Pulmonary Surfactant Proteins A and D Are Potent Endogenous Inhibitors of Lipid Peroxidation and Oxidative Cellular Injury*

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The lung is composed of a series of branching conducting airways that terminate in grape-like clusters of delicate gas-exchanging airspaces called pulmonary alveoli. Maintenance of alveolar patency at end expiration requires pulmonary surfactant, a mixture of phospholipids and proteins that coats the epithelial surface and reduces surface tension. The surfactant lining is exposed to the highest ambient oxygen tension of any internal interface and encounters a variety of oxidizing toxicants including ozone and trace metals contained within the 10 kl of air that is respired daily. The pathophysiological consequences of surfactant oxidation in humans and experimental animals include airspace collapse, reduced lung compliance, and impaired gas exchange. We now report that the hydrophilic surfactant proteins A (SP-A) and D (SP-D) directly protect surfactant phospholipids and macrophages from oxidative damage. Both proteins block accumulation of thiobarbituric acid-reactive substances and conjugated dienes during copper-induced oxidation of surfactant lipids or low density lipoprotein particles by a mechanism that does not involve metal chelation or oxidative modification of the proteins. Low density lipoprotein oxidation is instantaneously arrested upon SP-A or SP-D addition, suggesting direct interference with free radical formation or propagation. The antioxidant activity of SP-A maps to the carboxyl-terminal domain of the protein, which, like SP-D, contains a C-type lectin carbohydrate recognition domain. These results indicate that SP-A and SP-D, which are ubiquitous among air breathing organisms, could contribute to the protection of the lung from oxidative stresses due to atmospheric or supplemental oxygen, air pollutants, and lung inflammation.

Air breathing is made possible through the surface tension-lowering properties of lung surfactant, an oily film located at the boundary between the aqueous pulmonary epithelial lining fluid (ELF)§ and air in the lumen of the alveoli, the gas-exchanging units of the lung. By weight, surfactant is composed of 90% phospholipids and 10% protein, including the hydrophilic surfactant proteins A (SP-A) and D (SP-D), and the hydrophobic surfactant proteins B (SP-B) and C (SP-C) (1). After secretion into the ELF, the components of surfactant form membranes at the air-liquid interface that spread readily and compress poorly during cyclical respiratory expansion and contraction of the alveolus. These properties of surfactant result in enhanced lung compliance during inspiration, which reduces the work of breathing, and very low alveolar surface tension at end expiration, which helps to maintain airspace patency. Exposure of surfactant to ambient oxygen and potent environmental oxidants such as ozone results in peroxidation of unsaturated phospholipids, surfactant inactivation, airspace collapse, and impaired gas exchange (2). Antioxidant protection of surfactant phospholipids in the ELF has classically been attributed to low molecular mass components urate, ascorbate, and reduced glutathione and to proteinaceous antioxidants superoxide dismutase and catalase (3). The serum apolipoproteins apoE and apoAIV, which are intimately associated with lipid aggregates including chylomicrons, low density lipoproteins (LDL), and high density lipoproteins, have been reported to prevent lipid peroxidation and to protect against the development of atherosclerosis (4, 5). The objective of this study was to determine if the surfactant-associated proteins contribute to the prevention of lipid oxidation in the airspaces of the lung.

Surfactant proteins A and D are the pulmonary members of the collectin family of proteins, which also includes the serum mannose-binding proteins A (MBP-A) and C (MBP-C), bovine conglutinin, and CL-43 (6). Like all collectins, SP-A and SP-D have similar basic structural organization including an N-terminal segment and the collagen-like region and containing an Asn to Ser substitution at position 187 that prevents carbohydrate attachment; a collagen-like domain of Gly-X-Y repeats containing hydroxylated amino acids, an amphipathic helical “neck” region, and a C-terminal, C-type lectin domain (CLD) (7). Trimerization occurs by triple helix formation in the collagen-like domain and bundled α-helical coiled-coil formation in the neck region. At the level of quaternary structure, however, the pulmonary collectins diverge. Variably glycosylated subunits of SP-A (apparent molecular mass range 26–38 kDa) assemble into a hexamer of trimers that are disulfide-linked at the N terminus and laterally associated through the first portion of the colla-
gen-like domain, forming a flower bouquet-like structure with an estimated mass of 600,000 kDa (8). For SP-D, four trimers composed of glycosylated 42-kDa subunits form a 500,000-kDa cruciform-shaped oligomer joined by disulﬁde bonds at the N terminus (9). Both proteins bind calcium ions at two or three sites within the CLD (10, 11). Data from sedimentation, immuno- nistohistochemical, and ultrastructural analyses indicate that SP-D resides primarily in the aqueous compartment of the ELF, while SP-A is intimately associated with surfactant lipid membranes and aggregates (12).

