Pulmonary Surfactant Protein-A (SP-A) Restores the Surface Properties of Surfactant after Oxidation by a Mechanism That Requires the Cys\textsuperscript{6} Interchain Disulfide Bond and the Phospholipid Binding Domain*

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Reactive oxygen species produced by activated leukocytes in the alveolar epithelial lining fluid have been implicated in the inactivation of pulmonary surfactant and the impairment of lung function. Oxidation of bovine lipid extract surfactant (BLES), a therapeutic surfactant, with hypochlorous acid (H-BLES) or the Fenton reaction (F-BLES) led to temporal increases in conjugated dienes and formation of malondialdehyde and 4-hydroxy-2-nonenal. Electrospray ionization mass spectrometry revealed the appearance of lipid hydroperoxides, peroxides, lysophospholipids, and free fatty acids. Captive bubble tensiometer studies of H-BLES demonstrated prolonged adsorption times, film instability at low surface tensions during film compression, and reduced respreadability during film expansion. F-BLES exhibited prolonged adsorption times, a marked effect on increasing compressibility during compression, and a lesser effect on reducing respreadability on expansion. Addition of native bovine or rat surfactant-associated protein A (SP-A) reversed the effects of oxidation on surfactant biophysical properties. Studies using mutant recombinant rat SP-A indicated that an intact carbohydrate recognition domain and disulfide-dependent oligomeric assembly are critical for these effects, but the collagen-like region is not required. We conclude that SP-A can reverse the detrimental effects of surfactant oxidation on the biophysical properties of surfactant, by a mechanism that is dependent on interchain disulfide bond formation and the C-terminal domains of the protein.

Pulmonary surfactant is essential for normal lung function. By lowering surface tension (γ)\textsuperscript{1} during inspiration, surfactant reduces the work of breathing. Through its ability to generate very low tensions during expiration, surfactant stabilizes the alveoli at low lung volumes and limits transudation of interstitial fluid into the airspaces. The high spreadability of surfactant also limits differences between adjacent alveolar regions (1, 2). In addition to its surface-active properties, surfactant performs host defense and anti-inflammatory functions (3, 4).

The chemical composition of surfactant, which is highly conserved among different animal species, consists of ~80–90% phospholipids (PLs), ~3–8% neutral lipids (mainly cholesterol), and ~5–10% surfactant-associated proteins (SP-), including SP-A, SP-B, SP-C, and SP-D. The major surfactant PLs are dipalmitoyl phosphatidylcholine (DPPC; 30–45%), unsaturated phosphatidylcholine (PC; 25–35%), acidic PLs phosphatidylglycerol (PG) plus phosphatidylinositol (PI) (8–15%), and small amounts of phosphatidylethanolamine (PE) and sphingomyelin (1, 2, 5–8).

Surfactant lipids and proteins in the alveoli are exposed to oxidizing conditions, including direct exposure to oxidative air pollutants and to hyperoxia during supplemental oxygen therapy, as well as to endogenous reactive oxygen species from activated neutrophils and macrophages in the subphase. Although surfactant is resistant, to some extent, to oxidation because of the predominance of saturated PL (30–45% DPPC), unsaturated PLs represent ~55–70% of the weight of surfactant, including ~12% with two or more double bonds (8, 9). Whereas unsaturated PL species are essential to the fluidity of surfactant, the initial formation of the surfactant films, and the reincorporation of material into the film during expansion in the breathing cycle (1, 2, 8), they are also distinctively susceptible to oxidation. Surfactant oxidation and dysfunction likely contribute to the pathophysiology of several diseases including adult respiratory distress syndrome (10, 11), asthma (12, 13), chronic obstructive pulmonary disease (12), and infections (10). In addition to direct effects on the surface properties of surfactant, lipid peroxidation products can inhibit the synthesis and secretion of new surfactant (14) and reduce the viability of alveolar macrophages, and type II pneumocytes (15).

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¶ The abbreviations used are: γ, surface tension; γ\textsubscript{eq}, equilibrium surface tension; BLES, bovine lipid extract surfactant; C\textsubscript{10}, compressibility at 10 mN/m; CBT, captive bubble tensiometer; CRD, carbohydrate recognition domain; DPPC, dipalmitoyl phosphatidylcholine; ESI-MS, electrospray ionization mass spectroscopy; F-BLES, Fenton reacted BLES; H-BLES, hypochlorous acid reacted BLES; HNE, 4-hydroxy-2-nonenal; MDA, malondialdehyde; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipid; R\textsubscript{10}, respreadability at 10 mN/m; rSP-A, recombinant SP-A; SP-, surfactant-associated protein; wt rec, wild type recombinant; PE, phosphatidylethanolamine; SP, surfactant-associated protein; mN, milli-Newton.
In vitro studies indicate that the most abundant surfactant-associated protein, SP-A, facilitates the surface tension-lowering properties of surfactant PLS (16–18) and counteracts protein inactivation of surfactant in vitro (17, 19). Although not yet clearly delineated in vivo, these studies suggest that SP-A may enhance surfactant function in vivo under stress conditions associated with lung injury and proteinaceous pulmonary edema.

The structural domains of SP-A required for surface tension lowering effects have been mapped by targeted mutagenesis, which has revealed the importance of intact oligomeric assembly and the PL binding function of the COOH-terminal domains. The supraquaternary structure of SP-A consists of six trimeric subunits. Each monomer has four distinct domains: a short NH2-terminal domain containing one or two cysteines that function to stabilize the octadecamer by forming disulfide bonds between trimers; a collagen-like domain, a neck region, and a globular C-terminal domain. The collagen and neck portions of the monomers combine as triple helices to form trimers, and six trimers associate to form an octadecameric “flower bouquet-like” structure that resembles C1q, the first component of complement. The COOH-terminal domain contains both a carbohydrate recognition domain (CRD), and an overlapping PL binding domain (4, 20).

In the present study we hypothesized that SP-A may restore the surface-active ability of surfactant inactivated through oxidation. The effects of SP-A and mutant recombinant SP-As on the biophysical function of bovine lipid extract surfactant (BLES) were tested in the captive bubble tensiometer (CBT) before and after oxidation with Fenton chemistry or hypochlorous acid (BLES) were tested in the captive bubble tensiometer (CBT) before and after oxidation with Fenton chemistry or hypochlorous acid. The biophysical function of bovine lipid extract surfactant and proteinaceous pulmonary edema.

EXPERIMENTAL PROCEDURES

Reagents—All reagents were purchased from Sigma and/or VWR/Canlab unless otherwise noted. BLES was a kind gift of BLES Biochemicals, Inc., London, Ontario, Canada. BLES for these studies contained 80.1 ± 1.5% PC, 0.9 ± 0.12% lyso-PC, 13.2 ± 1.17% PG, 2.7 ± 0.37% PE, 0.9 ± 0.25% PI, 0.2 ± 0.09% phosphatidylerine, and 1.5 ± 0.12% sphingomyelin. The lower chloroform phase (n = 3).

