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The role of multilayers in preventing the premature buckling of the pulmonary surfactant

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ABSTRACT

The pulmonary surfactant is a protein-lipid mixture that spreads into a film at the air-lung interface. The highly-compacted molecules of the film keep the interface from shrinking under the influence of otherwise high surface tension and thus prevent atelectasis. We have previously shown that for the film to withstand a high film pressure without collapsing it needs to assume a specific architecture of a molecular monolayer with islands of stacks of molecular multilayers scattered over the area. Surface activity was assessed in a captive bubble surfactometer (CBS) and the role of cholesterol and oxidation on surfactant function examined. The surfactant film was conceptualized as a plate under pressure. Finite element analysis was used to evaluate the role of the multilayer stacks in preventing buckling of the plate during compression. The model of film topography was constructed from atomic force microscope (AFM) scans of surfactant films and known physical properties of dipalmitoylphosphatidylcholine (DPPC), a major constituent of surfactant, using ANSYS structural-analysis software. We report that multilayer structures increase film stability. In simulation studies, the critical load required to induce surfactant film buckling increased about two-fold in the presence of multilayers. Our *in vitro* surfactant studies showed that surface topography varied between functional and dysfunctional films. However, the critical factor for film stability was the anchoring of the multilayers. Furthermore, the anchoring of multilayers and mechanical stability of the film was dependent on the presence of hydrophobic surfactant protein-C. The current study expands our understanding of the mechanism of surfactant inactivation in disease.

1. Introduction

Lung compliance is dominated by the surface tension of the hydration layer covering the alveolar interphase [1]. This aqueous interface is covered by a molecular film of pulmonary surfactant. The surfactant prevents the interface from contracting under the influence of surface tension [1,2]. In cystic fibrosis (CF) and acute respiratory distress syndrome (ARDS), dysfunctional surfactant is unable to form a stable film leading to alveolar and/or small airway collapse, decreased compliance, and impaired gas exchange [3,4]. The cause for the unstable film has been ascribed to impaired lipid metabolism, exacerbated levels of neutral lipids, and oxidative damage of the surfactant lipids and proteins [4–8].

Surfactant chemical composition is conserved among most mammalian species, consisting of 80–90% phospholipids (*primarily dipalmitoylphosphatidylcholine*, *DPPC*), 2–8% neutral lipid, and 5–10% four surfactant specific proteins (SP-A, SP-B, SP-C, and SP-D) [9–11]. The high proportion of DPPC is thought necessary for the surfactant to be able to reach near zero surface tension during film compression, whereas, unsaturated phospholipids act as liquefiers for efficient adsorption and rapid film spreading [12].

Viable Multilayer formation is dependent on the presence of hydrophobic surfactant proteins SP-B and SP-C (8.7 kDa and 3.5 kDa, respectively) [13,14]. It was previously shown that cationic surfactant proteins

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stabilize the surfactant film by electrostatic interactions with the anionic lipid constituents, dipalmitoylphosphatidylglycerol (DPPG) and palmitoyloleoylphos-phatidylglycerol (POPG), preventing the loss of surfactant material [15,16].

The function and role of both SP-B and SP-C has been comprehensively studied [17–19]. SP-B and SP-C independently enhance film spreading and stability by facilitating the recruitment of phospholipids into the expanding film. SP-B and SP-C are essential for the formation of tubular myelin, promoting rapid phospholipid insertion into the air-liquid interface, influencing the molecular ordering of phospholipid layer, and the formation of multilayer structures [20]. SP-B and SP-C are also known to be crucial for film function and stability [17,18]. Surfactant protein B reduces surface tension by increasing the lateral stability of the phospholipid monolayer, whereas, SP-C mediates monolayer-multilayer transition [19]. SP-C also enhances re-spreading and stability of DPPC [18,21].

Functional *in vitro* studies show that because of the prolonged elevated pressure surfactant experiences, its composition and structural arrangements are essential in maintaining a functional film [2]. Thus, the mechanical function of surfactant depends on a molecular film architecture of a continuous lipid monolayer interspersed with stacks of lipid layers containing surfactant proteins B and C [1,22,23]. Absent or deformed multilayer stacks were consistently associated with dysfunction. Using computer modeling, the current study explores the role of multilayer stacks in mechanically stabilizing the film under pressure. Specifically, we examined buckling as the main mechanism of failure for a plate under lateral pressure.

Different groups have described their role in reversible surfactant monolayer-multilayer conversion, however, the role of multilayers in preventing premature buckling has not been examined. Here, we considered buckling as a principal failure mechanism. Our hypothesis was that surfactant dysfunction is due to impairment of multilayer formation or the anchoring of the multilayers in the monolayer. We conceptualized the surfactant film as a plate under pressure and used finite element analysis software to evaluate the role of the molecular multilayer stacks in preventing buckling of the plate. We compare results using modeling to experimental findings with various surfactant model systems.

