Surfactant lipid peroxidation damages surfactant protein A and inhibits interactions with phospholipid vesicles

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Abstract **The goal of these studies was to examine the effect of lipid peroxidation (LPO) on the function of surfactant protein A (SP-A). First, the optimal dialysis conditions for quantitative removal of EDTA and redoxactive metals from reagents were established. Surfactant phospholipids were incubated with free radical generators in the absence or presence of the SP-A or with BSA as a control. We found that SP-A inhibited copper-initiated LPO to an extent similar to BSA ($P < 0.05$). Exposure of SP-A to LPO was associated with an increase in the level of SP-A-associated carbonyl moieties and a marked reduction in SP-A-mediated aggregation of liposomes. LPO initiated by an azo-compound also resulted in enhanced protein oxidation and markedly inhibited SP-A-mediated liposome aggregation. The kinetics of aggregation of auto-oxidized and nonoxidized liposomes by nonoxidized SP-A was similar, suggesting that SP-A has similar affinities for oxidized and nonoxidized lipids. Oxidative inactivation of SP-A did not occur upon direct incubation of the protein with malondialdehyde alone. We conclude that exposure of SP-A to LPO results in oxidative modification and functional inactivation of SP-A by phospholipid radicals.**—Kuzmenko, A. I., H. Wu, J. P. Bridges, and F. X. McCormack. **Surfactant lipid peroxidation damages surfactant protein A and inhibits interactions with phospholipid vesicles.** ***J. Lipid Res.*** **2004.** 45: **1061–1068.**

Supplementary key words oxidation • radical • antioxidant • oxidative stress • ethylenediaminetetraacetic acid • dialysis

Surfactant is a mixture of proteins and saturated and unsaturated phospholipids that reduces surface tension in the lung (1). Surfactant protein A (SP-A) is a calcium-dependent phospholipid binding protein that is secreted into the distal airspaces by alveolar type II cells (2). In the alveolar lumen, SP-A reaches concentrations of 0.5–1.0 mg/ml (3) and localizes to surfactant membrane interfaces. There, it drives the formation of the lattice-like networks of surfactant lipids called tubular myelin (4) and assists in the defense of the lung from inhaled pathogens (5). The latter function was first suggested by sequence homology with mannose binding protein, identifying SP-A as a member of the collectin family of innate immune host defense proteins (6, 7). Recent studies suggest that SP-A directly permeabilizes (8, 9) and opsonizes pulmonary pathogens and enhances intracellular killing by macrophages (10). The strong affinity of the protein for the phospholipid components of surfactant, especially dipalmitoylphosphatidylcholine (11), suggests that the collectin may also serve primary surfactant functions. Alternatively, lipid interactions may provide a mechanism to array SP-A at surfactant membrane interfaces in an optimal position to interact with inhaled microorganisms (12). Genetically engineered mice lacking SP-A are deficient in tubular myelin and have subtle surfactant functional defects (13), but the most robust phenotypes include dysregulated inflammatory responses and defective pulmonary clearance upon intratracheal challenge with Gram-positive and Gram-negative bacteria, fungi, and viruses (14–16).

Oxidizing environmental exposures and lung inflammation result in surfactant dysfunction, reduced lung compliance, and impaired gas exchange. The alveolar air/liquid interface is among the most oxidizing compartments in the body, and unsaturated fatty acyl chains of surfactant phospholipids provide substrate for free radical chain reactions that may amplify injury. SP-A is oxidatively modified and functionally inactivated by exposure to high-oxygen tensions, such as ozone (17–19), peroxide (20), metals (21), and peroxynitrite (22). The role of surfactant lipid peroxidation (LPO) in surfactant protein damage and functional inactivation is incompletely understood.

The purpose of this study was to assess the effect of LPO on SP-A function.

MATERIALS AND METHODS

Reagents

BSA, butylated hydroxytoluene, Chelex 100, chloroform, cholesterol, cupric sulfate, cupric disodium EDTA, disodium EDTA,

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malonaldehyde bis(dimethyl acetal), malondialdehyde tetrabutylammonium salt (MDA), β-phenantrolinedisulfonic acid, thiobarbituric acid, and trichloroacetic acid were from Sigma-Aldrich (St. Louis, MO). HPLC-grade water and tetrabutylammonium phosphate were supplied by ACROS (Geel, Belgium). Hydrochloric acid, HPLC-grade methanol, and acetonitrile were from Fisher Scientific (Hanover Park, IL). 2,2′-Azobis(*N*,*N*′-dimethyleneisobutyramine)dihydrochloride (VA-044) was a gift from Wako Chemicals USA, Inc. (Richmond, VA). The OxyBlot™ Protein Oxidation Detection Kit was from Intergen (Purchase, NY). The BCA Protein Assay Reagent Kit was from Pierce (Rockford, IL). The lipid hydroperoxide determination kit was from Cayman Chemical (Ann Arbor, MI). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), L-phosphatidylcholine from egg yolk (EggPC), and 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (18:0-18:2PC) in chloroform were from Avanti Polar Lipids, Inc. (Alabaster, AL). The Spectra/Por cellulose membrane MWCO 3,500 (flat width 18 mm; diameter 11.5 mm; Spectrum Laboratories, Inc., Rancho Dominguez, CA) was used for dialysis. All other chemicals were of analytical grade.