In Vitro Oxidation—Reaction mixtures composed of 10 mg/ml surfactant lipids, plus Fenton reagents, HOCl/OCl or controls in buffer A (0.150 mM NaCl, 2 mM Tris-HCl, 1.5 mM CaCl2; pH 7.4) were incubated at 37 °C in a shaking water bath for 24 h. Hypochlorous acid was purchased as sodium hypochlorite (NaOCl, 10% w/v) from Sigma. Hypochlorous acid concentration was determined spectrophotometrically immediately before use by diluting the NaOCl stock solution 1:10 in 1 M NaOH and applying a molar extinction coefficient of pH 12 at 43.6 mol−1 cm−1 at 240 nm. Treatment with HOCl/OCl was carried out at a final concentration of 0.5 mM at pH 7.4. Because hypochlorous acid has a pKa of 7.46, the term HOCl/OCl used in this paper represents the mixture of hypochlorite and its corresponding free acid. For oxidation by the Fenton reaction, BLES was incubated with 0.65 mM FeCl2, 0.65 mM sodium EDTA, and 30 mM H2O2 in buffer A. These conditions approximate those used by a number of other investigators who conclude that they represent high physiological ranges (21).

Biochemical Analysis—Conjugated dienes formed during oxidation were measured via spectrophotometric monitoring of the absorbance at 235 nm (A235) after exposure to HOCl/OCl or Fenton reagents, as above. Aliquots of the oxidized BLES were diluted to 0.25 mg/ml surfactant and A235 determined every 10 min for 5.5 h against a buffer blank. Data are expressed as the changes in UV absorbance at 235 nm across time. The lipid peroxide content of the surfactant formed during oxidation was measured by using a lipid peroxidation assay kit (BIOXYTECH™ LPO-588; Oxis International, Inc.), which is based on the formation of a colored adduct that absorbs at A536, from the reaction of a chromogenic reagent with either malondialdehyde (MDA) and/or 4-hydroxy-2-nonenal (HNE). Data are expressed as nanomoles of MDA and HNE/mg of surfactant PLS.

Mass Spectroscopy—Molecular species of surfactant PLSs were analyzed by electrospray ionization mass spectroscopy (ESI-MS) with a triple quadrupole ESI-MS (model PI 365, Sciex Co., Concord, ON, Canada) using positive mode for PCs and negative mode for PGs employing a slight modification of the method of Postle et al. (13, 24). Briefly, the surfactant lipids were extracted with chloroform:methanol (1:1, v/v) by the method of Bligh and Dyer (25). The lower chloroform layers were dried under N2, and the samples were reconstituted in chloroform (1:1, v/v). Dimyristoyl-PC and dimyristoyl-PG (10 μg, 10:1), from Avanti Polar Lipids (Birmingham, AL), were added as internal standards to aliquots of each surfactant preparation containing ~80 μg of surfactant. Samples were dried under nitrogen and dissolved in 0.5 ml of methanol:chloroform (2:1, v/v) containing 5 mM NaOH. PC molecules modified with adducts were detected by the event charge parameters (M + Na+), whereas PG and PI molecular species were detected as their molecular ions under negative conditions, (M − H+). Spectra were obtained by signal averaging (typically 50 scans, 1 min) and processed by use of software supplied by the manufacturer.

SP-A—Bovine SP-A was extracted from natural pulmonary surfactant and purified by chromatography on mannose columns as previously described by Cockshutt et al. (17). Protein purity was tested by SDS-PAGE (12% gel) under reducing conditions. Rat SP-A was isolated from surfactant obtained from rats exposed to silica, using chromatography on mannose-Sepharose 6B columns. The various rat recombinant SP-As (rSP-A) were prepared as previously described (26–31) and are listed in Fig. 1. Wild-type recombinant SP-A (wt rec) lacks proline hydroxylation as it is produced in insect cells. The recombinant SP-Ahyp−M1−Pro (ΔM1−P80) lacking the NH2-terminal and collagen-like regions, is trimeric (31). The rSP-Ahyp−M1−Pro (ΔG8−P80) is a collagen deletion mutant containing the NH2 terminus, the neck region, and the CRD (29). The mutant rSP-Ahyp−Cys (ΔCys), which has Cys replaced by a serine, forms trimers but not larger oligomeric forms (29). The two CRD mutant proteins, rSP-Ahyp−K195A (E195A) and rSP-Ahyp−D215A (D215A), bear point mutations in amino acids predicted to contribute to coordination of a calcium ion involved in PL and carbohydrate binding (28). Although these SP-As have reduced ligand binding, they are isolated by mannose chromatography and consequently retain some carbohydrate binding activity (Fig. 1).

Captive Bubble Tensiometry—CBT assays were performed in triplicate, using 0.05 mg/ml samples diluted in buffer (0.150 mM NaCl, 2 mM Tris-HCl, 1.5 mM CaCl2). The effect of SP-A was tested by adding natural bovine or recombinant rat SP-A at 5% (w/w) relative to surfactant phospholipids. Samples were incubated in a shaking water bath for 1 h prior to measurements. All experiments were performed at 37 °C except those involving the recombinant rat SP-As because the lack of hydroxyprolines makes these proteins more stable at temperatures above 30 °C (32, 33). Therefore the effect of the recombinant SP-As was studied at 25 °C.

After filling the CBT chamber with a suspension of the desired surfactant in buffer, an air bubble, 5–8 mm in diameter, was introduced. The change in bubble shape was recorded to monitor the adsorption of the surface-active materials to the air-saline interface. After (γmin, mN/m) was attained, the chamber was sealed, and quasi-static or dynamic compression-expansion cycles were performed. Changes in bubble area were recorded during each individual experiment and the bubble shapes analyzed using custom designed software to calculate the surface tension of the film (34, 35).

For quasi-static experiments, the film was allowed to adsorb to γmin before cycling. Quasi-static compression-expansion curves were generated by increasing the pressure in the sample chamber by slowly turning the plunger 5–10° to generate ~10% changes in bubble volume per cycle. Following a 10° deflection, the film was allowed to relax and quasi-static cycles were conducted with an inter-cycle delay of 1.5 min on return to the original volume. Minimum surface tension (γmin) was established either as the lowest γ attainable without bubble clacking (observed as an instantaneous change to a small surface area with a higher γ) or near 1.0 mN/m. In the case of F-BLES, compression was limited to the 50% of the original surface area to avoid breaking the chamber by the high hydrostatic pressure.

For dynamic cycling, the surfactant was allowed to adsorb to near γmin, A quasi-static compression-expansion cycle was conducted to establish the surface area reduction required to attain γmin. Dynamic modes were implemented by cycling the bubble between 100 and 110% of the original surface area and this was used to achieve γmin at 30 cycles per minute.

