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Dysfunction of pulmonary surfactant mediated by phospholipid oxidation is cholesterol-dependent

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ABSTRACT

Pulmonary surfactant forms a cohesive film at the alveolar air-lung interface, lowering surface tension, and thus reducing the work of breathing and preventing atelectasis. Surfactant function becomes impaired during inflammation due to degradation of the surfactant lipids and proteins by free radicals. In this study, we examine the role of reactive nitrogen (RNS) and oxygen (ROS) species on surfactant function with and without physiological cholesterol levels (5–10%). Surface activity was assessed in vitro in a captive bubble surfactometer (CBS). Surfactant chemistry, monolayer fluidity and thermodynamic behavior were also recorded before and after oxidation. We report that physiologic amounts of cholesterol combined with oxidation results in severe impairment of surfactant function. We also show that surfactant polyunsaturated phospholipids are the most susceptible to oxidative alteration. Membrane thermodynamic experiments showed significant surfactant film stiffening after free radical exposure in the presence of cholesterol. These results point to a previously unappreciated role for cholesterol in amplifying defects in surface activity caused by oxidation of pulmonary surfactant, a finding that may have implications for treating several lung diseases.

1. Introduction

Pulmonary surfactant prevents alveolar collapse, maintains patency of small airways and reduces the work required for breathing [1]. Pulmonary surfactant consists of 80–90% (by weight) phospholipids, with a large proportion (~30–45%) being disaturated dipalmitoylphosphatidylcholine (DPPC) [2], whereas unsaturated phospholipids [3] account for the remaining 50–60% whereas the levels of polyunsaturated species are reported as > 10% of total phospholipids [2,4]. Neutral lipids, primarily cholesterol, make the remaining 2–10% of surfactant [5–7]. Surfactant also contains four surfactant-associated proteins – SP-A, B, C, and D [8]. A highly surface-active form of pulmonary surfactant, referred to as the large aggregate fraction, is enriched in the hydrophilic protein SP-A and hydrophobic proteins SP-B and SP-C.

The release of free radicals from activated leukocytes [9] and/or following exposure to environmental pollutants [10] may result in a highly oxidizing milieu within the lungs. Increased levels of reactive oxygen species and their byproducts have been detected in bronchoalveolar lavage fluid collected from patients with acute respiratory distress syndrome (ARDS), asthma, cystic fibrosis (CF), ventilator-induced lung injury (VILI), chronic obstructive pulmonary disease (COPD) and other disease states [9–11]. Several studies have shown that oxidation of pulmonary surfactant greatly impairs in vitro and in vivo function [11–16]. This has so far been largely attributed to oxidative alterations of susceptible residues in SP-B and SP-C, whereas peroxidation and hydrolysis of phospholipids were considered less important [17]. Another mechanism resulting in surfactant dysfunction is elevation of cholesterol to 15% w/w or more with respect to total phospholipids [5,18], even in the absence of other inhibitory surfactant alterations such as oxidation.

An unaddressed question arising from these studies concerns the

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relationship between cholesterol and oxidation-induced surfactant dysfunction. In particular, it remains unknown whether oxidative alterations to surfactant are affected by normal or increased levels of cholesterol to augment surfactant dysfunction or, conversely, whether oxidative surfactant dysfunction can be reversed by removal of cholesterol. We have shown previously that oxidation of a cholesterol-depleted pulmonary surfactant interacts with physiological levels of cholesterol to detrimentally affect in vitro surfactant function [5]. In addition, we have shown that lung lavage cholesterol level is elevated in pediatric CF patients, and that the interaction between the elevated cholesterol and oxidation in the presence of inflammation results in surfactant impairment [11]. In the present study, we assess in greater detail the contribution of phospholipid oxidation to cholesterol-mediated surfactant dysfunction. This is in an effort to better understand the biophysical basis for surfactant dysfunction seen in a variety of inflammatory respiratory diseases.

In this study we employed a captive bubble surfactometer (CBS) to assess the surface activity of bovine lipid extract surfactant (BLES) exposed to Fenton-like and Peroxynitrite-induced oxidation. BLES is typically depleted of cholesterol compared to completely natural surfactants [2]. In the CBS, the surfactant is spread at the air-buffer interface of a trapped bubble and its size varied while measuring surface tension. This procedure replicates surfactant performance in the lung during the breathing cycle [19]. Fenton reaction-like oxidation is an established method to mimic the oxidative environment in the inflamed lung [17]. We supplemented BLES, from which cholesterol is largely removed, with varying amounts of cholesterol to match physiological levels. Both surfactant monolayer fluidity and thermodynamic behavior were also recorded before and after oxidation.

2. Experimental procedure

2.1. Materials

BLES was a kind gift from BLES Biochemicals Inc. (London, ON, Canada). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG), and bovine heart cardiolipin (CL, predominantly 1′,3′-Bis[1,2-dilinoleoyl-*sn*-glycero-3-phospho]-*sn*-glycerol) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). All lipids were stored at −20 °C under nitrogen. Surfactant mixtures were stored at 4 °C under nitrogen and used within three days after preparation. Buffers containing HEPES were stored in the dark at 4 °C to avoid inadvertently generating H_2O_2.