Membrane vesicle preparation

Model surfactant lipids were used as substrates for lipid oxidation. Unsaturated liposomes (ULs) or saturated liposomes (SLs) were prepared by the method of Gregoriadis and Ryman (23). In brief, an unsaturated lipid mixture [DPPC, cholesterol, EggPC, 18:0-18:2PC (1:1:0.15:0.15, w/w/w/w)] or a saturated lipid mixture [DPPC, cholesterol (2.15:0.15, w/w)] was dissolved in $CHCl_3$-methanol (85:15, v/v). The organic solvents were evaporated to dryness in a rotary evaporator under an N_2 atmosphere at 20°C, and the lipid film was hydrated. Multilamellar liposomes were generated by vigorous vortexing for 5 min. Unilamellar vesicles were generated by probe sonication for 30 min at 45°C in a Fisher ultrasonicator (Fisher Scientific, Pittsburgh, PA) and centrifugation at 16,000 *g* for 30 min to remove multilamellar vesicles.

Lipid oxidation and thiobarbituric acid-reactive substances measurement

Stock solutions of 10 mM $CuSO_4$ were freshly prepared daily. Reaction mixtures composed of 1 mg/ml ULs, 10 μM $CuSO_4$, and proteins or controls were prepared in 3% Chelex-treated saline (0.9% NaCl) or PBS for 24 h at 20°C. The mixtures were incubated at 37°C in a shaking water bath for 24 h (24). Control reactions that included ULs only, $CuSO_4$ only, or BSA only were also performed. In some experiments, alternative oxidant stimuli were used. Auto-oxidized unilamellar ULs were prepared by incubation in room air for 7 days at 20°C. Surfactant lipids were also exposed to the hydrophilic free radical generator, 10 mM VA-044, for 1 h at 20°C. Oxidation was monitored by measuring thiobarbituric acid-reactive substances (TBARS) using a method adapted from Gelvan and Saltman (24). Samples and malondialdehyde standards were developed by the addition of a solution composed of 0.375% thiobarbituric acid, 15% trichloroacetic acid, and 0.25 N HCl at a volume ratio of 1:2 (sample-developer). After incubation at 95°C for 30 min and centrifugation at 16,000 *g* for 15 min, an aliquot was read in a spectrophotometer using a 540 nm filter. An absorption scan (500–570 nm) of both the malondialdehyde-thiobarbituric acid adducts and the lipid aldehyde-thiobarbituric acid adducts indicated that the absorbance at 540 nm was representative of the peak obtained at the thiobarbituric acid absorption maximum at 532 nm (not shown).

Purification and analysis of human SP-A

SP-A was isolated from bronchoalveolar lavage of patients with the lung disease alveolar proteinosis by the method of Suwabe, Mason, and Voelker (25). Briefly, the surfactant pellet was washed with 1 mM $CaCl_2$ in 150 mM NaCl and SP-A was released by incubation with 2 mM EDTA in 150 mM NaCl. The recalcified sample was adsorbed to a mannose-Sepharose column. After elution with 2 mM EDTA in 5 mM Tris, the sample was dialyzed for 48 h against 5 mM Tris and 100 mM NaCl followed by dialysis for 24 h against 5 mM Tris. The EDTA content of all protein reagents was measured by the method of Kord, Tumanova, and Matier (26) using reverse-phase (RP) HPLC. Samples were pretreated with 25 mM $CuSO_4$ for 15 min at 25°C and then 20 μl was loaded onto the column [Supelco RP-LC18 25 cm × 4.6 mm inner diameter, 120 Å, 5 μm particle size (Bellefonte, PA)]. Samples were eluted using mobile-phase acetonitrile (35%) in 5 mM tetrabutylammonium phosphate (pH 6.5) at a flow rate of 1 ml/min at 25°C. Detection was by ultraviolet (UV) absorbance at 254 nm. The retention time for the Cu-EDTA complex was 5 min. Spectrophotometric determination of EDTA content was made by the method of Kratochvil and White (27), with modifications. The assay used in our work is based on the inhibition of iron-enhanced absorption of β-phenantrolinedisulfonic acid in the presence of EDTA. All assays were carried out in 96-well plates. Briefly, protein samples were incubated with 30 μM $FeSO_4$ in deionized water at room temperature for 5 min and 0.5 mM β-phenantrolinedisulfonic acid was added. Formation of Fe(II)-β-phenantrolinedisulfonic acid complexes was determined by the absorbance changes at 540 nm. Disodium EDTA was used for calibration.