The compressibility (C) and respreadability (R) of the film at a desired γ were calculated using (CRγ) = 1A/(dA/dγ) for the compression or expansion curve, respectively, where A is surface area and dA/dγ is the

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slope at a desired γ. C refers to the ability of the film to reduce γ during surface area reduction. This property represents the elasticity of the film, plus the insertion or reinsertion of phospholipid molecules into the expanding film. Insertion occurs through adsorption while reinsertion occurs from the surface-associated surfactant reservoir (2, 36).

**Statistical Analysis**—All experiments were performed at least three times with individual freshly prepared samples. Standard error of the mean was obtained from samples of n ≥ 3 or greater. Statistical comparisons were conducted using SPSS Inc. (Chicago, IL) software. Comparisons between two groups were made using paired Student’s t test and comparisons between larger groups were conducted by analysis of variants followed by a Tukey post-hoc test. Probability values below 0.05 are considered significant.

**RESULTS**

**Effect of Free Radical Exposure on Surfactant Phospholipids**—Both hypochlorous acid and the Fenton reaction were effective in oxidizing BLES, as shown by increased primary (conjugated dienes, Fig. 2A) and secondary lipoperoxidation products (MDA and HNE, Fig. 2B). The conjugated dienes formed during oxidation (Fig. 2A) were monitored by reading the absorbance at 235 nm after the exposure of BLES to oxidation. In the absence of oxidants (closed circles), in the presence of Fe²⁺/EDTA (closed triangles) or H₂O₂ (open circles), small, similar increases in A₂₃₅ were observed that remained relatively constant over the 2-h period shown in Fig. 2A. The levels did not change further over the remaining 3.5 h of the experiment (data not shown). In the presence of HOCl/OCl⁻ (open triangles), A₂₃₅ increased rapidly, reaching maximal levels at 20–30 min, after which it declined. Higher differences in A₂₃₅ were evident with the Fenton reaction compared with hypochlorous acid. Differences between the oxidation reactions and the control incubations disappeared at 60 min for BLES:HOCl/OCl⁻ and 90 min for BLES:Fenton. The decrease in absorbance, observed between 90 and 100 min, is consistent with the consumption of the susceptible unsaturated fatty acids and the production of secondary products.

The secondary peroxidation products formed during oxidation were measured after 24 h incubation as the content of MDA and HNE in the surfactant sample (Fig. 2B). The Fenton reaction, which acts through generation of reactive hydroxyl radicals (37), generated 4.5 times more MDA and HNE than the control while there was a 2.0-fold increase after reaction
The presence of the quaternary ammonia group in PC results in highly efficient detection. PEs at m/z 710–740 were detectable only to a very limited extent.

Major peaks observed for control BLES at the higher m/z ratios (500 to 850) were 16:0/16:0-PC (m/z 756.8), 16:0/16:1-PC (m/z 754.8), 16:0/18:1-PC (m/z 782.8), 16:0/18:2-PC (m/z 780.8), 16:0/18:0-PC (m/z 784.8), and 16:0A, 16:0-PC (A indicates al- kyl) (m/z 742.7) (Fig. 3). Phospholipids with fatty acids of higher molecular mass also contribute to the spectrum, namely 18:0/18:2-PC or 18:1/18:1-PC (m/z 808.7) and 18:0/18:1-PC (m/z 810.7), 18:0/18:0-PC (m/z 812.7) and 16:0/20:4-PC (m/z 824.8).

It is important to note that ESI-MS analysis determines molecular mass/charge and not PL molecular structure, but interpretation in the context of known surfactant composition strengthens the molecular species analysis in terms of fatty acyl distribution. For example, the ion detected at m/z 808 is probably a mixture of 18:3/18:1-PC and 18:0/18:2-PC because these species have identical molecular masses, but the ion at m/z 756 must be 16:0/16:0-PC because other possibilities, such as 14:0/18:0-PC, are not present in surfactant at appreciable levels (6, 7, 9, 40, 41). In addition, although the location of specific unsaturated fatty acids cannot be assigned by ESI-MS, it is known that unsaturated fatty acids predominantly occupy the sn-2 position (9).

Oxidation by either of the systems led to the appearance of new species, indicated by ions that appear between m/z 700 and 900, such as 16:0/peroxy 16:1 PC (m/z 786.8) and 18:0/peroxy 18:1 PC (m/z 798.1), or 16:0/peroxy 18:1 PC (m/z 814.8) and 18:0/ peroxy 18:2 PC (m/z 840.9). The emergence of these new species was accompanied in some cases by a decrease in the intensity of the peaks of their corresponding unsaturated PLs (Fig. 3B; Table I). The Fenton reaction, as was expected, generated greater increases in these hydroperoxide products and larger reductions in the parent species. The effects of hypochlorite on the peroxide formation from PL moieties were weaker.

The most intense peaks in the low mass region of BLES spectra corresponded to lyso-PCs, including 16:0 lyso-PC (m/z 518.5) as well as 18:1 lyso-PC (m/z 544.6), which were detected with low intensity. Oxidation of BLES by HOCl/OCl or Fenton reaction led to increased generation of lyso-PC. That the tendency for the formation of lyso-PL was a consequence of the PL oxidation was reinforced by the fact that the Fenton reaction enhanced the appearance of lyso-PLs to a greater extent than HOCl/OCl.

ESI-MS negative mode analysis of BLES revealed palmitoyl, oleoyl-PG, and DPPG as the major peaks with palmitoyl, palmitoleoyl-PG, stearoyl, linoleoyl + oleoyl, oleoyl-PG, and stearoyl, oleoyl-PG at significant levels (Fig. 4A, Table I). Small amounts of unsaturated species of PI and PS were also detected in negative-mode ESI-MS but not quantified.

BLES oxidation with HOCl/OCl or the Fenton reaction resulted in the appearance of peroxyl derivatives of palmitoyl, palmitoleoyl-PG, and palmitoyl, oleoyl-PG. Fenton oxidation also resulted in a decline in dienoic and, to a lesser extent, monoenoic species.

As in the case of the neutral PCs, significant levels of shortened chain PGs were not detected. Oxidation did result in a relative increase in lyso-PGs, particularly with the Fenton reaction. Of additional interest was the increase in free fatty acids, again to a higher degree with the Fenton reaction. The ESI-MS intensity yield of lyso-PLs and fatty acids relative to PLs under our conditions has not been established, although it is apparent free fatty acids can “fly” to a greater extent than PLs. Consequently, with these particular compounds, these studies provide only a qualitative indication of the underlying chemistry.