The unifying functional theme for the collectins is innate host defense, and several laboratories have reported that the pulmonary collectin aggregates and opsonize diverse microbial species and enhance the clearance of microorganisms from the lungs of mice (13). However, the pulmonary collectins also perform specialized roles in the structure and function of pulmonary surfactant. Experimental data suggest that SP-A protects the surface activity of surfactant from serum protein inhibitors (14), contributes to stability of surfactant aggregates such as tubular myelin (15), and inhibits the activity of some phospholipases A2 (16, 17), while SP-D appears to be required for the maintenance of surfactant homeostasis and lung structure (18, 19). Almost all reported SP-A and SP-D ligand binding properties require calcium, but neither protein has been reported to have enzymatic activity or to be associated with metals other than calcium. Here we demonstrate that SP-A and SP-D also have potent, direct phospholipid and cellular antioxidant properties.

MATERIALS AND METHODS

Lipid Preparations—Substrates for lipid oxidation included mixtures of natural and synthetic glycerophospholipids that are found in pulmonary surfactant and human LDL. The model surfactant lipids, composed of egg phosphatidylcholine, dipalmitoylphosphatidylcholine, cholesterol, and 1-deoxy-2-linoleoyl-sn-glycero-3-phosphocholine (1:10:15:0.15, w/w/w/w, respectively) (Avanti Polar Lipids) were mixed in chloroform and dried to a film under nitrogen. Following resuspension in phosphate-buffered saline or 0.15 M NaCl, multilamellar vesicles were generated by vigorous vortexing for 5 min. LDL were isolated from the plasma of normal blood donors by density gradient ultracentrifugation (20). LDL were isolated from rats that were fed a high cholesterol diet for 4 weeks and were fasted overnight before sacrifice. LDL isolation was performed as described previously (21–23). Wild-type and mutant recombinant rat SP-A, SP-D, and MBP were purified from the supernatant by maltose-Sepharose affinity chromatography as described previously (21). The purity of all surfactant proteins or control proteins were placed in quartz cuvettes and allowed to oxidize at room temperature over 335 min. Conjugated diene formation was assessed by measuring absorbance at a wavelength of 234 nm in a spectrophotometer.

Antioxidant Properties of SP-A and SP-D

Antioxidant activity was measured by using a spectrophotometric assay (24). Samples and 0–10 μM 1,1,3,3-tetramethylinodiolide standards were developed by the addition of a solution composed of 0.375% thiobarbituric acid, 15% trichloroacetic acid, and 0.25 mM HCl at a volume ratio of 1:2:0.5:0.01 of sample/developer. Following incubation at 95 °C for 30 min and centrifugation at 14,000 rpm 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-TBA adducts and the lipid-aldehyde TBA adducts indicated that the absorbance at 540 nm was representative of the peak obtained at the absorption maximum at 532 nm (25). Continuous assessment of lipid oxidation occurred during LDL oxidation was monitored spectrophotometrically (26). Mixtures of 50 μg/ml LDL, 10 μM copper, and various amounts of surfactant proteins or control proteins were placed in quartz cuvettes and allowed to oxidize at room temperature over 335 min. Conjugated diene formation was assessed by measuring absorbance at a wavelength of 234 nm in a spectrophotometer.

Cell Oxidation Experiments—A murine macrophage cell line (RAW 264.7) was adhered to 24-well plates (2 × 10⁶ cells/well) in Ham’s F-12 medium containing 10% FBS overnight at 37 °C in a 10% CO₂ atmosphere. After washing, the cells were incubated with 40 μM tert-butylhydroperoxide (t-BOOH) in serum-free Ham’s F-12 for 24 h in the presence of various concentrations of the surfactant proteins or BSA. Viability was assessed by exclusion of the vital dye, trypan blue.