Protein oxidation and detection of carbonyls

Protein was oxidized in the presence of multilamellar ULs and 10 μM $CuSO_4$ for 24 h at 37°C or 10 mM VA-044 for 1 h at 20°C. Multilamellar SLs were used as a control. Proteins were analyzed for oxidative modification of amino acid side groups using the Oxyblot Detection Kit (28). SP-A was incubated for 24 h alone or in the presence of ULs, $CuSO_4$ alone, or $CuSO_4$ and ULs at 37°C. Reaction mixtures were composed of 250 μg/ml SP-A, 250 μg/ml SP-A plus 1 mg/ml ULs, 250 μg/ml SP-A plus 10 μM $CuSO_4$, or 250 μg/ml SP-A plus 1 mg/ml ULs and 10 μM $CuSO_4$. SP-A treated in this manner was incubated with 2,4-dinitrophenylhydrazine to derivatize protein-associated carbonyls to 2,4-dinitrophenylhydrazone (DNP). After size fractionation by 8–16% SDS-polyacrylamide gel electrophoresis under reducing conditions, protein species were electrophoretically transferred to nitrocellulose membranes. The membrane was sequentially incubated with a rabbit anti-DNP IgG and a horseradish peroxidase-conjugated goat anti-rabbit IgG. Blots were developed by horseradish peroxidase-dependent oxidation of a chemiluminescent substrate and visualized using autoradiography.

Lipid aggregation assay

Lipid aggregation was performed as described by Hawgood et al. (29), with minor modifications. Unilamellar ULs were prepared as described above. SP-A-mediated aggregation of unilamellar liposomes was measured by monitoring the changes in light scattering using a Beckman DU-640 spectrophotometer. Briefly, unilamellar ULs (400 μg/ml lipid) were preincubated for 2 min in aggregation buffer (50 mM Tris, pH 7.4, 1 mM EDTA, and 150 mM NaCl) with SP-A (10 μg/ml), and $CaCl_2$ (5 mM) was added to initiate liposome aggregation. Absorbance at 425 nm was followed for 25 min at 20°C. Aggregation activity was monitored at 425 nm and expressed as initial rate of absorbance change and as maximal absorbance.

Conjugated diene measurements

Measurement of conjugated dienes was performed by HPLC analysis using a Supelco RP-LCSi column (25 cm × 4.6 mm inner diameter, 120 Å, 5 μm particle size) and Class VP Chromatography Data Acquisition System software (Shimadzu, Columbia, MD).

The RP-HPLC method used to analyze the conjugated diene concentration was that described by Noguchi et al. (30). Samples were eluted using mobile-phase methanol-40 mM phosphate buffer (pH 7.4) (90:10, v/v) at a flow rate of 1 ml/min at 20°C. Detection was by UV absorbance at 234 nm. The retention time for conjugated dienes was 7 min.

Lipid hydroperoxide determination

A commercially available kit (Cayman Chemical) was used to assess lipid hydroperoxide concentration in the surfactant pellet from oxygen-exposed mice and in model surfactant lipids after copper-initiated peroxidation.

Protein determination

Protein was analyzed using the BCA Protein Assay Reagent Kit (Pierce). Absorbance was read at 570 nm against a sample blank.

Statistical analysis

The two-tailed Student's *t*-test was used for comparisons between experimental groups. The level of significance was set at $P < 0.05$. All data are presented as means ± SEM unless otherwise noted.

RESULTS

Preparation of EDTA-free human SP-A

SP-A reagents used in oxidation assays must be free from copurifying antioxidants. EDTA is routinely used in the purification of collectins to elute the proteins from carbohydrate affinity columns. We have found that EDTA does not freely dialyze under low-ionic-strength conditions, which are often used in the preparation of collectins. An audit of our SP-A reagents demonstrated concentrations of EDTA contaminants that varied between 50 and 650 μM. Experiments were performed to determine the optimal dialysis conditions for the removal of EDTA, and the results are shown in **Table 1**. Two methods of EDTA detection were used, an HPLC technique and a spectrophotometric technique, as described in Materials and Methods. Dialysis of a 2 mM solution of EDTA in water against low-ionic-strength buffer was ineffective, leading to a residual EDTA concentration after 3 days of exposure to two daily changes of 2,000 buffer volumes (12,000 volumes total) of one-third of the starting concentration. Dialysis buffers containing HEPES at 5 and 25 mM concentrations performed similarly. Dialysis against 150 mM NaCl resulted in 99% removal of EDTA by 24 h. We conclude that dialysis of EDTA in low-ionic-strength buffer is inefficient.