2.2. In vitro oxidation

Clinical surfactant (BLES), at a concentration of 27 mg/ml surfactant phospholipids, was exposed to hydroxyl radicals generated from Fenton-like chemistry for 24 h to produce oxidized BLES (oxBLES) (as described by Manzanares et al. [17]). DPPC, POPC, PLPC, DPPG, and CL were suspended in saline with 1.5 mM $CaCl_2$ and exposed to identical oxidizing conditions and are indicated as oxDPPC, oxPOPC, oxPLPC, oxDPPG and oxCL Oxidation of surfactant phospholipids was confirmed by measuring the formation of the secondary lipoperoxidation products malondialdehyde (MDA) and 4-hydroxyalkenal (4-HAE) in 1.0 mg/ml oxBLES (BIOXYTECH LPO-586, OxisResearch, Burlingame, CA, USA) and via High-resolution ESI-QTOF mass spectrometry (MS).

2.3. Peroxynitrite-mediated oxidation

Peroxynitrite was synthesized in a quenched-flow reactor as previously described [15]. Solutions of 0.6 M $NaNO_2$ and 0.6 M HCl/0.7 M H_2O_2 were pumped at 10 ml/s into a tee-junction and mixed in a 3-mm diameter × 10-cm glass tube. The acid catalyzed reaction of nitrous acid with H_2O_2 to form peroxynitrous acid was quenched by pumping 1.5 M NaOH at the same rate into a second tee-junction at the end of the glass tubing. The solution was frozen at −20 °C for as long as a week. The top yellow layer, formed due to freeze fractionation, contained −170-220 mM peroxynitrite as determined by absorbance at 302 nm in 1 M NaOH (nm = 1670 $M^{-1}\cdot cm^{-1}$). Interference by other absorbing compounds (e.g., nitrate) was corrected by subtracting the final absorbance after adding peroxynitrite to 100 mM potassium phosphate (pH 7.4). Peroxynitrite was inactivated (decomposed) by incubation in a HEPES solution [15].

Reactive nitrogen species (RNS) mediated surfactant oxidation was performed based on Radi et al. methods [20]. 100 µl of 27 mg/ml BLES was dried (using nitrogen gas) and resuspended in no-sucrose Goerke's buffer (140 mM NaCl, 10 mM HEPES, 2.5 mM $CaCl_2$, pH 6.9), 30 µl of 0.05 mol/l phosphate buffer, and 1 µl of 30% peroxynitrite. The phosphate buffer (1 M monosodium phosphate, 1 M disodium phosphate) was prepared in 500 ml of distilled water with 78.0 g of monosodium phosphate and 89.0 g of disodium phosphate. The pH was adjusted to 6.9 with the addition of phosphoric acid. The solution was then diluted with distilled water to 1.0 L with a molar concentration of 0.5 M. In a separate tube, an aliquote of buffer was diluted to a 1:10 ratio with molar concentration of 0.05 M. 3 µl of peroxynitrite was then added to the mixture (final concentration of 30 mM peroxynitrite). The reaction was incubated for 15 min, during which time the solution was bubbled with nitrogen gas. The solution was vortexed every 3 min during this period. Afterwards, an organic extraction was performed by using Bligh-Dyer organic extraction (chloroform:methanol 2:1), and the solution was dried completely using nitrogen gas. BLES oxidized in this manner was resuspended in no-sucrose Goerke's buffer prior to testing.

2.4. Surfactant preparation

To ensure accurate mixing, additional lipids were added to lipid extracts of BLES or oxBLES as organic solutions. The mixtures were then dried under nitrogen and resuspended in sucrose-free Goerke's buffer to a phospholipid concentration of 27 mg/ml. Phospholipid concentration was assessed using a phosphate assay (Gentaur Molecular Products, Kampenhout, Belgium) after liberation of free phosphate [21]. Total phospholipid concentration was 96.8 ± 6.8% (n = 6) after lipid extraction and 98.6 ± 11.1% (n = 12) after oxidation. BLES contained 2.60% cholesterol (w/w phospholipids) as determined using an enzymatic assay for cholesterol (Amplex Red Cholesterol Assay, Invitrogen, Eugene, OR, USA).

2.5. Surface activity assessment

Surface activity of surfactant was determined with a computer-controlled captive bubble surfactometer (CBS) as described in detail by Gunasekara et al. [22], with two important changes: 1) A transparent capillary was used to deposit a ~1.0 µl bolus of 27 mg/ml surfactant near the air-buffer interface. The subphase of the CBS consisted of Goerke's buffer containing 10% *w/v* sucrose to facilitate deposition of the surfactant bolus at the air-buffer interface. The quantity and concentration of surfactant were chosen to minimize effects caused by insufficient surfactant or bulk phase adsorption. This amount of native BLES reliably reproduces minimum and maximum surface tensions as measured in situ at functional reserve capacity and total lung capacity (< 1 mN/m and ~30 mN/m, respectively) [22]. In a subset of experiments, a transparent capillary was used to deposit 54.0 µl of 0.5 mg/ml surfactant to assess differences in surfactant function caused by changes in concentration. 2) A conditioning step was performed to ensure consistent surface activity in subsequent compression-expansion cycles. The bubble was first quasi-statically compressed to ~20% of the maximum volume and subsequently expanded to set minimum and maximum volumes. Afterwards, the bubble volume was dynamically cycled