RESULTS

Effects of Pulmonary Collectins on Lipid Oxidation—The ability of SP-A and SP-D to inhibit the copper-induced oxidation of a mixture of saturated and unsaturated lipids found in surfactant was assessed by using a spectrophotometric assay (27). Briefly, carbonyl-containing protein adducts in reaction mixtures of 150 μg/ml LDL, 10 μM CuSO₄, and putative anti-oxidant proteins or controls were prepared in phosphate-buffered saline or 0.9% saline. The mixtures were incubated at 37 °C in a shaking water bath for 4 h for LDL and 24 h for surfactant lipids (28). Control reactions that included LDL only, copper only, or protein controls of bovine serum albumin (BSA), rat IgG, recombinant MBP, rat serum, or human complement C1q were also performed. In some experiments, alternative oxidant stimuli were used. Ferric chloride (FeCl₃) (50 μM) or ferric pyrophosphate (FePP) (1 μM) plus ascorbic acid (0.25 mM) was incubated with the model surfactant lipids for 24 h in 0.9% saline. The surfactant lipid oxidizer, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) (Aldrich) was incubated with surfactant lipids or LDL for 0.5–3 h. In all cases, thiorbituric acid-reactive substances (TBARS) were measured by using a method adapted from Gelvan and Saltman (25).

Assessment of Lipid Oxidation—Stock solutions of 10 μM CuSO₄ were freshly prepared daily. Reaction mixtures composed of 1 mg/ml surfactant lipids or 150 μg/ml LDL, 10 μM CuSO₄, and putative antioxidant proteins or controls were prepared in phosphate-buffered saline or 0.9% saline. The mixtures were incubated at 37 °C in a shaking water bath for 4 h for LDL and 24 h for surfactant lipids (28). Control reactions that included LDL only, copper only, or control proteins were placed in quartz cuvettes and allowed to oxidize at room temperature over 335 min. Conjugated diene formation was assessed by measuring absorbance at a wavelength of 234 nm in a spectrophotometer.

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dependent manner with maximal protection observed at doses equal to or greater than 0.5 µg/ml (Fig. 1a). The IC\textsubscript{50} for protection by SP-D was 0.1 µg/ml (IC\textsubscript{50} ~ 0.2 nM, assuming a mass of 500 kDa), or approximately 35-fold lower than SP-A, and 100-fold lower than the antioxidant serum lipoprotein apoAIV (IC\textsubscript{50} ~ 50 nM) (4). The inhibitory concentrations for both lung proteins were well below their physiologic ELF levels, estimated to be 300–1800 µg/ml for SP-A and 36–216 µg/ml for SP-D (12). Alkylation of SP-A with iodoacetamide completely blocked the antioxidant effects of the protein, even when the modified protein was added at concentrations that were 5-fold greater than the inhibitory level (Fig. 1b). There was no protection from oxidation by control proteins albumin, rat serum, or the structurally similar molecule, C1q, at concentrations of 50 µg/ml (Fig. 1b). However, BSA exhibited dose-dependent inhibition at 100, 200, and 500 µg/ml, and rat serum partially inhibited oxidation at 500 µg/ml. A BSA concentration of 200 µg/ml or rat serum concentration of 500 µg/ml was required to achieve the same level of inhibition of oxidation that occurred with about 5.0 µg/ml SP-A or 0.1–0.2 µg/ml SP-D. These data indicate that the two hydrophilic surfactant proteins, SP-A and SP-D, protect surfactant lipids from copper-induced oxidation in vitro, at physiologically relevant concentrations.

Both SP-A and SP-D exhibited very similar antioxidant activity when LDL particles, which include unsaturated phospholipids, triglycerides, cholesterol, and cholesterol esters, were used as the substrates for lipid oxidation (Fig. 2). The IC\textsubscript{50} values for inhibition of copper-induced oxidation of LDL for SP-A and SP-D were 4.8 µg/ml (7.9 nM) and 0.1 µg/ml (0.3 nM), respectively, and complete inhibition of oxidation occurred at 10 and 1 µg/ml, respectively. In contrast, 20 µg/ml of the highly homologous collectin, recombinant rat mannose-binding protein A (rMBP) did not inhibit LDL oxidation (Fig. 2b). Rat serum, albumin, C1q, and IgG had also no effect on LDL lipid oxidation.
oxidation at concentrations of 50 μg/ml (Fig. 2b). Only approximately 2.5 μg/ml SP-A or 0.05 μg/ml SP-D was required to provide the same level of antioxidant protection as 500 μg/ml of rat serum. In the absence of oxidation inhibitors, the absolute TBARS level following a 4-h incubation with 10 μM copper was over 3 times greater for LDL than for surfactant lipids. For this reason, LDL was used as the lipid substrate for kinetic experiments and mutagenesis studies of the collectin antioxidant activities.