Effects of human SP-A on LPO

The ability of SP-A to inhibit the $CuSO_4$-induced oxidation of a mixture of model surfactant lipids was assessed by measuring the accumulation of TBARS during a 24 h coincubation at 37°C (**Fig. 1**). LPO of multilamellar ULs was dramatically increased in the presence of 4 or 10 μM $CuSO_4$. Native human SP-A inhibited the oxidation of surfactant lipids in a dose-dependent manner. Albumin also inhibited $CuSO_4$-induced surfactant lipid oxidation in a dose-dependent manner, and although the antioxidant activity of SP-A was slightly greater than that of BSA at the same concentration ($P < 0.05$), the difference was modest and unlikely to be physiologically relevant.

Oxidative damage of SP-A by LPO

To determine if SP-A becomes modified during LPO, we used a Western blot analysis technique that detects carbonyl adducts (28). Oxidation of SP-A in the presence or absence of $CuSO_4$ and in the presence or absence of model surfactant liposomes was performed for 24 h at 37°C. After derivatization of protein-associated carbonyls with 2,4-dinitrophenylhydrazine and immunoblotting with an anti-DNP adduct antibody, dense bands that corresponded to the molecular mass of SP-A were detected (26–38 kDa). The data shown in **Fig. 2** indicate that oxidation of SP-A occurs in the presence of $CuSO_4$ alone and during autoxidation of lipids but is greatest in the presence of LPO induced by $CuSO_4$.

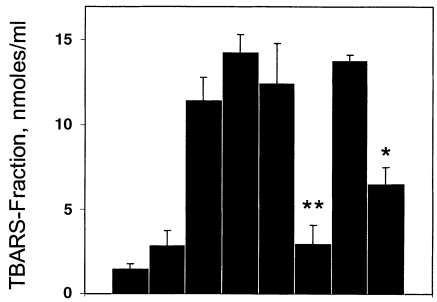
Effect of LPO-induced protein damage on lipid aggregation

We next assessed the effect of LPO on SP-A function. As a control, SP-A was exposed to $CuSO_4$ or SLs separately or in combination before testing in a liposome aggregation assay. There was no effect of SP-A preincubation with multilamellar SLs alone, $CuSO_4$ alone, or a mixture of multilamellar SLs plus $CuSO_4$ on the kinetics of lipid aggregation

TABLE 1. Residual EDTA concentration after dialysis of a 2 mM EDTA solution in cellulose membranes (MWCO 3,500) against two daily changes of 2,000 volumes of various buffers

	Time of Dialysis					
	24 h		48 h		72 h	
Buffer	Spectrophotometry[a]	HPLC[b]	Spectrophotometry	HPLC	Spectrophotometry	HPLC
5 mM Tris, pH 7.4	851.9 ± 126.2	642.9 ± 18.1	770.9 ± 43.4	773.8 ± 118.8	635.2 ± 32.5	476.0 ± 22.8
25 mM Tris, pH 7.4	649.1 ± 126.2	N/A	454.2 ± 17.7	N/A	392.7 ± 95.4	N/A
150 mM NaCl	2.5 ± 1.3	2.2 ± 1.2	1.6 ± 0.6	0.5 ± 0.2	0.1 ± 0.0	0.2 ± 0.1
5 mM Tris and 150 mM NaCl, pH 7.4	46.8 ± 10.5	7.9 ± 1.8	10.7 ± 1.0	10.7 ± 0.3	5.1 ± 1.9	10.0 ± 1.3
5 mM HEPES, pH 7.4	758.9 ± 79.2	N/A	436.6 ± 32.4	N/A	444.1 ± 88.8	N/A
25 mM HEPES, pH 7.4	551.7 ± 254.0	346.0 ± 146.3	277.5 ± 93.6	24.2 ± 6.8	44.4 ± 15.1	9.2 ± 0.2

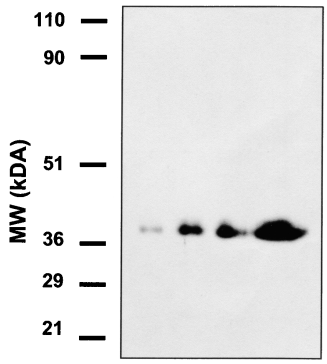
N/A, not available. Data shown are mean EDTA concentrations (μM) ± SEM.
[a] Modified method of Kratochvil and White (27).
[b] Method of Kord, Tumanova, and Matier (26).



