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Pulmonary surfactant inactivation by β-D-glucan and protective role of surfactant protein A

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ABSTRACT

Pulmonary fungal infections lead to damage of the endogenous lung surfactant system. However, the molecular mechanism underlying surfactant inhibition is unknown. β-D-glucan is the major component of pathogenic fungal cell walls and is also present in organic dust, which increases the risk of respiratory diseases. The objective of this study was to characterize the interaction of this D-glucopyranose polymer with pulmonary surfactant. Our results show that β-D-glucan induced a concentration-dependent inhibition of the surface adsorption, respreading, and surface tension-lowering activity of surfactant preparations containing surfactant proteins SP-B and SP-C. Our data support a new mechanism of surfactant inhibition that consists in the extraction of phospholipid molecules from surfactant membranes by β-D-glucan. As a result, surfactant membranes became more fluid, as demonstrated by fluorescence anisotropy, and showed decreased T_m and transition enthalpy. Surfactant preparations containing surfactant protein A (SP-A) were more resistant to β-D-glucan inhibition. SP-A bound to different β-D-glucans with high affinity (K_d = 1.5 ± 0.1 nM), preventing and reverting β-D-glucan inhibitory effects on surfactant interfacial adsorption and partially abrogating β-D-glucan inhibitory effects on surfactant's reduction of surface tension. We conclude that β-D-glucan inhibits the biophysical function of surfactant preparations lacking SP-A by subtraction of phospholipids from surfactant bilayers and monolayers. The increased resistance of SP-A-containing surfactant preparations to β-D-glucan reinforces its use in surfactant replacement therapy.

Introduction

Fungi are the cause of opportunistic infections, predominantly in immunosuppressed patients after solid organ transplantation, patients infected with human immunodeficiency virus, or patients with haematological cancer [15,50]. Fungal colonization of the lungs decreases the survival rate of lung transplant recipients [50] and has powerful effects on asthma development [3]. Fungal infections also induce surfactant dysfunction [2,21,26]. Although the inhibition of surfactant in *Pneumocystis carinii* pneumonia has been related to pulmonary inflammation [60], it has been suggested that direct interaction between surfactant and some components of these microorganisms could contribute to surfactant dysfunction [58]. In this regard, Wang et al. showed that lung surfactant exposure to disrupted *P. carinii* organisms or extracted hydrophobic fungal components significantly reduced the surface activity of lung surfactant [58].

Glucans are the major structural components of the cell wall of many pathogenic fungi [59], including *Aspergillus*, *Candida*, and *Pneumocystis*. Structurally, glucans are polymers of D-glucopyranose, joined by glycosidic linkages between the hemiacetal oxygen at C-1 and C-2, C-3, C-4, or C-6 on the next glucose residue. In the fungal cell wall, the major glucan is (1→3)-β-D-glucan, but other glucans, such as (1→6)-β-glucan, mixed (1→3)-β- and (1→4)-β-linked glucans, and α(1→3)- and α(1→4)-linked glucans have also been found in various fungal cell walls [5,29].

β-D-glucan is also present in organic dust [27,40], a mixture of components from microbial cell walls of Gram-negative and -positive bacteria, mold, and particulate matter (PM) [22]. Exposure to organic dust or PM increases the risk of respiratory diseases, including airway inflammation, toxic pneumonitis, and asthma [22,27,40]. (1→3)-β-D-glucan present in cotton dust produces agglutination of pulmonary surfactant, suggesting direct interaction between glucans and surfactant components [18].

Surfactant is necessary to keep the alveolus open, thereby allowing

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gas exchange. Without surfactant, the alveoli collapse since the surface tension at the air–water interface exerts a collapsing pressure [10,63]. Thus, one of the main pulmonary surfactant functions is to form a stable lipid monolayer at the air–liquid interface that efficiently lowers surface tension to almost 0 mN/m on expiration [10,63]. Pulmonary surfactant is composed of 90 wt% lipids and 10 wt% proteins. The lipids consist mainly of phospholipids (~90–95 wt%) with a small amount of cholesterol and α-tocopherol (~5–10 wt%). Among phospholipids, phosphatidylcholine (PC) is the most prevalent class, accounting for ~80 wt% of the total phospholipids, and dipalmitoylphosphatidlycholine (DPPC) is the most predominant molecular species, accounting for ~45–55 mol% of the total PC [10]. The acidic phospholipids, phosphatidylglycerol and phosphatidylinositol, are mainly unsaturated and account for 8–10 wt% of the total surfactant phospholipid pool [10,63].