**Kinetic Analysis of Collectin Antioxidant Activity**—The temporal relationship between the addition of SP-A or SP-D and the inhibition of LDL oxidation was determined spectrophotometrically by continuously monitoring conjugated diene accumulation associated with exposure to copper (26). This assay is based on the oxidation-dependent rearrangement of 1,4-penta dienyl double bonds of LDL lipids to 1,3-butadienyl double bonds, which absorb in the ultraviolet range. In the presence of 10 μM copper at room temperature, LDL particles resist oxidation for up to 100 min as endogenous antioxidants such as α-tocopherol are consumed (Fig. 3). At that point, the rate of oxidation increases in proportion to the concentration of initiating radicals, reaching a plateau when all unsaturated fatty acids are consumed. When SP-A (Fig. 3c) or SP-D (Fig. 3d) proteins were included at zero time, they blocked the accumulation of conjugated dienes in a dose-dependent manner. With increasing surfactant protein concentrations, the predominant change was a decrease in the slope during the rapid oxidation phase, consistent with inhibition of free radical chain initiation or with free radical chain termination. The concentrations of SP-A and SP-D that prevented conjugated diene formation were very similar to those that were required to block TBARS formation. When fully suppressive concentrations of SP-A (10 μg/ml) (Fig. 3c) or SP-D (1 μg/ml) (Fig. 3d) were added at various time points during the propagation phase of oxidation, conjugated diene formation was completely arrested at the point of addition. The kinetics of oxidation inhibition by the collectins were distinctly different from those of 2 mM EDTA, which blocked conjugated diene accumulation only after a significant lag period (Fig. 3d, inset). These data indicate that SP-A and SP-D directly interfere with lipid oxidation.

**Effects of Pulmonary Collectins on Oxidant-induced Cell Death**—Exposure of cultured mammalian cells to t-BOOH promotes a variety of toxic events including depletion of glutathione, mitochondrial dysfunction, and peroxidation of membrane lipids (28). To determine if SP-A and SP-D protect cells from oxidative stress, RAW 264.7 murine macrophages were exposed to 40 μM t-BOOH for 24 h in the presence of SP-A or SP-D at concentrations from 0.01 to 50 μg/ml (Fig. 4). Cell death was assessed by staining cells with the vital dye, trypan blue. We found that both SP-A and SP-D protected RAW cells from t-BOOH-induced death in a dose-dependent fashion that was half-maximal at concentrations of 0.52 μg/ml for SP-A and 0.56 μg/ml for SP-D and that reached a plateau at a concentration of approximately 1 μg/ml for both proteins. These data indicate that both SP-A and SP-D protect macrophages from oxidant stress.

**Pulmonary Collectin Domains in Prevention of Lipid Oxidation**—To determine the domain(s) of SP-A that are responsible for oxidation inhibition, the effect of the addition of 2 mM EDTA (d, inset) at 65 min (closed triangles) and 125 min (closed circles) during the propagation phase of oxidation was also shown, for comparison with the collectins. LDL oxidation in the absence of added collectin (open triangles) or copper (open squares) is also shown.
for protection from copper-induced oxidation of LDL and synthetic lipids, we tested the activity of mutant recombinant SP-A containing deletions in N-terminal domains in the TBARS assay (Fig. 5). Wild type recombinant SP-A inhibited copper-induced oxidation of LDL to the half-maximal point at 2.1 μg/ml and to basal levels at 5.0 μg/ml. A mutant SP-A containing a deletion of the collagen-like region (ΔG8–P80) (21) but retaining the N-terminal segment and interchain disulfide bonds was nearly as active as the wild type recombinant protein (IC\textsubscript{50} = 2.8 μg/ml). A non-disulfide-cross-linked trimeric construct solely composed of the neck and CLD region of the protein (ΔN1–P80) (23) was also a potent antioxidant, with an IC\textsubscript{50} of 6.7 μg/ml. Although carbohydrates are known to have antioxidant properties, the protection of lipids by SP-A was not attributable to the oligosaccharide attached to Asn187, since a nonglycosylated neck and CLD construct containing an N187S mutation also inhibited both LDL and surfactant lipid oxidation (ΔN1–P80,N187S) (Figs. 1b and 2b). We conclude that the antioxidant activity of SP-A is localized to the polypeptide sequences in the C-terminal (neck + CLD) domains of the protein.