Lipid	+	+	+	+	+	+	+	+
Cu, μM	0	2	4	10	10	10	10	10
SP-A, μg/ml	-	-	-	-	10	50	-	-
BSA, μg/ml	-	-	-	-	-	-	10	50

Fig. 1. Surfactant protein A (SP-A) inhibits copper-induced lipid peroxidation (LPO). Surfactant multilamellar unsaturated liposomes (ULs; 1 mg/ml) were incubated for 24 h at 37°C with $CuSO_4$ in the absence or presence of the indicated concentrations of surfactant protein SP-A or BSA. Data are means ± SEM (n = 3). * and ** $P < 0.01$. TBARS, thiobarbituric acid-reactive substances.

(**Fig. 3A**). Human SP-A was oxidized by incubation with multilamellar ULs, $CuSO_4$ alone, or a mixture of $CuSO_4$ and multilamellar ULs for 24 h at 37°C. Treated or untreated SP-A was then equilibrated with unilamellar ULs, and the kinetic profile of liposome aggregation was monitored by measurement of light scattering after the addition of calcium (Fig. 3B). The maximal aggregation end point for copper plus UL-oxidized SP-A was significantly lower than that for untreated SP-A controls (Fig. 3C). Also, the initial rate of lipid aggregation by the copper plus UL-treated SP-A was significantly lower than that for untreated SP-A (Fig. 3D). There was no effect of SP-A preincubation with ULs alone or $CuSO_4$ alone on the kinetics of lipid aggregation (Fig. 3B–D) at the concentrations used. These data indicate that $CuSO_4$-induced LPO oxidatively damages SP-A and blocks protein-lipid interactions.



SP-A	+	+	+	+
Lipid	-	+	-	+
Cu	-	-	+	+

Fig. 2. LPO results in oxidative damage to SP-A. Modification of SP-A that occurred during exposure to 10 μM $CuSO_4$ or 10 μM $CuSO_4$ plus phospholipids for 24 h at 37°C was determined by Western analysis using an antibody to 2,4-dinitrophenylhydrazone-derivatized carbonyl moieties. MW, molecular mass.

Oxidative modification of SP-A by VA-044-initiated LPO and lipid aggregation

To determine if the LPO-induced SP-A modification and inactivation was specific for $CuSO_4$-initiated oxidation, we also examined LPO induced by the 2,2′-azobis compound, VA-044. VA-044 thermally decomposes and produces free radicals. The formation of conjugated dienes during 10 mM VA-044-initiated LPO of ULs and SLs for 1 h at 20°C was determined by HPLC (**Fig. 4**). As expected, SLs did not produce detectable conjugated dienes, either spontaneously or during incubation with VA-044 (Fig. 4). Spontaneous oxidation of ULs at 20°C over 7 days resulted in a marked induction of conjugated dienes, which was approximately twice that obtained upon 1 h of exposure to VA-044 and more than four times the detectable conjugated dienes in the UL preparation at baseline. Exposure of SP-A to VA-044-induced oxidation of SLs or ULs resulted in partial and marked reduction in SP-A-induced lipid aggregation, respectively, as assessed by the kinetics of aggregation (**Fig. 5A**), the initial rate of aggregation (Fig. 5B), or the maximal aggregation end point (Fig. 5C). These results indicate that free radical-initiated LPO functionally inactivates SP-A to a greater extent than oxidation induced in the absence of lipids.

Effect of LPO on unilamellar vesicle aggregation by nonoxidized SP-A

One potential interpretation of the results described above is that SP-A has reduced affinity for oxidized lipids, which in turn interfere with collectin-mediated aggregation. To address this issue, the aggregation function of SP-A was tested using unoxidized and preoxidized lipid vesicles. Auto-oxidation of ULs was performed at 20°C in air for 7 days in the absence of SP-A, $CuSO_4$, or VA-044. LPO was monitored by measurement of conjugated diene formation by HPLC (Fig. 4). The oxidized liposomes were equilibrated with SP-A, and calcium was added. SP-A-mediated aggregation of oxidized ULs was not significantly different from that of nonoxidized vesicles (**Fig. 6**).

Effect of MDA on unilamellar vesicle aggregation by SP-A

Experiments were performed to investigate the extent to which the direct modification of SP-A by aldehydes generated during LPO interferes with SP-A-induced aggregation. Based on the MDA standards used for the TBARS assay, LPO that occurs during spontaneous oxidation of unsaturated multilamellar vesicles for 24 h yields ~15 μM MDA equivalents. SP-A exposed to LPO under these conditions is much less active in aggregating phospholipid liposomes (Fig. 3). SP-A was treated for 24 h at 37°C in phosphate-buffered saline or 0.9% saline (pretreated with 3% Chelex 100 to remove contaminating metals, pH 7.4) with 10, 30, or 60 μM MDA in the absence of lipids and then tested for the ability to aggregate liposomes. Two forms of MDA were used to determine the impact of MDA on SP-A (**Fig. 7**), one with blocked aldehyde groups [malonaldehyde bis(dimethyl acetal)] and one with open aldehyde groups (malondialdehyde tetrabutylammonium salt). There was no difference in lipid aggregation by MDA-