Four surfactant proteins have been reported to exist in this material: the hydrophobic proteins SP-B and SP-C, which are inserted in surfactant membranes, and the collectins SP-A and SP-D [10]. SP-B and SP-C enhance the adsorption, spreading, and stability of surfactant lipids required for the reduction of surface tension in the alveolus and, together with surfactant phospholipids, are the major constituents of replacement surfactants used for treatment of respiratory distress syndrome in preterm babies [10,63]. Lung collectins, SP-A (3–4% of the total mass of surfactant) and SP-D (0.5 wt%), are mainly involved in immune innate host defense [11,12,16,33]. They are characterized by an N-terminal collagen-like domain and a globular C-terminal domain that includes a C-type carbohydrate recognition domain (CRD) [11,12]. SP-A, but not SP-D, is mainly associated with surfactant lipids. SP-A's ability to bind lipids provides SP-A with special non-immune functions. Thus SP-A: i) improves the adsorption and spreading of surfactant membranes onto an air-liquid interface [10,63], ii) protects surfactant biophysical activity from the inhibitory action of serum proteins and meconium [10,63], iii) mitigates cholesterol-mediated surfactant disfunction both in vitro and in vivo [10,25,63]; and iv) acts as a scavenger of other surfactant inhibitors [8,43], blocking their effects on surfactant membranes [43]. The absence of SP-A in replacement surfactants has been related to reduced surface activity [6] and increased susceptibility to inactivation [63] with respect to native pulmonary surfactants, which contain SP-A.

We hypothesize that direct interaction between lung surfactant and the fungi component β-glucans could contribute to surfactant dysfunction in fungal infections and overexposure to organic dust or PM. Thus, this study was undertaken to investigate whether the surface activity of pulmonary surfactant can be inhibited by (1→3) (1→4)-β-D-glucan (βG) and to uncover the potential mechanism of βG-induced surfactant inhibition. In addition, we investigated whether SP-A can protect pulmonary surfactant against βG-induced inactivation and we evaluated the mechanism by which SP-A might exert its protective effects.

Materials and methods

Materials

Water soluble (1→3)(1→4)-β-linked glucan (PS-BA-N1500, M_r = 1.5 $\times 10^6$) was acquired from Putus Macromolecular Sci. & Tech. Ltd (China). Size distribution of βG in **buffer A** (5 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.5 mM $CaCl_2$) was determined by dynamic light scattering using a Zetasizer Nano S (Malvern Instruments, Malvern, UK) equipped with a 633-nm HeNe laser as previously described [25]. βG showed two peaks with mean sizes of 56 ± 10 nm and 419 ± 58 nm. (1→3)(1→6)-β-D-glucan was generously supplied as a sample by Putus Macromolecular Sci. & Tech. Ltd. Soluble (1→3)-β-D-glucan phosphate was provided by Prof. D. L. Williams (Department of Surgery, East Tennessee State University) and prepared as previously described [59]. Dipalmitoylphosphatidylcholine (DPPC) and 1-palmitoyl-2-oleoyl-sn--glycero-3-phosphocholine (POPC) were obtained from Avanti Polar Lipids (Birmingham, AL).

The fluorescent lipid probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was purchased from Thermo Fisher Scientific (Waltham, MA). The organic solvents used to dissolve the pulmonary lipid extract were HPLC grade. Water used in monolayer experiments and analytical procedures was deionized and doubly distilled in glass; the second distillation being from dilute potassium permanganate solution.

Isolation of SP-A

Surfactant protein A was isolated from bronchoalveolar lavage of patients with alveolar proteinosis using sequential butanol and octylglucoside extraction [16,33,45,46]. The purity of SP-A was checked by one-dimensional SDS-PAGE in 12% acrylamide under reducing conditions and mass spectrometry. SP-A consisted of supratrimeric oligomers of at least 18 subunits. The oligomerization state of SP-A was assessed by electrophoresis under nondenaturing conditions, electron microscopy, and analytical ultracentrifugation as reported elsewhere [45,46]. Each subunit had an apparent molecular mass of 36,000 Da. Endotoxin content of isolated human SP-A was about 300 pg endotoxin/mg SP-A, as determined by Limulus amebocyte lysate assay (Bio-Whittaker, Walkersville, MD).