**Analysis of Antioxidant Mechanisms of the Pulmonary Collectins**—Several experiments were performed to evaluate the role of collectin-mediated copper chelation in the prevention of lipid oxidation (Figs. 6 and 7). First, the ability of SP-A to inhibit the oxidation of surfactant lipids in the presence of 2 mM lipid oxidation (Figs. 6 and 7). First, the ability of SP-A to inhibit the oxidation of surfactant lipids in the presence of 2 mM lipid oxidation (Figs. 6 and 7). First, the ability of SP-A to inhibit the oxidation of surfactant lipids in the presence of 2 mM lipid oxidation (Figs. 6 and 7). First, the ability of SP-A to inhibit the oxidation of surfactant lipids in the presence of 2 mM lipid oxidation (Figs. 6 and 7). First, the ability of SP-A to inhibit the oxidation of surfactant lipids in the presence of 2 mM lipid oxidation (Figs. 6 and 7). First, the ability of SP-A to inhibit the oxidation of surfactant lipids in the presence of 2 mM lipid oxidation (Figs. 6 and 7). First, the ability of SP-A to inhibit the oxidation of surfactant lipids in the presence of 2 mM lipid oxidation (Figs. 6 and 7). First, the ability of SP-A to inhibit the oxidation of surfactant lipids in the presence of 2 mM lipid oxidation (Figs. 6 and 7).
treated SP-A. Carbonyl modification of iodoacetamide-treated SP-D was more difficult to assess due to lower levels of SP-D in the mixture and the complexity of bands near 40 kDa. We

Finally, we addressed the possibility that copurifying or contaminating factors were responsible for the antioxidant effects of the collectins. All proteins, including rat SP-A and rat SP-D, mouse SP-D, and wild type and mutant recombinant collectins were greater than 95% pure of contaminating proteins based on inspection of SDS-polyacrylamide gels, as previously reported (23, 29). It is important to note that SP-A and SP-D purified from different sources, including rat lavage, mouse lavage, and cell culture systems, were all efficacious despite substantial differences in the purification schemes. All reagents were extensively dialyzed to remove EDTA or other diffusible contaminants. The maximum possible concentration of EDTA in our reagents after dialysis and dilution in the reaction mixture was less than 1 pm, much lower than the 5 μM we had determined to be required to inhibit surfactant lipid or LDL peroxidation by 10 μM copper (data not shown). To examine the role of nondialyzable factors in the antioxidant properties of SP-A, we purified human SP-A from the bronchoalveolar lavage of patients with alveolar proteinosis, a rare lung disease that results in accumulation of large amounts of surfactant proteins and lipids in the airspaces. The AP-SP-A is purified by a sedimentation method that does not include butanol extraction, and it is more easily obtained in large quantities than SP-A from rodents. The extensively dialyzed AP-SP-A completely inhibited copper-induced oxidation of surfactant lipids at a concentration of 10 μg/ml (to 17.2 ± 2.6% of control TBARS). Duplicate concentrated samples of AP-SP-A (36 μg/ml) had undetectable levels of ubiquinone or the lipid-soluble antioxidants lutein, zeaxanthin, cryptoxanthin, lycopene, α-carotene, β-carotene, retinol, retinyl-palmitate, α-tocopherol, δ-tocopherol, and γ-tocopherol (not shown).

**DISCUSSION**

Our results indicate that SP-A and SP-D have potent, direct antioxidant properties. The surfactant proteins protect unsaturated phospholipids and growing cells from oxidative injury at concentrations that are well within their physiologic ranges. The antioxidant activity of SP-A is found in the C-terminal region of the protein, which includes the C-type lectin domain. The C-type lectin family is noted for functional diversity as pattern recognition-dependent opsonins, cell adhesion molecules, cell surface receptors, and anti-freeze proteins (7). The antioxidant function may be unique to the pulmonary collectin subgroup of the C-type lectins, however, since neither the structurally related complement protein, C1q, nor the highly homologous serum collectin, MBP-A, had antioxidant activity in our study.