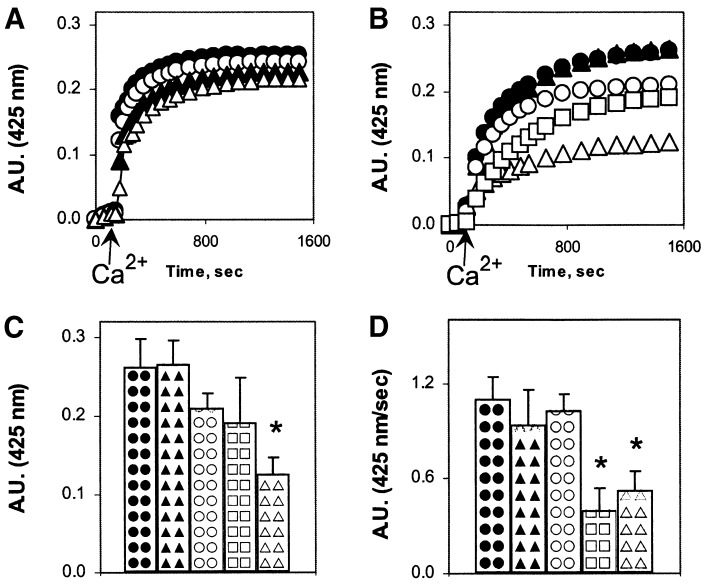


Fig. 3. LPO-mediated oxidative damage of SP-A blocks interaction of the protein with unilamellar phospholipids. SP-A was preincubated alone, with multilamellar phospholipids, with copper, or with multilamellar phospholipids plus copper for 24 h at 37°C and then tested in an aggregation assay. Kinetics of unilamellar liposome aggregation with SP-A (closed circles), SP-A preincubated with multilamellar phospholipids (closed triangles), SP-A preincubated with 10 μM $CuSO_4$ (open circles), SP-A preincubated with multilamellar phospholipids and 4 μM $CuSO_4$ (open squares), and SP-A preincubated with multilamellar phospholipids and 10 μM $CuSO_4$ (open triangles) are shown for saturated liposomes (SLs) in A and ULs in B. Initial rate of aggregation for UL-treated SP-A (C) and maximal aggregation (D) are also shown. Data are means ± SEM (n = 3). * $P < 0.05$. A.U., absorbance units.

treated SP-A, as assessed by aggregation profile after incubation with malonaldehyde bis(dimethyl acetal) (Fig. 7A) or malondialdehyde tetrabutylammonium salt (Fig. 7B). These data suggest that LPO-induced functional inactivation of SP-A cannot be reproduced by direct incubation with MDA and that the oxidation of SP-A by lipid radicals that are generated during LPO is primarily responsible for the loss of lipid-aggregating function of the protein.

DISCUSSION

The data presented in this study indicate that human SP-A inhibits copper- or free radical-initiated surfactant LPO at physiologically relevant concentrations by a mechanism that includes oxidative modification of SP-A. Oxidative damage to SP-A that occurs during LPO inhibits liposomal aggregation by the protein. Loss of the lipid aggregation function of human SP-A after LPO is most likely caused by damage initiated by lipid radicals and not by adduct formation with the aldehydic product of LPO, MDA, or differential affinity of SP-A for oxidized versus nonoxidized lipids.

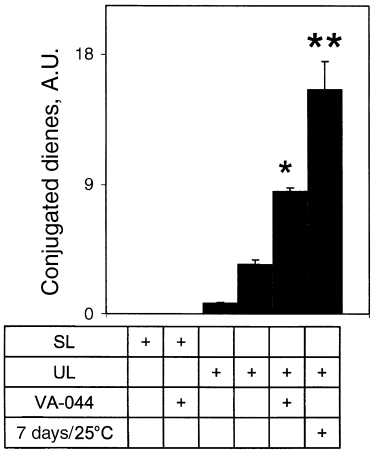


Fig. 4. Formation of conjugated diene by incubation of multilamellar SLs or ULs with VA-044 or air. Multilamellar ULs were preincubated with or without VA-044 for 1 h at 20°C or auto-oxidized by incubation for 7 days at 20°C in air. An HPLC method was used to analyze conjugated diene concentrations. Data are means ± SEM (n = 3–4). * $P < 0.001$. ** $P < 0.02$.

LPO can be initiated in the presence of redox-active metals (e.g., copper or iron) or azo-compounds (31). The mechanism of metal-initiated LPO is based on the decomposition of lipid hydroperoxides in the presence of metal ions, which leads to the formation of peroxyl ($LOO^{\bullet}$) and alkoxyl ($LO^{\bullet}$) radicals (32), whereas azo-compounds initiate LPO by the generation of free radicals during thermal decomposition. In either case, the generation of highly reactive oxygen species can trigger chain reactions of lipid oxidation that damage proteins.

