar surface tension in the guinea pig but not in the rat. Exp Lung Res 25: 229–244, 1999
31. Schoel WM, Goerke J, Schürch S: The captive bubble method for the evaluation of pulmonary surfactant: Surface tension, area and volume calculations. Biochim Biophys Acta 1200: 281–290, 1994
32. Schürch S, Bachofen H, Goerke J, Possmayer F: A captive bubble method reproduces the *in situ* behavior of lung surfactant monolayers. J Appl Physiol 67: 2389–2396, 1989
33. Schürch S, Possmayer F, Cheng S, Cockshutt AM: Pulmonary SP-A enhances adsorption and appears to induce surface sorting of lipid extract surfactant. Am J Physiol 263: L210–L218, 1992
34. Rouser G, Fleischer S, Yamamoto A: Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis. Lipids 5: 494–496, 1970
35. Vallyathan V, Leonard S, Kuppusamy P, Pack D, Chzhan M, Sanders SP, Zweier JL: Oxidative stress in silicosis: Evidence for the enhanced clearance of free radicals from whole lungs. Mol Cell Biochem 168: 125–132, 1997
36. Borg DC: Applications of electron spin resonance in biology. In: W.A. Pryor (ed). Free Radicals in Biology, vol. 1. Academic Press, New York, 1976, pp 69–147
37. Qanbar R, Cheng S, Possmayer F, Schürch S: Role of the palmitoylation of surfactant-associated protein C in surfactant film formation and stability. Am J Physiol 271: L572–L580, 1996
38. Rice-Evans CA: Techniques in free radical chemistry. Elsevier, New York, 1991
39. Cifuentes J, Ruiz-Oronoz J, Myles C, Nieves B, Carlo W, Matalon S: Interaction of surfactant mixtures with reactive oxygen and nitrogen species. J Appl Physiol 78: 1800–1805, 1995
40. Stults JT, Griffin PR, Lesikar DD, Naidu A, Moffat B, Benson BJ: Lung surfactant protein SP-C from human, bovine and canine sources contains palmityl cysteine thioester linkages. Am J Physiol 261: L118–L125, 1991
41. Taneva S, Keough KMW: Pulmonary surfactant proteins SP-B and SP-C in spread monolayers at the air–water interface: III. Proteins SP-B plus SP-C with phospholipids in spread monolayers. Biophys J 66: 1158–1166, 1994
42. Davis IC, Zhu S, Sampson JB, Crow JP, Matalon S: Inhibition of human surfactant protein A function by oxidation intermediates of nitrite. Free Radic Biol Med 33: 1703–1713, 2002
43. Matalon S, Holm BA, Baker RR, Whitfield MK, Freedman BA: Characterization of antioxidant activities of pulmonary surfactant mixtures. Biochim Biophys Acta 1035: 121–127, 1990
44. Ghio AJ, Fracia PJ, Young SL, Piantadosi CA: Synthetic surfactant scavenges oxidants and protects against hyperoxic lung injury. J Appl Physiol 77: 1217–1223, 1994
45. Bridges JP, Davis HW, Damodarasamy M, Kuroki Y, Howles G, Hui DY, McCormack FX: Pulmonary surfactant proteins A and D are potent endogenous inhibitors of lipid peroxidation and oxidative cellular injury. J Biol Chem 275: 38848–38855, 2000