2. Methods and materials

2.1. Preparation of the surfactants

Bovine lipid extract surfactant (BLES) with a phospholipid concentration of 27 mg/ml was a gift by the manufacturer (BLES Biochemical, London, Ontario). Cholesterol was purchased from Sigma Chemicals (St. Louis, MO). A solution of 1:1:1 ratio of methanol, chloroform, and BLES by volume was first vortexed and then centrifuged at 100 g for 5 min. The methanol/water phase was discarded and the BLES in chloroform was retained and none, 5% *w*/w, or 20% *w*/w of cholesterol, with respect to phospholipids, was added. Each solution was then dried under N_2 and resuspended with buffer (140 mM NaCl, 10 mM HEPES, and 2.5 mM CaCl2; pH 6.9) to obtain an aqueous suspension of BLES and cholesterol at a concentration of 27 mg/ml phospholipids.

2.2. In vitro oxidation

BLES was exposed to hydroxyl radicals generated from Fenton-like chemistry for 24 h to produce oxidized BLES (oxBLES) as previously described [24]. Oxidation of surfactant phospholipids was confirmed by mass spectrometry and measuring the formation of secondary lipoperoxidation products malondialdehyde (MDA) and 4-hydroxyalkenal (4-HAE) (BIOXYTECH LPO-586, OxisResearch, Burlingame, CA, USA).

2.3. Surface tension assessment

Surface activity of surfactant was determined with a computer-controlled captive bubble surfactometer (CBS). Initially, the chamber of the CBS was filled with buffer solution (140 mM NaCl, 10 mM HEPES, 10% sucrose, and 2.5 mM CaCl2; pH 6.9), where a small (~0.046–0.05 mL) bubble was introduced into the chamber, and a transparent capillary used to deposit ~1.0 μL of 27 mg/mL BLES near the air-buffer interface. The bubble shape was recorded by a video camera (Pulnix TM 7 CN) for later analysis. The chamber was kept at 37 °C throughout the experiment. The initial 5 min adsorption (film formation) period followed the introduction of the surfactant into the chamber. The chamber was then sealed and the bubble rapidly (1 s) expanded to a maximum volume of 0.13 mL. This amount of native BLES (1.0 μL) reliably regenerates 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). The bubble was first quasi-statically compressed to ~20% of the maximum volume and subsequently expanded to 0.13 mL (100%), setting minimum and maximum volumes. Subsequently, Bubble volumes were rapidly and continuously cycled over the same volume range at a rate of 20 cycles/min; reflecting physiological lung function. Interfacial area and surface tension were calculated using the bubble height and diameter. Four quasi-static cycles and a second set of dynamic cycles were carried out afterwards. Measurements of surface tension and interfacial area were made on this second series of cycles.

2.4. Isotherms and film deposition for the microscopy

A Langmuir Wilhelmy balance (LWB) (750 cm^2 area, Nima Technology, Coventry, England) was initially cleaned with distilled H_2O at 65 °C; the water was then removed the trough cleaned with Kim wipe soaked with acetone, then methanol, hexane, and chloroform solutions in the order specified. About 450 ml of distilled water was added to the trough until the meniscus of the buffer is level to the inner rim of the trough. A Wilhelmy paper plate was then placed on the metal links of the pressure sensor, and the plate was lowered into the buffer until the end of the plate was submerged in the trough solution. Then the trough was filled with distilled water. A temperature of 34 °C was established and either a suspensions of bovine lipid extract surfactant (BLES) or 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) added to the fully open air-water interface until a surface tension >12 mN/m was observed. A temperature of 34 °C was chosen after performing differential scanning calorimetry on BLES batches. Prior to film deposition, film-pressure-area isotherms were recorded by reducing and expanding the interfacial area at a rate of 150 cm^2/min. Maximum pressure was set to 50 mN/m. The open area was defined as 696 cm^2. For microscopy, the surfactant film was deposited on glass coverslips by the Langmuir-Blodgett technique [25]. Prior to use the coverslips were cleaned in "Piranha" solution (3:1 v/v H_2SO4: 30% H_2O_2) for about 10–30 min. The slides were then rinsed in HPLC-grade water three times and were kept in Goerke's buffer of 140 mM NaCl, 10 mM HEPES, 5 mM CaCl2, pH 6.9.

2.5. Microscopy

AFM topographical images were collected in air in intermittent contact mode with a NanoWizard II AFM (JPK Instruments, Berlin, Germany) using silicon cantilevers NCH-20 (NanoWorld, Neuchâtel, Switzerland) with typical spring constants of 21–78 N/m, and resonance frequencies of 260–460 kHz. The AFM was fitted on a Zeiss Axio Observer (Zeiss, Jena, Germany).

2.6. Modeling boundaries and parameters

2.6.1. Computer modeling parameter

Surfactant test parameters were based on the known basal thickness of the phospholipid monolayer 2.5 nm and the thickness of the