Isolation and analysis of pulmonary surfactant and the lipid organic extract of surfactant

Native pulmonary surfactant (NPS) from porcine lungs was obtained as described previously [13,44]. The organic extract of porcine pulmonary surfactant, containing surfactant lipids, SP-B, and SP-C, was obtained by chloroform/methanol extraction [44]. Surfactant membranes were prepared from this organic extract, hereafter referred to as LES (lipid extract surfactant). Total phospholipid concentration in LES and NPS was determined by phosphorus analysis.

Surfactant liposomes

Multilamellar (MLVs) and large unilamellar (LUVs) vesicles of LES were formed in buffer A as described in [42,43]. Briefly, MLVs were prepared by hydrating the dry proteolipid film in buffer A, and allowing it to swell for 1 h at 45 °C. LUVs were obtained from the MLV suspension by sonication at 45ºC during 4 min at 390 W/cm^2 (burst of 0.6 s, with 0.4 s between bursts) in a UP 200 S sonifier with a 2 mm microtip [41-43]. The mean hydrodynamic diameter of the resulting LUVs was 171 ± 9 nm as determined by dynamic light scattering.

Interfacial adsorption assays

The effect of βG on the ability of porcine NPS and LES to adsorb onto and spread at an air-water interface was analyzed with a highly sensitive Wilhelmy-like surface microbalance (NIMA Technologies, Coventry, United Kingdom) coupled to a small Teflon dish, as previously reported [13,41-44,9]. Multilamellar vesicles of NPS and LES (150 μM phospholipids, 112 μg/ml) were injected into the hypophase chamber of the Teflon dish, which contained 1.5 ml of 150 mM NaCl, 25 mM HEPES buffer, pH 7.0, and 0.5 mM $CaCl_2$, in the absence and presence of different βG concentrations and/or SP-A. We used SP-A concentrations as low as 2.5 wt% of the total mass of surfactant lipids since greater SP-A concentrations (5, 10, or 20 wt%) had no further effect on surface adsorption [42]. Interfacial adsorption was measured following the change in surface tension as a function of time at 25.0 ± 0.1ºC.

Monolayer experiments

Monolayer experiments were performed using two different thermostated and magnetically stirred Langmuir-Blodget troughs: a 102 microfilm balance with a total area of 100 cm^2, and a 302RB ribbon barrier film balance, both from NIMA Technologies (Coventry,

England). The ribbon barrier trough was used to minimize film leakage during compression. This trough has a maximum area of 195 cm^2 and a minimum area of 40 cm^2. Surfactant lipids and synthetic phospholipids were dissolved in chloroform/methanol 3:1 (v/v). Phospholipid and LES films were formed over a buffer A subphase and measurements were performed at 25.0 ± 0.1ºC.

Surface pressure-area isotherms: Compression isotherms of mixed DPPC/POPC monolayers, with and without βG, were recorded upon film compression at 50 cm^2/min as previously described [8]. Compression isotherms were analyzed in terms of the compressibility modulus, C_S^{-1}, defined as:

$$C_s^{-1} = -A\left(\frac{d\pi}{dA}\right)_T \quad (1)$$

where A is the molecular area and π the surface pressure.

Cyclic compression-expansion isotherms: Hysteresis curves of solvent-spread porcine LES interfacial films were recorded in the Langmuir-Blodgett trough equipped with a ribbon barrier. Surfactant lipids were spread at the air-water interface to a standard interfacial concentration of 43 $Å^2$/phospholipid molecule. The organic solvent was allowed to evaporate for 15 min before recording seven successive cycles of compression/expansion with a compression ratio of 4.9:1 and a speed of 3.5 min per completed cycle with no lag time between consecutive cycles. To evaluate the effect of βG on cyclic compression-expansion isotherms, the glucan was injected into the subphase once the monolayer was formed (π = 30 mN/m), to yield a final glucan concentration of 0.6 or 1 nM. β-glucan was allowed to interact with the monolayer for 30 min before compression of the interfacial film. On the other hand, to assess the possible protective role of physiological concentrations of SP-A (2.5 wt% of the total mass of surfactant lipids), the protein, alone or mixed with βG, was injected into the subphase and allowed to interact with the surfactant film for 30 min before recording the hysteresis curves. Compression isotherms were analyzed in terms of C_S^{-1} as described above.