Effect of Free Radical Exposure on Surfactant Function—The
effects of free radical exposure on surfactant function were studied with the CBT (16, 35). With this apparatus, the ability of a surfactant to adsorb to the interface is assessed, after which surface area compression-expansion isotherms are performed, either quasi-statically or dynamically, to evaluate the surface properties of the sample under conditions that mimic...
alveolar mechanics during respiration. Changes in bubble shape are analyzed to obtain the surface tension of the film during surface area manipulation and to generate isotherms. Figs. 5–7 represent adsorption times and quasi-static and dynamic isotherms. Oxidation by exposure to either of the two free radical systems resulted in retardation of initial film formation such that times to attain equilibrium γ (23 mN/m) were increased from 10 min (control) to 120 min (H-BLES) and 140 min (F-BLES). Addition of SP-A to oxidized BLES improved the adsorption rates to levels similar to the control BLES (Fig. 5).

Although breathing is a dynamic process, quasi-static cycling experiments provide important information about film behavior, by making it possible to study re-spreading and/or re-
adsorption. During the inter-cycle delay, phospholipids reinsert into the film, promoting a drop in \( \gamma \) allowing the film to return to near equilibrium. This phenomenon can be studied only in the quasi-static mode. Quasi-static compression of BLES (control) adsorbed at 0.05 mg/ml results in an initial highly compressible “squeeze-out” plateau to \( \sim 18 \) mN/m, followed by a steep decline to near 0 mN/m. Compressibility at \( \gamma_{20} \) (\( C_{20} \)) was 0.078 \pm 0.020. As reported previously, control BLES adsorbed at 50 \( \mu \)g/ml required \( \sim 60\% \) surface area reduction to lower \( \gamma \) from equilibrium (23 mN/m) to near zero during the first compression (16, 35, 42).

Upon surface area expansion of compressed BLES films, \( \gamma \) increases sharply with a respreadability at \( \gamma_{10} (R_{10}) \) of 0.011 \pm 0.002, which improves above 15 mN/m to a \( R_{10} \) of 0.800, mean \( \pm \) S.E. Surface tension remains close to equilibrium during the latter stages of expansion until the original surface area is reached. The difference in respreadability during film expansion relative to compressibility during surface area reduction resulted in a moderate hysteresis. By the fifth quasi-static compression, the squeeze-out plateau was barely evident. Because of this alteration in surface properties, \( \gamma \) could be reduced to near zero with \( \sim 20\% \) surface area reduction. Compressibility at \( \gamma_{10} \) was improved to 0.009 \pm 0.000, although with cycles 1 and 5, there is an increase in compressibility at very low surface tensions, resulting in a slight hook to the isotherm. Upon bubble expansion, \( \gamma \) increases sharply, although \( R_{10} \) was slightly higher than the 0.011 observed during the first cycle. The increase in respreadability at \( \gamma_{10} \) indicates an improved elasticity of the film, because neither insertion of PL through adsorption from bulk phase nor lipid reinsertion from the surface-associated reservoir should occur at \( \gamma \) values below equilibrium (2).

The addition of SP-A, at a wt ratio of 5% PL, resulted in a

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**TABLE I**

Percent of major PC and PG species present in BLES, F-BLES, and H-BLES

| Lipid       | m/z  | % of major PCs |
|-------------|------|----------------|
|             |      | BLES           | F-BLES | H-BLES |
| 14:0, 0:0   | 700  | 0.43 \pm 0.05  | 0.37 \pm 0.00 | 0.31 \pm 0.00 |
| 14:0, 16:1  | 726  | 8.57 \pm 0.10  | 9.28 \pm 0.74 | 5.85 \pm 0.09 |
| 16:0, 16:1  | 740  | 0.53 \pm 0.00  | 0.51 \pm 0.00 | 0.35 \pm 0.00 |
| 16:0, 16:0  | 742  | 4.13 \pm 0.10  | 6.39 \pm 0.87 | 2.84 \pm 0.04 |
| 16:0, 16:1  | 754  | 12.05 \pm 0.25 | 9.34 \pm 0.12 | 11.77 \pm 0.19 |
| 16:0, 16:0  | 756  | 37.54 \pm 0.95 | 40.81 \pm 0.62 | 35.68 \pm 0.84 |
| 16:0, 18:1  | 768  | 1.94 \pm 0.29  | 3.17 \pm 0.69 | 2.08 \pm 0.03 |
| 16:0, 18:2  | 780  | 4.22 \pm 0.48  | 0.90 \pm 0.27 | 4.04 \pm 0.06 |
| 16:0, 18:1  | 782  | 21.22 \pm 0.27 | 18.43 \pm 1.09 | 23.79 \pm 0.40 |
| 16:0, 18:0  | 784  | 1.81 \pm 0.06  | 1.79 \pm 0.11 | 3.27 \pm 1.83 |
| 18:0, 18:2  | 794  | 0.51 \pm 0.09  | 0.51 \pm 0.05 | 0.68 \pm 0.01 |
| 18:0, 18:1  | 796  | 1.27 \pm 0.11  | 3.61 \pm 0.39 | 1.53 \pm 0.02 |
| 18:1, 18:2  | 806  | 1.10 \pm 0.12  | 0.47 \pm 0.01 | 0.65 \pm 0.01 |
| 18:0, 18:1  | 808  | 2.53 \pm 0.10  | 1.10 \pm 0.22 | 3.26 \pm 0.05 |
| 18:0, 18:0  | 810  | 1.71 \pm 0.47  | 1.89 \pm 0.29 | 2.72 \pm 0.04 |
| 18:0, 18:0  | 812  | 0.45 \pm 0.11  | 1.38 \pm 0.12 | 1.09 \pm 0.01 |

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**FIG. 5** Effect of bovine SP-A on the initial film formation by oxidized BLES. Bar graph represents the time required for the film to achieve equilibrium surface tension. All measurements were performed in triplicate at 37 °C for 0.05 mg/ml samples reconstituted in buffer A, as indicated under “Experimental Procedures.” The effect of SP-A was tested by adding natural bovine SP-A at 5% (w/w) relative to surfactant PLs. Comparisons among the samples were conducted using Tukey’s studentized range (highly significant difference) test. Letters represent comparisons among samples. Means with the same letter are not significantly different.

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Values are mean \( \pm \) S.E. for mol % (\( n \geq 3 \)). We have used dimyristoyl-sn-glycero-3-phosphocholine, and dimyristoyl-sn-glycero-3-phosphoglycerol, as internal standards for PC and PG, respectively. These synthetic lipids are not present in biological samples. Molecular species are designated as \( n:a,m:b \), where \( n,a,m,b \) are number of carbons, and \( a \) and \( b \) are number of double bonds for the fatty acids.
slight improvement in film surface properties during the first cycle (Fig. 6, top right, BLES:SP-A). The squeeze-out plateau observed during the initial stage of film compression was reduced so that <40% surface area reduction was sufficient to reduce γ to near zero. Compressibility and respreadability at γ<sub>10</sub> were also slightly improved compared with those in the absence of SP-A, and hysteresis was reduced compared with cycle 1 in the absence of SP-A. By the 5<sup>th</sup> compression-expansion cycle, little difference could be noted between samples with and without SP-A. These observations reinforce the concept that SP-A is not essential during normal surface activity manipulations.