The mechanism by which pulmonary collectins protect lipids from oxidation was examined using several approaches. Although BSA and rat serum inhibited oxidation of lipids and LDL particles, the concentrations required were 50–500-fold higher than for SP-A and SP-D, respectively. Collectin-mediated copper chelation probably does not account for the antioxidant properties, since there was a greater than 104-fold molar excess of copper to collectin in reactions that demonstrated complete inhibition of oxidation. In addition, neither the occupation of the known SP-A metal binding sites by coincubation with 2 mM Ca2+, extensive dialysis against 20 mM iodoacetamide, nor presaturation of SP-A or SP-D with excess copper blocked the collectin antioxidant activities. SP-A also protects lipids from oxidation by iron, indicating that the antioxidant property of the protein is not specific to copper. The stoichiometry of the reaction indicates that substrate sequestration is an implausible antioxidant mechanism, since at inhibitory concentrations the molar ratios of surfactant phospholipid/protein were >104 for SP-A and >105 for SP-D. We considered that the surfactant proteins may have altered the accessibility of the lipid vesicles to copper or free radicals by causing aggregation, but SP-D does not aggregate phosphatidylethanolamine vesicles, and experiments were done under calcium-free and physiologic pH conditions, which do not support aggregation by SP-A (30, 31). The proteins do not themselves become modified during lipid oxidation and therefore do not protect lipids by functioning as “suicide” sinks for covalent attack by reactive lipid intermediates. SP-A and SP-D arrest LDL oxidation almost instantaneously when added during the propagation phase, suggesting that the collectins directly interfere with lipid oxidation by inhibiting the formation of lipid radicals or by acting as free radicals.
radical chain terminators. The very low concentrations of the proteins that are required for protection suggest that the surfactant proteins may inhibit lipid oxidation through a catalytic mechanism, such as by functioning as peroxidases, but as yet there is no direct evidence for enzymatic activity in the collectin family.

An alternative hypothesis is that the collectins perform a chain termination function through their capacity to recognize, approximate, and extinguish lipid radicals. The antioxidant activity of SP-A maps to the region spanning amino acids 80–226, containing the neck and CLD of the protein. The CLD of SP-A contains the phospholipid binding site (22, 32, 33). The observation that SP-A binds much more avidly to saturated phosphatidylcholine than to unsaturated phosphatidylcholine indicates that the lipid binding pocket of the protein is sensitive to specific configurations of fatty acid side chains (34). We speculate that SP-A may bind lipid peroxyradicals through the recognition of the peroxy group and/or the characteristic trans (1,3-buta dienyl) double bond configuration present in oxidized lipids and extinguish lipid oxidation chain reactions by the approximation of two or more lipid radicals. This mechanism could potentially account for the rapid quenching of conjugated diene formation during the propagation phase of LDL peroxidation. In addition, the finding that SP-A does not inhibit lipid peroxidation induced by the water-soluble free radical initiator AAPH is consistent with this model, since the radical species generated by the compound are not lipids. Thus, even if SP-A is able to inhibit the free radical chain reaction between unsaturated phospholipids in the target substrate, the constant flow of AAPH-derived radicals that are not recognized or intercepted by SP-A could produce persistent lipid peroxidation in the presence of the protein.

The pulmonary collectins also protect growing cells from oxidant stress. The mechanism of t-BOOH-induced cell death is thought to be dependent on metal ions (35), mediated by free radicals generated through the iron-dependent Fenton reaction (36) or by copper-induced oxidation of lipid hydroperoxides (37). SP-A and SP-D are large hydrophilic molecules that almost certainly exert their antioxidant effects in the extracellular compartment. Based on the results of our lipid oxidation experiments, we believe that the lung collects protect cells by interfering with the formation of free radicals or by free radical chain termination in the extracellular space or within the plasma membrane. It is possible, however, that the proteins alter the cellular response to oxidant stress by binding to cell surface molecules and activating critical signaling pathways.

The lung is exposed to oxidant stress through inhalation of oxygen present in the atmosphere; exposure to ozone, trace metals, and other components in air pollution; and oxidant species released from macrophages and neutrophils in the ELF. In addition, the ELF from normal subjects has been shown to contain chelatable redox-active iron that is derived from endogenous sources (38). Although surfactant is inherently resistant to oxidation due to the predominance of saturated phospholipids, oxidizable cholesterol and unsaturated phospholipids represent approximately 35% of the weight of surfactant, and 15% of all surfactant phospholipids contain two or more double bonds (39). The properties of SP-A and SP-D to inhibit the propagation of free radical chain reactions may prevent waves of lipid oxidation from spreading through the expansive (approximately 100 m²) surfactant lining. Since SP-A is intimately associated with surfactant lipids and aggregates in the airspace and since SP-D is found primarily free in the ELF, we speculate that the pulmonary collectins perform complementary functions to protect the lipid interfaces and the aqueous compartment of the ELF from oxidative stress.