Monolayer relaxation kinetics: Relaxation kinetics were obtained with both Langmuir-Blodget troughs by recording the trough surface area as a function of time [8,9]. To that end, the organic solutions of LES or synthetic phospholipids were spread onto a buffer A subphase by microsyringe. After 15 min, the monolayers were compressed to a preset surface pressure of 30 or 47 mN/m, which was kept constant by automatically adjusting the surface area of the trough through the movement of barriers. Once this surface pressure was reached, different amounts of either βG or buffer were injected into the subphase. A relaxation curve was obtained by recording the trough surface area during the relaxation period.

To characterize the effect of βG on LES relaxation kinetics, data were analyzed by fitting to Eq. 2:

$$-\log(A/A0) = k\cdot\sqrt{t} \quad (2)$$

where A and A_0 are the trough areas at a given time t and at t = 0, respectively, and k is the rate of the desorption process [8,9].

Fluorescence experiments

Fluorescence experiments were carried out in buffer A at 25 ± 0.1 °C as described previously [16,41-43,45,46,9], using a SLM-Aminco AB-2 spectrofluorimeter equipped with Glam Prism polarizers and a thermostated cuvette holder (Thermo Spectronic, Waltham, MA, USA). Quartz cuvettes of 5 × 5-mm path length were used.

Fluorescence Emission Anisotropy: The required amounts of phospholipids from lipid organic extract of surfactant were mixed with DPH at a probe/phospholipid molar ratio of 1:200 (final phospholipid concentration of 1 mg/ml) as previously described [41-43,9]. After LUV formation in buffer A, different concentrations of βG were added in the absence and presence of physiological concentrations of SP-A (2.5 wt%). Excitation and emission wavelengths were set at 360 and 430 nm, respectively. For each sample, fluorescence emission intensity data in parallel and perpendicular orientations with respect to the exciting beam were collected 10 times each and then averaged. Anisotropy, r, was calculated as

$$r = (I_{ll} - G\cdot I_{\perp})/(I_{ll} + 2G\cdot I_{\perp}) \quad (3)$$

where I_{ll} and $I_{\perp}$ are the parallel and perpendicular polarized intensities measured with the vertically polarized excitation light, and G is the monochromator grating correction factor.

Binding studies: Binding studies were performed with soluble (1→3)(1→4)-β-D-glucan, (1→3)(1→6)-β-D-glucan, and (1→3)-β-D-glucan phosphate. Tryptophan fluorescence emission spectra of SP-A were recorded in the absence and presence of increasing concentrations of β-D-glucans in buffer A. SP-A samples (with and without β-D-glucan) and blank samples (β-D-glucan alone) were excited at 295 nm and emission spectra recorded from 300 to 400 nm.

The apparent dissociation constants, K_d, for the SP-A/β-D-glucan complexes were obtained by analyzing the tryptophan fluorescence change when 10 μg/ml (15 nM) SP-A reacted with various concentrations of β-D-glucans (0–20 nM). Each titration data point was performed in separated samples, and tryptophan fluorescence emission was monitored 10 min after β-D-glucan addition. The change in the fluorescence of SP-A at 338 nm and 25ºC was monitored as a function of β-D-glucan concentration, and the titration data were analyzed by nonlinear least-squares fitting to the Hill equation, as previously reported [16]:

$$\Delta F/\Delta F_{\max} = [L]^{nH}/\left([L]^{nH} + K_d\right) \quad (4)$$

where ΔF is the change in fluorescence intensity at 338 nm relative to the intensity of free SP-A; ΔF_{max} is the change in fluorescence intensity at saturating β-D-glucan concentrations; K_d is the apparent equilibrium dissociation constant; [L] is the molar concentration of free β-D-glucan; and nH is the Hill coefficient.