The addition of Fe<sup>2+</sup>/EDTA alone, or H<sub>2</sub>O<sub>2</sub> alone, had no observable effect on quasi-static and dynamic profiles for any of the samples studied (data not shown). Incubation of surfactant with HOCl/OCl (Fig. 6, middle panel) or complete Fenton reagents (Fig. 6, bottom panel) caused a reproducible deterioration in surface activity, resulting in significantly higher γ<sub>max</sub> and γ<sub>min</sub>. With H-BLES, the squeeze-out plateau during the first compression was similar to or smaller than control. However, C<sub>10</sub> was significantly increased to 0.029 and the lowest γ<sub>min</sub> attainable was elevated to ~5 mN/m and increased further during cycling. Attempts at lowering γ further through surface area reduction resulted in “clicking.” Clicking refers to an almost instantaneous decrease in bubble surface area, resulting in an increase in γ that may be due to the loss of PL from the bubble surface (34). During the initial surface expansion with H-BLES, γ increases sharply to ~25 mN/m. This indicates that, compared with control, film elasticity and respreading are extremely poor. Whether the decrease in slope at γ ~ 25 mN/m results from PL respreading or adsorption of surfactant from the bulk phase cannot be concluded directly from the expansion curve. However, it is clear from the adsorption studies that adsorption is slow at this particular γ, suggesting that respreading is the major mechanism. As a result of the poor respreadability, γ rises to ~40 mN/m. Poor insertion-reinsertion of PL molecules into the expanding film results in a large hysteresis. Because of the poor PL reincorporation, compression 5 initiates with γ ~ 35 mN/m. After a relatively sharp decline, a small squeeze-out plateau initiating at γ = 25 mN/m is observed, with a shape similar to that of the first compression cycle. The C<sub>10</sub> was 0.0048 ± 0.001, which is superior to that noted with the first compression. However, the film remains unstable and cannot achieve γs below 10 mN/m. R<sub>10</sub> during surface area reduction increases to 0.003 and an improvement in lipid incorporation is noted at γ ~ 15 mN/m, so the respreadability at γ<sub>20</sub> improves to 0.037 ± 0.01. However, little further change is observed in respreadability at γ ~ 25.
and $\gamma$ increases almost linearly to $\sim 40 \text{ mN/m}$, again suggesting poor PL insertion even above equilibrium surface tension. Nevertheless, because of the improved characteristics during film compression and expansion, minimal hysteresis is evident.

Addition of bovine SP-A to the HOCl/OCl-treated sample leads to a marked improvement in film performance. The length of the squeeze-out plateau is markedly reduced so that $\gamma$ decreases more readily during compression (Fig. 6, middle, right panel). The presence of SP-A improves the ability to achieve low surface tensions, although with the initial compression there is a slight tendency toward higher compressibility. Inclusion of SP-A also improves respreadability at $\gamma_{10}$ to 0.001 and PL reinsertion increases markedly at $\gamma = 15$, so that $\gamma_{\text{max}}$ is lowered from $>40$ to $<30 \text{ mN/m}$ at maximum surface area. The overall compression-expansion pattern approaches that of BLES:SP-A (Fig. 6, upper right), although hysteresis remains somewhat increased.

The ability of SP-A to restore surface activity with H-BLES is also evident for the 5th quasi-static cycle, which has a shape close to that of BLES:SP-A, except that enhanced PL incorporation into the monolayer during expansion is not observed until equilibrium $\gamma$ is attained, leading to a slight increase in hysteresis.

Subjecting BLES to the Fenton reaction (Fig. 6, lower left panel) results in a greater deterioration in surface activity than HOCl/OCl. The squeeze-out plateau is broadened considerably so that a 50% reduction in surface area results in lowering $\gamma$ to only $\sim 10 \text{ mN/m}$. Respreadability is not hampered as much as with the HOCl/OCl treatment so that, although film expansion initiates from a higher $\gamma$, the final $\gamma$ attained remains below 40 mN/m. This $\gamma$ is still significantly higher than that observed with BLES alone. By the 5th cycle, there is a decrease in the squeeze-out plateau so that $\gamma_{\text{min}}$ remains at $\sim 10 \text{ mN/m}$, despite being initiated at $\gamma = 38 \text{ mN/m}$. However, due the high compressibility during adsorption and poor PL insertion/reinsertion above equilibrium, hysteresis remains high.

As in the case of H-BLES, addition of bovine SP-A to F-BLES results in a large improvement in surface activities (Fig. 6, lower right panel). The sample recovers its ability to attain low surface tensions with $\sim 60\%$ surface area reduction in the first quasi-static cycle and improved respreadability is also observed. A further small improvement in surface properties is observed during the 5th cycle. Nevertheless, hysteresis remains somewhat higher than observed with control BLES:SP-A.

Dynamic cycling of surfactant films in the CBT more closely mimics normal respiratory compression-expansion within the

![Figure 7](http://www.jbc.org/)

**FIG. 7.** Effect of bovine SP-A on dynamic compression-expansion cycling of oxidized surfactant films. Adsorbed films were cycled at a rate of 30 cycles/min between 100 and 110% of the original bubble surface area and the area required to achieve $\gamma_{\text{min}}$ during the first quasi-static compression. Overcompression of the bubbles at low $\gamma$ was avoided. The data are the average of three separate experimental samples. Treatment with HOCl/OCl (middle left panel, H-BLES) or Fenton reagents (bottom left panel, F-BLES) caused a reproducible change in surface activity, with significantly higher minimum and maximum surface tensions. The effects of SP-A addition to the surfactants is shown in the right panels.
alveolar airspace. For these experiments, adsorbed films were cycled at a rate of 30 cycles/min between 100 and 110% of the original bubble surface area and the area required to achieve \( \gamma_{\text{min}} \) during the first quasi-static compression. Overcompression of the bubbles at low \( \gamma \) was avoided. The data were averaged from three separate experiments, and isotherms for cycles 1 and 21 are shown in Fig. 7. In general, the overall results obtained from dynamic cycling were similar to those obtained in quasi-static cycling. However, the \( \gamma_{\text{max}} \) levels observed during film expansion were higher. This was more evident with the hypochlorous oxidized samples; the \( \gamma_{\text{max}} \) values attained during expansion increased to \( \sim 60 \) mN/m, and a marked deterioration was observed on consecutive dynamic cycles. These observations are consistent with the reduced re-spreadability observed with \( \text{HOCI}'/\text{OCl} \) during quasi-static cycling. Addition of natural bovine SP-A to the oxidized BLES restored the BLES ability of both oxidized samples to attain surface tensions near zero with less surface area reduction during the film compression and remain close to equilibrium during the film expansion (middle and bottom right panels). The overall pattern of compression-expansion isotherms of these samples approached those for non-oxidized BLES.