Recent data from collectin-deficient animal models suggest a role for the surfactant proteins in the modulation of oxidant stress. The SP-D-deficient gene-targeted mouse, which also had reduced SP-A levels, has enhanced oxidant production in the lung and develops a surfactant lipid clearance defect (18, 19, 40). The lack of SP-D increased hydrogen peroxide production by isolated alveolar macrophages, but a direct, acellular antioxidant role for SP-D was not examined in those studies (18). On the other hand, the SP-A-deficient gene-targeted mouse, which had normal SP-D levels, did not have a clear surfactant homeostasis phenotype (41). We speculate that combined SP-A and SP-D deficiency in the SP-D null mouse results in surfactant oxidation that overwhelms the clearance capacity of surfactant metabolizing enzymes but that sufficient SP-D is present in the SP-A null mouse to protect the surfactant system from oxidative injury. This issue will be most clearly resolved by directly examining the surfactant phospholipids from the single knockout animals for evidence of peroxidation and by determining the surfactant phenotype of the combined SP-A/SP-D gene-targeted animals under development in several laboratories.

The recent observation that all of the major vertebrate groups have lung collectins in their airspaces, including the most primitive amphibious fish, underscores the fundamental importance of these proteins to pulmonary function (38, 42). We conclude that the harmful effects of air breathing on the oxidation-sensitive cellular and molecular components at the environmental interface of the lung may be mitigated by the presence of surfactant proteins A and D.