There have been many studies that describe the oxidative modification of proteins during biological processes. Oxidized proteins accumulate with aging (33–35), after lung injury or toxic inhalation (36), and in various pathological states, including diabetes (37) and Alzheimer's disease (38). Oxidative modification and functional inactivation of proteins may occur by direct interaction with the oxidizing substance or indirectly through interaction with free radicals or reactive oxidation products, such as the aldehydic intermediates MDA (39) and 4-hydroxynonenal (40).

Lipid hydroperoxides generated during clinically achievable oxidant exposures were measured to assess the physiological relevance of our findings. We found that exposure of mice to 90% oxygen for 3 days led to the formation of 2.6 ± 0.7 nmol/ml lipid hydroperoxides in the surfactant pellet isolated by lavage. This value is lower than, but of the same order of magnitude as, that of lipid hydroperoxides generated during copper-initiated (4 μM $CuSO_4$) oxidation of lipids in vitro, which was measured at 16.6 ± 2.3 nmol/ml. The concentration of lipid hydroperoxides in the bronchoalveolar lavage fluid of oxygen-exposed mice

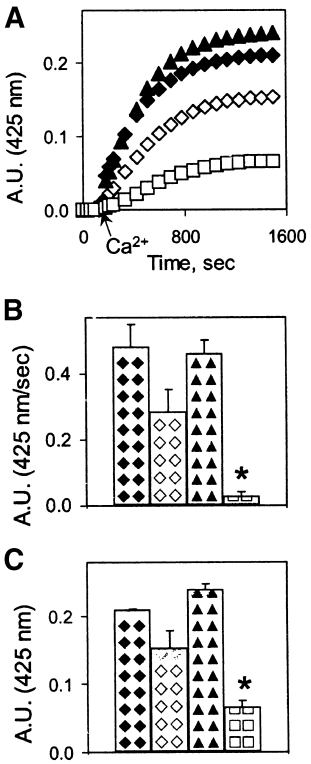


Fig. 5. LPO-induced oxidative damage to SP-A is independent of the initiating stimulus. SP-A was preincubated with SLs or ULs with or without VA-044 for 1 h at 20°C. The kinetics of aggregation with SP-A preincubated with surfactant unilamellar SLs (closed diamonds), SP-A preincubated with unilamellar SLs and VA-044 (open diamonds), SP-A preincubated with ULs (closed triangles), and SP-A preincubated with ULs and VA-044 (open squares) are shown in A. Initial rate of aggregation (B) and maximal aggregation (C) are also presented. Data are means ± SEM (n = 3–4). * $P < 0.001$.

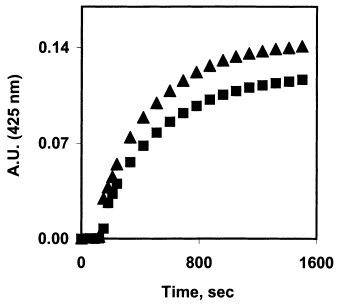


Fig. 6. SP-A does not preferentially aggregate nonoxidized liposomes. Effect of lipid peroxidation on UL aggregation by untreated SP-A. Aggregation of unilamellar ULs by unoxidized SP-A was measured immediately after liposome preparation and after liposome auto-oxidation for 7 days at 20°C in air. Kinetics of aggregation with SP-A and ULs (closed diamonds) and SP-A and ULs incubated for 7 days at 20°C in air (closed squares) are presented. Data are means (n = 3–4).

may be even higher, because oxidized lipids may also partition into surfactant vesicles that do not sediment during centrifugation and are therefore not assayed by the method employed.

Our results indicate that concentrations of SP-A that are within the predicted physiological range in the alveolar space (0.2–1.0 mg/ml) are able to inhibit LPO. On a weight basis, the antioxidant effect of SP-A was slightly greater than that of albumin, which has also been reported to inhibit oxidation (41). However, albumin is manyfold more abundant than SP-A in the airspace. It is possible that the proximity of SP-A to lipids in the airspace provides physiologically relevant antioxidant protection for surfactant membranes.

Finally, it is important to note that there are redundant antioxidant defenses in the alveolar space. Previous studies have shown that natural lung surfactant mixtures contain significant antioxidant activity attributable to glutathione, superoxide dismutase, and catalase (42). One objective of this study was to determine antioxidant activity related to the surfactant protein only using a model system composed of purified SP-A and analytical-grade lipids.