**Domains of SP-A Required for Enhancing the Surface Properties of Oxidized Surfactant**—The structural basis for the restorative effects of SP-A on the surface activity of oxidized BLES was assessed using a set of six mutant recombinant rat SP-As produced in insect cell systems. These include the wt rec protein, and mutant recombinant SP-As containing a C6S substitution that disrupts the critical NH\(_2\)-terminal interchain disulfide bond (C6S), a deletion of the collagen domain (\( \Delta \)G8-P80), a deletion of the collagen-like domain and the NH\(_2\)-terminal segment (\( \Delta \)N1-P80), and point mutations in residues that are critical for coordination of calcium, carbohydrate, and PL binding (E195A and D215A). Cross-linking analyses and gel filtration chromatography under physiologic ionic strength conditions have revealed that the \( \Delta \)N1-P80 protein is trimeric (31), the \( \Delta \)G8-P80 and C6S proteins are predominantly trimeric and hexameric, and the wt rec, E195A, and D215A proteins are composed of trimers, hexamers, and nonamers (not shown).

Bar graphs summarizing the adsorption times required to achieve equilibrium \( \gamma \) generated for control BLES and oxidized BLES alone and in the presence of native rat and the six rSP-As, are presented in Fig. 8. Overall effects of SP-As on BLES oxidized by \( \text{HOCI}'/\text{OCl} \) or the Fenton reaction were very similar. Native rat SP-A was comparable with bovine SP-A in improving the adsorption time of oxidized BLES (Figs. 5 versus 8). The recombinant wild type also decreased the adsorption rates of oxidized BLES, albeit less effectively than the natural SP-A, from \( \sim 110 \) min for control BLES to \( \sim 35 \) min for F-BLES and \( \sim 45 \) min for H-BLES. The collagen deletion mutant \( \Delta \)G8-P80 (31) was the most active in restoring a rapid adsorption time to oxidized BLES, whereas \( \Delta \)N1-P80 (31), which differs from the \( \Delta \)G8-P80 only in the absence of the NH\(_2\)-terminal segment and interchain disulfide bonds, was ineffective in promoting initial film formation. Isolated disruption of the C6S interchain disulfide bond (29) also reduced restoration of adsorption, as did point mutations replacing amino acids thought to be important in calcium and carbohydrate binding (E195A and D215A) (43).

Investigation of the ability of rat native and recombinant SP-As to reverse the effects of \( \text{HOCI}'/\text{OCl} \) and the Fenton reaction on BLES was extended to expansion-compression cycling of surfactant films under quasi-static and dynamic modes (Fig. 9, A and B). As in the case of film formation, the ability of the various SP-As to counteract the effects of BLES oxidation on \( \gamma_{\text{max}} \) and \( \gamma_{\text{min}} \) by either system showed a number of simi-
larities and so these results will be described together. Recombinant wild-type and ΔG8-P80 were as effective as native rat SP-A in restoring the ability of oxidized BLES to achieve $\gamma_s$ below 5 mN/m during the initial film compression. This activity was maintained throughout successive cycles, undergoing either quasi-static or dynamic manipulation. However, the ability of these SP-As to restore $\gamma_s$ reduction to near 0 mN/m was better with H-BLES than F-BLES.

The ΔN1-P80 was much less effective than ΔG8-P80 in restoring surface properties to oxidized BLES, highlighting the importance of the NH2-terminal segment and interchain disulfide bond to this activity. Furthermore, the $\gamma_{\text{min}}$ and $\gamma_{\text{max}}$ values increased during quasi-static and dynamic cycling with ΔN1-P80, as occurs with oxidized BLES by itself. This effect was more evident with H-BLES where ΔN1-P80 exhibited only a small effect on $\gamma_{\text{min}}$ during quasi-static cycling. Nevertheless, samples containing ΔN1-P80 demonstrate significantly better $\gamma_{\text{min}}$ and $\gamma_{\text{max}}$ values with F-BLES. C6S restored $\gamma_{\text{min}}$ to low values with H-BLES but had less effect on $\gamma_{\text{max}}$ particularly with dynamic cycling. The lack of effectiveness of C6S in restoring $\gamma_{\text{max}}$ was also apparent in experiments using F-BLES.

The E195A and D215A mutations reduced the restorative activity of SP-A on the surface properties of F-BLES and H-BLES during quasi-static or dynamic cycling, although both mutant recombinant SP-As improved the ability of the oxidized surfactant to attain lower $\gamma_{\text{min}}$ and $\gamma_{\text{max}}$ values. E195A protein tended to show better overall effects, but the differences were small.

Comparison of the percentage of surface area compressions required to achieve $\gamma$ near zero during quasi-static cycling (Fig. 10, A and B) provides a highly discriminative method for grading the abilities of native and rSP-As to restore the surface activity of oxidized BLES. In some cases such as oxidized BLES control, it was necessary to extrapolate the experimental values to obtain the values plotted. Addition of native rat SP-A restored the % area reductions required for H-BLES to attain values similar to control BLES, but recovery was less complete with F-BLES. The rat recombinant wild-type and the collagen-deletion mutant ΔG8-P80 were as active as rat SP-A in re-establishing the ability of BLES oxidized by either system to achieve low $\gamma$s during quasi-static cycling. With H-BLES little or no protective effect was observed with rSP-As containing NH2-terminal segment mutations that affect oligomeric assem-
bly (ΔN1-P80, C6S) or wild-type CRD (E195A, D215A). Except for ΔN1-P80, all of these mutants showed intermediate abilities to restore deterioration induced by the Fenton reaction. Collectively, these data indicate that the CRD and the Cys6 interchain disulfide bond are critical for the salutary effects of SP-A on the surface activity of oxidized surfactant.

**DISCUSSION**

**Effect of Reactive Oxygen Species on Pulmonary Surfactant**—Considerable evidence accumulated through extensive in vitro and in vivo investigations has shown that reactive oxygen species such as HOCl/OCl, H2O2, superoxide radical, and singlet oxygen radical, released in the alveolar lining layer by activated inflammatory cells, can react with and inhibit pulmonary surfactant (44, 45). The present studies confirm that exposure of BLES, a therapeutic modified natural surfactant, to HOCl/OCl and the Fenton reaction at concentrations employed by others with other surfactants and considered in the high physiological range (21–23) generates primary conjugated dienes (Fig. 2A), secondary lipoperoxidation (MDA, HNE, Fig. 2B) products despite the relatively low levels of polyunsaturated PL. In contrast to low and high density lipoproteins, where prolonged lag phases in diene formation are observed (46, 47), peroxidation of BLES occurs rapidly. This can be attributed to the lack of endogenous antioxidants, including ascorbic acid and α-tocopherol, which are removed from BLES during processing (9).