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REFERENCES
1. Goerke, J. (1998) Biochim. Biophys. Acta 1408, 79–89
2. Matalon, S., Holm, B. A., and Notter, R. H. (1987) J. Appl. Physiol. 62, 756–761
3. Masneé, W., and Rahman, I. (1999) Am. J. Respir. Crit. Care Med. 160, (suppl.) 58–65
4. Qin, X., Swartefeger, D. K., Zheng, S., Hui, D. Y., and Tso, P. (1998) Am. J. Physiol. 274, H1836–H1840
5. Hayek, T., Oknine, J., Brook, J. G., and Aviram, M. (1994) Biochem. Cell Biol. 72, 1567–1574
6. Lawson, P. R., and Reid, K. B. (2000) Immunol. Rev. 173, 66–78
7. Dickerman, K. (1999) Curr. Opin. Struct. Biol. 9, 585–590
8. Voss, T., Eistetter, H., Schafer, K. P., and Engel, J. (1988) J. Mol. Biol. 201, 219–227
9. Crouch, E., Chang, D., Rust, K., Persson, A., and Heuser, J. (1994) J. Biol. Chem. 269, 15848–15853
10. Haageman, H. P., Sargeant, T., Hauschka, P., Benson, B. J., and Hawgood, S. (1990) Biochemistry 29, 8894–8900
11. Hakansson, K., Lim, N. K., Hoppe, H. J., and Reid, K. B. (1999) Structure Fold Des. 7, 255–264
12. Wright, J. J. (1997) Physiol. Rev. 77, 931–962
13. Crouch, E. C. (1998) Am. J. Respir. Cell Mol. Biol. 19, 177–201
14. Cockshutt, A. M., Weitz, J., and Possmayer, F. (1999) Biochemistry 38, 8424–8429
15. Suzuki, Y., Fujita, Y., and Kogishi, K. (1989) Am. Rev. Respir. Dis. 140, 75–81
16. Fisher, A. B., Dodia, C., and Chandler, A. (1994) Am. J. Physiol. 267, L335–L341
17. Touqui, L., and Arbibe, L. (1999) Mol. Med. Today 5, 244–249
18. Wert, S. E., Yoshida, M., LeVine, A. M., Ikegami, M., Jones, T., Ross, G. F., Fisher, J. H., Korthagen, T. R., and Whitsett, J. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5972–5977
19. Botas, C., Poulain, F., Akiyama, Y., Brown, C., Allen, L., Goerke, J., Clements, J., Carlson, E., Gillespie, A. M., Epstein, C., and Hawgood, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11869–11874
20. Dethloff, L. A., Gilmore, L. B., Brody, A. R., and Hook, G. E. R. (1986) Biochem. J. 233, 111–118
21. McCormack, F. X., Pattanajivitai, S., Stewart, J. J., Possmayer, F., Inckley, K., and Voelker, D. R. (1997) J. Biol. Chem. 272, 27971–27979
22. McCormack, F. X., Stewart, J. J., Voelker, D. R., and Damodarasamy, M. D. (1997) Biochemistry 36, 13963–13971
23. McCormack, F. X., Damodarasamy, M., and Elhalwagi, B. M. (1999) J. Biol. Chem. 274, 3173–3183
24. Suwabe, A., Mason, R. J., and Voelker, D. R. (1996) Arch. Biochem. Biophys. 327, 285–291
25. Gelvan, D., and Saltman, P. (1990) Biochim. Biophys. Acta 1035, 353–360
26. Esterbauer, H., Gebicki, J., Puhl, H., and Jurgens, G. (1992) Free Radical Med. Biol. 13, 341–390
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27. Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., Ahn, B. W., Shaltiel, S., and Stadtman, E. R. (1990) *Methods Enzymol.* **186**, 464–478
28. Sestili, P., Brambilla, L., and Cantoni, O. (1999) *FEMS Lett.* **457**, 139–143
29. Ogawara, Y., McCormack, F. X., Mason, R. J., and Voelker, D. R. (1994) *J. Biol. Chem.* **269**, 29785–29792
30. Efrati, H., Hawgood, S., Williams, M. C., Hong, K., and Benson, B. J. (1987) *Biochemistry* **26**, 7986–7993
31. Hawgood, S., Benson, B., and Hamilton, R. J. (1985) *Biochemistry* **24**, 184–190
32. Kuroki, Y., McCormack, F. X., Ogawara, Y., Mason, R. J., and Voelker, D. R. (1994) *J. Biol. Chem.* **269**, 29783–29800
33. McCormack, F. X., Kuroki, Y., Stewart, J. J., Mason, R. J., and Voelker, D. R. (1994) *J. Biol. Chem.* **269**, 29801–29807
34. Kuroki, Y., and Akino, T. (1991) *J. Biol. Chem.* **266**, 3068–3073
35. Miyata, M., and Smith, J. D. (1996) *Nat. Genet.* **14**, 55–61
36. Buettner, G. R. (1993) *Arch. Biochem. Biophys.* **300**, 535–543
37. Patel, R. P., Svistunenko, D., Wilson, M. T., and Darley-Usmar, V. M. (1997) *Biochem. J.* **322**, 425–433
38. Gutteridge, J. M., Munday, S., Quinlan, G. J., Chung, K. F., and Evans, T. W. (1996) *Biochem. Cell Biol.* **74**, 1024–1027
39. Postle, A. D., Mander, A., Reid, K. B., Wang, J. Y., Wright, S. M., Moustaki, M., and Warner, J. O. (1999) *Am. J. Respir. Cell Mol. Biol.* **20**, 90–98
40. Korfhagen, T. R., Shefetlevich, V., Burhans, M. S., Bruno, M. D., Ross, G. F., Wett, S. E., Stahlman, M. T., Jobe, A. H., Ikegami, M., Whitsett, J. A., and Fisher, J. H. (1998) *J. Biol. Chem.* **273**, 28438–28443
41. Korfhagen, T. R., Bruno, M. D., Ross, G. F., Huelman, K. M., Ikegami, M., Jobe, A. H., Wett, S. E., Stripp, B. R., Morris, R. E., Glasser, S. W., Bachurski, C. J., Iwamoto, H. S., and Whitsett, J. A. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 9594–9599
42. Sullivan, L. C., Daniels, C. B., Phillips, I. D., Orgeig, S., and Whitsett, J. A. (1998) *J. Mol. Evol.* **46**, 131–138
Pulmonary Surfactant Proteins A and D Are Potent Endogenous Inhibitors of Lipid Peroxidation and Oxidative Cellular Injury
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Pulmonary surfactant proteins A and D are potent endogenous inhibitors of lipid peroxidation and oxidative cellular injury.

James P. Bridges, Harold W. Davis, Mamatha Damodarasamy, Yoshio Kuroki, Gabriel Howles, David Y. Hui, and Francis X. McCormack

The purification procedure for all surfactant proteins used in this study included affinity adsorption to carbohydrate columns, elution with EDTA, and dialysis to remove EDTA. We recently discovered that EDTA exhibits atypical dialysis behavior, and despite extensive dialysis, our final SP-A and SP-D preparations contained EDTA. Our conclusion that SP-A and SP-D inhibit surfactant lipid peroxidation at protein concentrations that are physiologically plausible has not changed, but the inhibitory potency of the proteins is less than we originally reported.

We intend to submit a methods article to another journal that outlines a scheme for purification of EDTA-free SP-A and SP-D.