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Effect of serum proteins on an exogenous pulmonary surfactant: ESR analysis of structural changes and their relation with surfactant activity

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ABSTRACT

The study of the structural changes in surfactant microviscosity and bilayer organization would help to understand the mechanisms by which surfactant could be inactivated by serum components. The in vitro effects of human serum, albumin and gamma-globulin on dynamic and structural properties of surfactant suspensions and their heavy fractions were evaluated by electronic spin resonance and surface tension measurements. Our results showed that albumin and serum modified the aggregation state, transforming the active into inactive subtype, but only serum decreased the fluidity in the polar region and inactivated surfactant. In contrast, albumin and gamma-globulin generated a greater proportion of fluid-like disordered phase, without loss of surface activity. Statistical analysis showed that surface activity correlated with the fluidity in the polar area but not with that in the hydrophobic region. We concluded that one or more serum components different from albumin or gamma-globulin cause a structural change in the surfactant bilayer, increasing the rigidity in the polar area, which would be critical for proper physiological activity.

1. Introduction

Pulmonary surfactant is a complex mixture of phospholipids (80–90%), neutral lipids (5–10%) and at least four specific proteins (5–10%) (SP-A, SP-B, SP-C and SP-D), which is synthesized and secreted by alveolar type II cells (Creuwels et al., 1997). Pulmonary surfactant forms a thin film on the liquid layer lining the alveolar air spaces. Rapid adsorption of pulmonary surfactant in the air–liquid interface is essential for normal breathing. Surfactant deficiency is the leading cause of acute infant respiratory distress syndrome (IRDS), and thus transbronchial surfactant application has become the gold standard for treating this disease (Clements and Avery, 1998). Exogenous surfactants often provide immediate relief of symptoms and improve oxygenation and gas exchange (Günther et al., 2001; Poulain and Clements, 1995; Veldhuizen et al., 1996). However, in certain diseases such as meconium aspiration syndrome and respiratory distress syndrome, substances not normally present in the alveolar fluid make the replacement therapy ineffective. Plasma proteins leaking into the airspaces inhibit the surfactant and raise the alveolar surface tension, a mechanism that might be of pathophysiological importance in adult respiratory distress syndrome (Dargaville and Mills, 2005; Finer, 2004; Poulain and Clements, 1995).

Different theories have been proposed to explain the mechanism of lung surfactant inactivation by serum proteins, but much remains unknown and efforts to rationally construct surfactant formulations appropriate for therapeutic uses in diseases in which the exogenous surfactants are currently ineffective have thus far been frustrating (Ochs et al., 2006; Matthay and Zemans, 2011).

It is known that, in vivo, two surfactant subtypes, with different physiological capabilities, may coexist. They can be separated by centrifugation and are known as subtype heavy or active (present in the pellet) and subtype light or inactive (present in the supernatant) (Gross et al., 2000; Ueda et al., 1994; Veldhuizen et al., 1993). The ratio between small and large surfactant aggregates increases in several types of lung injury (Maitra et al., 2002; Veldhuizen et al., 1996). We have previously found that although the presence of proteins causes disaggregation of the surfactant structures (i.e. induces the transformation of the active into the inactive subtype), this effect was not enough to inactivate the exogenous surfactant. Our results also indicate that surfactant inactivation by serum is probably due to a physical interaction between the surfactant and one or more serum components different from albumin (Martínez Sarrasague et al., 2011).

In the last years, several studies have provided evidence that a particular lateral structure occurs in native membranes of surfactant. Lipid phases, such as gel/fluid and fluid ordered/fluid disordered, coexist in these membranes, a coexistence that allows explaining specific physicochemical properties of the membrane (Nag et al., 2002; Scherfeld et al., 2003). de la Serna et al. (2004) have stated that even though lung surfactant has cholesterol

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concentration of up to 20–22 mol%, there is no clear understanding of how this molecule impacts the lateral structure of the native material (Hook et al., 1984; Lange and Steck, 1985).

Although the mechanism through which pulmonary surfactant spreads at the air–cell interface and the factors that influence that process are not yet resolved, it is known that fluidity is a major factor determining its ability to spread. The study of the structural changes in surfactant microviscosity and bilayer organization would help to understand the mechanisms by which surfactant is absorbed at the air–liquid interface and could be inactivated by serum components (Budai et al., 2003). Among spectroscopic techniques, electron spin resonance (ESR) has proved to be one of the most useful tools applicable in this field. Its dynamic sensitivity is optimally matched to the timescale of the rotational motions of the lipids in biological membranes (Ahlin et al., 2000; Budai et al., 2004; de Sousa Neto et al., 2009; Moser et al., 1989). ESR analysis allows quantifying both the stoichiometry and the selectivity of the interactions of different spin-labeled lipids with the protein and to study the dynamics of the protein-associated lipids. It also reflects changes in the layer mobility. Spin probes produce an ESR spectrum that yields information about the molecular environment of the label (Budai et al., 2003, 2004; Lange and Steck, 1985; Marsh and Horvath, 1998). In the present study, 5-doxyl stearic acid (5DSA) and 16-doxyl stearic acid (16DSA) were chosen as spin probes, which are oriented like the lipids in the surfactant layers. The radical in the 5- or 16-position of the alkyl chain can thus determine local motional profiles near the polar head group (5DSA) or at the end of the hydrophobic chain of the lipid (16DSA) (Nusair et al., 2012).