**ESI-MS Analysis of Control and Oxidized BLES**—ESI-MS is a highly sensitive technique that has recently been applied to the study of PL molecular species in surfactant from a number of animal species, including human (reviewed in Ref. 6). Applying ESI-MS to control and oxidized BLES demonstrate increases in the levels of hydroxide, hydroperoxide, and other truncated PCs and PGs, consistent with previously reported studies with low and high density lipoproteins (48–50). Higher levels of PL peroxidation products were observed with the Fenton reaction than with HOCl/OCl, but with both systems the amounts were relatively low (Table I). Interestingly, ESI-MS detected lyso-PC, lyso-PG, and free fatty acids (Figs. 3 and 4). Precise ion yields for these derivatives must still be established, but it is evident that these lipid species are markedly elevated over control BLES, particularly with the Fenton reaction. Lyso-PCs can also be generated through a phospholipase A-independent mechanism during myeloperoxidase-mediated PC degradation (38).

The positive and negative ESI-MS spectra presented here demonstrate that reaction of BLES with either HOCl/OCl or Fenton reaction results in the degradation of PCs and PGs. Phospholipids containing two or more double bonds appear particularly susceptible, but it is evident that monoenoic species are also reactive. Fenton reactions generated higher levels of degradation products, consistent with a difference between this system and hypochlorous acid. In contrast, alkyl-ether-PCs were relatively stable, consistent with previous reports on the ability of these lipids to resist oxidation. It has been suggested that plasmenyl ether-PCs may serve to protect surfactant in the alveolar space (51). Taken together with the results from positive mode ESI-MS, it is clear that both hypochlorous acid and the Fenton reaction attack unsaturated PCs and PGs. These observations provide further evidence indicating fundamental differences in the manner in which HOCl/OCl and the
Fenton reaction destroy the ability of pulmonary surfactant to maintain surface active properties consistent with normal lung function.

**Interfacial Effects of Surfactant Oxidation and Reversal by SP-A**—Exposing BLES to reactive oxygen species led to a marked inhibition in surface active properties assessed as (i) a reduced ability of the reacted surfactants to adsorb rapidly to the surface, (ii) elevated $\gamma_{\text{min}}$ and $\gamma_{\text{max}}$ during quasi-static and dynamic cycling, (iii) increases in the surface area reduction required to achieve $\gamma_{\text{min}}$, and (iv) a marked progressive deterioration in these functions upon cycling, relative to control non-reacted BLES.

The two systems produced markedly different effects during compression-expansion cycling. HOCl/OCI treatment did not affect film compressibility during quasi-static compression but induced film instability at low $\gamma_s$. It also resulted in poor respreadability below and above $\gamma_{\text{eq}}$ during film expansion. The Fenton reaction greatly increased compressibility at $\gamma_{\text{eq}}$ and also limited PL insertion-reinsertion above equilibrium $\gamma$. Similar overall differences were observed during dynamic cycling. The basis for this dissimilarity in behavior is presently unknown. However, it appears possible that they could arise from differential effects on the two low molecular weight hydrophobic proteins (SP-B and SP-C) and the phospholipids. Studies on the oxidation of low density lipoproteins and high density lipoproteins have shown that HOCl/OCI is effective in oxidizing proteins, whereas the Fenton reaction more specifically oxidizes lipids containing unsaturated fatty acyl groups (48, 52).

Addition of Fenton-oxidized palmitoyl, linoleoyl-PC, or palmitylproteins, whereas the Fenton reaction more specifically oxidized proteins than surfactant from normal controls (32). Pressure volume relationships of lungs from SP-A $^{−/−}$ and SP-A $^{+/−}$ mice were similar to those of SP-A $^{+/+}$ mice. The discrepancy between the in vivo and in vitro properties of surfactants containing $\Delta G8$-$P80$ may be related to the temperatures at which the biophysical surfactant assays were performed (37 versus 25 °C).

In general, the CRD and NH$_2$-terminal domain mutations studied demonstrated partial activities relative to wt rec in restoring the biophysical activities with either oxidized BLES. A notable exception was $\Delta N1$-$P80$, which retained reasonable activity in achieving low $\gamma_{\text{min}}$ during cycling with H-BLES, but poorer activity with F-BLES. Altering C6S and removing the entire N-terminal region disrupts oligomeric assembly of trimeric subunits, resulting in a predominance of trimers (53). These results strongly suggest that the oligomeric structure is important for the salutary effects of SP-A on surface activity of BLES oxidized either by HOCl/OCI or the Fenton reaction.

Although initially controversial, considerable evidence has accrued indicating that the CRD of SP-A possesses both the carbohydrate-binding and PL-binding motifs (20). The two CRD mutants E195A and D215A used here reportedly lack the ability to bind or aggregate PL liposomes and exhibit reduced binding of carbohydrate beads or calcium (28). Nevertheless, some carbohydrate binding activity must remain, because the SP-A and SP-C are purified on the basis of calcium-dependent binding on mannose columns. CRD mutants E195A and D215A possessed intermediate activity in reducing the effects of oxidation by either system. However, these rSP-A tended to show poorer activity toward H-BLES, reinforcing the notion that hypochlorous acid and the Fenton reaction have different effects. The intermediate effects suggest that some PL binding activity is also retained. PL-binding and carbohydrate-binding motifs of SP-A have not been precisely mapped and crystallographic analysis will be necessary to define these regions. Nevertheless, it is clear that considerable overlap occurs between these two regions, because the tandem mutations E195Q,R197D disassociate the lipid binding and aggregating functions of SP-A, and convert SP-A carbohydrate binding specificity from a preference for mannose to galactose (26).

It will be important to determine whether the effects observed here derive from direct interactions between SP-A and surfactant lipids, particularly DPPC, or involve cooperative interactions with other surfactant proteins. Although SP-A has only slight effects in enhancing DPPC adsorption by itself, it can interact with DPPC monolayers to improve compressibility with such films (54, 55). SP-A also improves compressibility with DPPC/cholesterol spread films, possibly by reducing DPPC/cholesterol interactions and generating aggregated DPPC domains. SP-A binds DPPC ripple phase and, in the presence of DPPC, can form long strands that could contribute to tubular myelin formation (53, 56). SP-A binds to the liquid condensed phase of spread surfactant monolayers (57). Because SP-B and SP-C preferentially locate in liquid expanded phases, this would suggest that SP-A and SP-B could occupy different interchains disulfide bond of SP-A are critical for restoring pre-oxidation surface properties to H-BLES and F-BLES, and that the collagen-like region of SP-A is dispensable for the function.