The antioxidant effect of SP-A was less pronounced than previously reported (43). The differences are attributable to unexpected EDTA contamination in the original preparations used, which resulted from atypical dialysis behavior of SP-A in low-ionic-strength and Tris-containing buffers (44). The residual EDTA concentration after dialysis of 2 mM EDTA against 2,000 volumes of low-ionic-strength buffers was approximately one-third of the original concentration, and several protein samples in our original publications contained EDTA in concentrations as high as 500–600 μM (Table 1). Given the dual valency of EDTA for copper, these EDTA concentrations are high enough to interfere with the SP-A functional assays using typical calcium (e.g., 2 mM) or copper (e.g., 10 μM) concentrations, even after dilution from concentrated protein

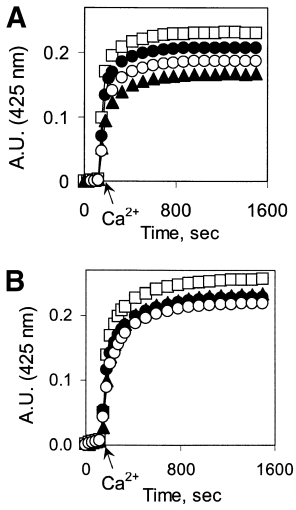


Fig. 7. Direct exposure to LPO aldehydes does not result in functional inactivation of SPA. Incubation with malonaldehyde bis(dimethyl acetal) (A) or malondialdehyde tetrabutylammonium salt (MDA; B) does not affect lipid aggregation by SP-A. SP-A was preincubated alone or with MDA for 24 h at 37°C. Kinetics of UL aggregation with SP-A alone (open squares), SP-A preincubated with 10 μM MDA (closed triangles), SP-A preincubated with 30 μM MDA (closed circles), and SP-A preincubated with 60 μM MDA (open circles) are shown in A. Data are means ± SEM (n = 3).

stocks. Quantitative removal of EDTA from SP-A reagents by dialysis against 12,000 volumes of salt-containing buffers in this study revealed that the IC_{50} for the inhibition of LPO by SP-A reported previously was overestimated by ~8-fold (43). As C-type lectins are frequently purified by carbohydrate affinity column chromatography, EDTA elution, and dialysis against low-ionic-strength buffers, unexpected EDTA concentration may have affected other studies. Another consideration is that protease sensitivity is in some cases calcium dependent and contamination by EDTA may affect SP-A stability during storage (45). For collectin purification procedures that include EDTA, we recommend analysis of the EDTA content of all reagents to be used in chelator-sensitive assays. Effective removal of EDTA can be achieved by dialysis against 150 mM NaCl-containing buffer or by gel exclusion chromatography.

Protein carbonyl adducts are markers for protein oxidation (46). The observed increase in carbonyl content of the copper- and LPO-exposed proteins suggest that the intrinsic antioxidant activity of SP-A is associated with scavenging of chain-perpetuating lipid peroxyl radicals. Oxidative modification of SP-A also occurs with copper alone, but it increases dramatically in the presence of LPO. These data are consistent with our hypothesis that SP-A is vulnerable to oxidation in the lipid-rich environment of the alveolar lining fluid.

One functional activity of SP-A, the ability of the protein to aggregate phospholipid liposomes, decreased to a greater extent upon exposure to LPO than to copper alone. This result indicates that lipid peroxyradicals amplify the oxidative damage initiated by copper. The damaging effect of LPO was independent of the initiating stimulus, because VA-044-initiated LPO decreased lipid aggregation to a greater extent than VA-044 alone. These experimental results indicate that lipid radicals functionally inactivate SP-A.

Lipid hydroperoxides generated during LPO may affect the interaction between SP-A and phospholipid liposomes, either by interfering with binding or by directly modifying the protein. To address the former possibility, the SP-A-mediated aggregation of auto-oxidized liposomes containing increased concentrations of lipid hydroperoxides was compared with that of nonoxidized liposomes, and no differences were found. LPO also leads to the formation of reactive aldehydes that have been shown to derivatize proteins (47, 48). The addition of MDA at two to four times higher concentrations than levels reached during LPO under the conditions used had no effect on lipid aggregation rate or end point. These data suggest that the effect of LPO on the functional activity of SP-A is not attributable to direct modification by MDA and further implicate lipid peroxyradicals in protein damage.

SP-A and surfactant phospholipids are intimately associated in the alveolar space. The phospholipid concentration in the alveolar lining fluid is very high and, despite the relative abundance of saturated lipids in surfactant, provides abundant unsaturated substrate for oxidation in this compartment. We speculate that SP-A bound at surfactant membrane interfaces may limit the propagation of free radical chain reactions in the alveolar lining layer. In the presence of LPO, however, SP-A is modified and functionally inactivated. Protein damage to SP-A may have important implications for surfactant dysfunction or susceptibility to infection in patients exposed to oxidizing conditions, such as the use of high oxygen tensions during mechanical ventilation.

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