The analysis by ESR of the deleterious action of serum proteins might help to understand the nature of the *surfactant–protein* interaction, and to establish *structure–function* relations To achieve these objectives, the present study focused on evaluating the in vitro effects of human serum, bovine serum albumin (BSA) and gamma globulin on dynamic and structural properties of surfactant suspensions and their respective heavy subtypes.

2. Materials and methods

2.1. Exogenous pulmonary surfactant (EPS)

Prosurf is an active pharmaceutical ingredient (API) produced at industrial scale in Argentina (Nialtec S.A., Buenos Aires, Argentina). This API has been used by the pharmaceutical industry (GeMePe SA and Richet SA Laboratories) for the elaboration of therapeutic surfactants. Prosurf is a sterile chloroform solution that contains lipids and proteins extracted by means of bronchoalveolar lavage from bovine lungs, with slightly hypertonic solution (Hager and De Paoli, 2001). Prosurf is composed of phospholipids (PL) 94.8%; DPPC (dipalmitoylphosphatidylcholine) 46% of total phospholipids; cholesterol 4.4%; and proteins (SP-B, SP-C) 0.8%.

Chloroform was evaporated at low pressure and below 40 °C; the pellet was resuspended in sterile saline solution (0.9% NaCl) at 50 °C obtaining a final phospholipid concentration of 30 mg/ml. This final suspension, fractionated in sterile vials, constitutes the exogenous pulmonary surfactant (EPS).

To obtain EPS with different cholesterol concentrations, adequate aliquots of cholesterol chloroform solution were added to Prosurf before solvent was evaporated.

2.2. Exogenous proteins

Human serum was obtained from healthy adult donors. A pool of these human serum was used and the concentration of its main components were: total proteins 6.8 g/dl, albumin 3.7 g/dl, gamma globulin 0.9 g/dl, cholesterol 182 mg/dl, triglycerides 80 mg/dl and phospholipids 175 mg/dl.

Bovine serum albumin (BSA) and cholesterol were purchased from Sigma. Gamma globulin was purchased from Spectrum Chemical Mfg. Corp.

2.3. Chemicals

The spin derivatives of stearic acid, 5- and 16-doxyl stearic acids (5DSA and 16DSA, respectively), were purchased from Sigma.

2.4. Chemical determinations

Phospholipid and protein concentrations were measured by the Stewart (1980) and Lowry (1951) methods, respectively. Cholesterol was determined by the enzymatic method (Allain et al., 1974).

All the reagents were of analytical grade.

2.5. Samples

EPS was diluted with saline solution (0.9% NaCl) to a final PL concentration of 10 mg/ml. Samples added with proteins were incubated for 20 min at 37 °C with either BSA, gamma globulin or serum to obtain a final protein concentration of 5, 10 or 20 mg/ml. EPS (10 mg/ml of PL) without added proteins was used as control.

Serum, pure solutions of gamma globulin or albumin (4–60 mg/ml) and EPS (5–30 mg of PL/ml) were also used as controls in ESR analysis.

2.6. Heavy and light subtypes

2.6.1. Isolation

The surfactant subtypes were obtained by centrifugation at 10,000 × *g* for 20 min at room temperature. The supernatants containing the light subtype were separated, and the pellets with the heavy subtype were washed and resuspended to initial volume with saline solution (0.9% NaCl).

2.6.2. Quantification

The percentage of each subtype was estimated as: (PL concentration in the fraction/PL concentration in the non-fractionated EPS) × 100, measured by chemical determination, and by ESR, as described below.

2.7. Electronic spin resonance spectroscopy

The use of hydrophobic spin probes in the study of membranes is well known; however, the method has been little used to study EPS. ESR spectroscopy allows the investigation of structural and dynamic aspects.

The ESR spectrum of the nitroxyl ring in 5DSA and 16DSA is sensitive to the local host environment (Nusair et al., 2012).

2.7.1. ESR samples

An adequate quantity of the spin probe in ethanolic solution was dried onto the sides of the incubation tubes under a stream of N_2 gas. Samples were added and incubated with the spin probe for 10 min at room temperature. The final concentration of the spin probe was 1.74 μM. Each sample was then placed into a capillary tube, and each capillary was placed into a quartz ESR sample tube and centered in a rectangular microwave cavity for ESR measurement.

2.7.2. ESR measurements

ESR measurements were performed using a Bruker EMX-Plus, X-band spectrometer (Germany).