Previous studies using the pulsating bubble surfactometer demonstrated that $\Delta G8$-$P80$ improved the ability of BLES at low concentrations to achieve low $\gamma_{\text{min}}$ values on the pulsating bubble surfactometer (29). However, surfactant isolated from transgenic mice engineered to express $\Delta G8$-$P80$ in the lungs of SP-A null (SP-A $^{−/−}$) mice showed high equilibrium $\gamma$, $\gamma_{\text{min}}$, and $\gamma_{\text{max}}$ values and were more susceptible to inhibition by serum proteins than surfactant from normal controls (32). Pressure volume relationships of lungs from SP-A $^{−/−}$ and SP-A $^{+/−}$ mice were similar to those of SP-A $^{+/+}$ mice.
regions of the surfactant monolayers. These observations would indicate that SP-A could have independent effects on oxidized surfactant films.

In contrast, there is considerable evidence that SP-A can have cooperative effects with SP-B. SP-A can enhance surfactant adsorption, reduce film compressibility, increase respreadability, and limit the surface area reductions required to attain low yss with surfactant extracts and model surfactant systems containing SP-B but not with systems containing only SP-C (5, 16). SP-A and SP-B, but not SP-C, are required for reconstitution of tubular myelin and SP-A can contribute to the formation and maintenance of large aggregates (5, 58). Whether SP-A reversal of the effects of surfactant oxidation requires SP-B or SP-C must be determined through reconstitution studies. In addition to counteracting effects of surfactant PL oxidation, SP-A can also counteract inhibitory effects of non-surfactant proteins (17, 18). Studies comparing SP-A domain and structural requirements for these two prosurfactant activities are currently in progress.

Summary and Conclusions—The present study examined the biochemical and biophysical effects of free radical exposure on a lipid extract surfactant, BLES. BLES was oxidized using hypochlorous acid or the Fenton reaction. After oxidation the surfactants displayed biochemical modifications, and primary and secondary lipoperoxidation products were identified. ESI-MS analysis of oxidized BLES revealed the presence of hydroxy and hydroperoxy derivatives of unsaturated PCs and PGs, especially with the Fenton reaction. Oxidation led to an increase in lyso-PC and lyso-PG and free fatty acids, again to a greater extent with the Fenton reaction. Surface activity was markedly impaired. Oxidation by either system greatly hampered surfactant adsorption and the ability to attain low yss during film compression. Hypochlorous acid treatment had little effect on compressibility at yss and but increased film instability, limiting the ability to attain yss below 7 mN/m during cycling. Exposure to HOCl/OCI markedly hampered respreadability during film expansion, particularly at yss and at yss above equilibrium. Exposing BLES to the Fenton reaction led to increased compressibility at yss such that the oxidized surfactants could not reduce yss to below 10 mN/m, even with large compression ratios. These results show that, despite similar apparent effects on PLs, BLES oxidation by HOCl/OCI or Fenton reaction may display distinct effects on the surface properties of surfactant films. Regardless of the difference in the effects on biophysical function, addition of bovine or rat SP-A restored the ability of oxidized surfactants to attain yss near zero during film compression and to spread during film expansion. The mechanisms by which SP-A rescues surfactant function are not understood. Mutagenesis studies demonstrate that an intact CRD and Cys<sup>8</sup> disulphide bond are critical for restoration of the surface activity of oxidized surfactant and indicate that the phospholipid binding properties and oligomeric assembly of SP-A are essential for this function.

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REFERENCES
1. Keough, K. M. W. (1998) in Lung Surfactant: Cellular and Molecular Processes (Rooney, J. A., ed) pp. 1-27, R. G. Landes Company, Austin, TX.
2. Possmayer, F. (2003) in Fetal and Neonatal Physiology (Polin, R. A., and Fox, W. W., eds) W. B. Saunders Company, Philadelphia, PA, in press.
3. Crouch, E., and Wright, J. R. (2001) Annu. Rev. Physiol. 63, 521-554.
4. McCormack, F. X., and Whittet, J. A. (2002) in J. Clin. Invest. 109, 707-712.
5. Possmayer, F., Nag, K., Rodriguez, K., Qanbar, R., and Schurch, S. (2001) Comp. Biochem. Physiol. A Mol. Integr. Physiol. 129, 209-220.
6. Postle, A. D., Heely, E. L., and Wilton, D. C. (2001) Comp. Biochem. Physiol. A Mol. Integr. Physiol. 129, 65-73.
7. Schiller, J., Hammerschmidt, S., Wirtz, H., Arnhold, J., and Arnold, K. (2001) Chem. Phys. Lipids 112, 67-79.
8. Veldhuizen, R., Nag, K., Orgeig, S., and Possmayer, F. (1998) Biochem. Biophys. Acta 1408, 90-108.
9. Yu, S., Harding, P. G., Smith, N., and Possmayer, F. (2003) Lipids 18, 522-529.
10. Gunther, A., Siebert, C., Schmidt, R., Zeiger, S., Grimminger, F., Tabat, M., Tempferfeld, B., Walmrath, D. M. H., and Seeger, W. (1996) Am. J. Respir. Crit. Care Med. 153, 176-184.
11. Lewis, J. F., and Veldhuizen, R. A. (2000) Am. Rev. Respir. Dis. 167, 613-616.
12. Smith, W., Sedgewick, R., Facione, J., Fabel, H., and Siegel, J. (1997) Eur. Respir. J. 10, 482-491.
13. Wright, S. M., Hocky, P. M., Enhorning, G., Strong, P., Reid, K. B., Holgate, S. T., Djukanovic, R., and Postle, A. D. (2000) J. Appl. Physiol. 89, 1292.
14. Warburton, D., Buckley, S., Cosio, L., and Forman, H. J. (1989) Am. J. Physiol. 257, L217-L220.
15. Ullson, C., Harrison, K., Ahmad, C. B., Ahmad, S., White, C. W., and Murphy, R. C. (2002) Chem. Res. Toxicol. 15, 896-906.
16. Rodríguez-Capote, K., Nag, K., Schurch, S., and Possmayer, F. (2001) Am. J. Physiol. Lung Cell Mol. Physiol. 281, L231-L242.
17. Cockshutt, A. M., Wetz, J., and Possmayer, F. (1999) Biochemistry 39, 8424-8429.
18. Venkitaraman, A. R., Hall, S. B., Whitsett, J. A., and Notter, R. H. (1999) Chem. Phys. Lipids 20, 185-194.
19. Holm, B. A., Venkitaraman, A. R., Enhorning, G., and Notter, R. H. (1999) Chem. Phys. Lipids 20, 185-194.
Pulmonary Surfactant Protein-A (SP-A) Restores the Surface Properties of Surfactant after Oxidation by a Mechanism That Requires the Cys^6 Interchain Disulfide Bond and the Phospholipid Binding Domain
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