Differential scanning calorimetry

Calorimetric measurements were performed in a Microcal MCS differential scanning calorimeter (Microcal Inc., Northampton, MA) at a heating rate of 0.5 °C/min and under an extra constant pressure of 2 atm as previously reported [41-43,9]. Porcine LES multilamellar vesicles (1 mM) in the absence or presence of different concentrations of βG and/or 2.5 wt% SP-A were loaded in the sample cell of the microcalorimeter with buffer A in the reference cell. Three calorimetric scans were collected from each sample between 25 and 60 °C. The standard Microcal Origin software was used for data acquisition and analysis. The excess heat capacity functions were obtained after subtraction of the buffer-buffer baseline.

Statistical analyses

Data are presented as means ± SD. Differences in means between groups were evaluated by one-way ANOVA followed by the Bonferroni multiple-comparison test. For comparison of two groups, Student t-test was used. An α level ≤ 5% ($P \leq 0.05$) was considered significant.

Results

Inhibition of surfactant interfacial adsorption by β-D-glucan

To evaluate the effect of βG on the ability of pulmonary surfactant bilayers to adsorb onto and spread at an air/liquid interface, we recorded the interfacial adsorption kinetics of LES in the absence and presence of βG. Pure MLVs of LES quickly adsorbed to the air/liquid interface, increasing the surface pressure up to an equilibrium value of

47 mN/m (Fig. 1A), as previously shown [42,43]. The maximum surface pressure of LES decreased with increasing amounts of βG in a dose-dependent manner, which indicates that βG inhibits the capability of surfactant membranes to adsorb onto and spread at an air/liquid interface. This inhibition was not due to competitive adsorption since βG showed no interfacial activity (Fig. 1A, B, dashed lines). Interestingly, at the concentrations tested, βG did not affect the adsorption kinetics of NPS (Fig. 1B), nor the equilibrium surface pressure attained. Given that LES is devoid of surfactant protein A, our results suggest that the resistance of NPS to inactivation by βG may be due to the presence of SP-A.

SP-A binds to β-D-glucans

SP-A has been shown to bind to several fungal pathogens [7]. Fungal cell walls consist of mannan, glucan, and chitin components cross-linked in a network. Binding of SP-A to *A. fumigatus*, *C. neoformans*, and *P. carinii* can be inhibited by mannose, maltose, glucose, or mannan, depending on the pathogenic fungus [7]. Here we evaluated the binding of SP-A to β-D-glucan, which can be related to SP-A capability to bind fungal pathogens and to protect pulmonary surfactant against β-D-glucan-induced inactivation. The binding of SP-A to soluble β-D-glucans was followed by changes in intrinsic fluorescence emission intensity of SP-A in buffer A on excitation at 295 nm, before and after addition of increasing amounts of β-D-glucans (Fig. 2A). In the absence of calcium, the emission spectrum of SP-A showed no changes upon addition of increasing amounts of βG (data not shown). However, in the presence of 0.5 mM $CaCl_2$, addition of increasing amounts of βG resulted in a significant decrease in the amplitude of the SP-A emission spectrum, without changes in its maximum emission wavelength (Fig. 2A). This indicates that SP-A binds to βG in a calcium-dependent manner.

Protective effects of SP-A on the inhibitory effects of β-D-glucan on surfactant adsorption

We studied the effect of SP-A on the capability of surfactant bilayers to adsorb onto and spread at an air/liquid interface in the absence and presence of βG. Fig. 3 shows that incubation of LES (112 μg/ml) with 2.5 wt% SP-A (2.8 μg/ml) prevented β-D-glucan-induced inhibition of surfactant adsorption ([LES + SP-A] + βG , *prevention*), in agreement with the resistance of native surfactant against β-D-glucan inhibition shown in Fig. 1B. Moreover, addition of SP-A to LES membranes pre-incubated with βG reversed β-D-glucan-inhibition of surfactant adsorption ([LES + βG] + SP-A, *reversion*). Also, preincubation of SP-A with β-D-glucan blocked βG effects on the surface adsorption of LES (LES + [βG + SP-A], *blockage*). Taken together, our results show that SP-A can counteract the inhibitory effect of membrane-associated βG, and abrogates the effect of free βG, hampering its interaction with surfactant membranes.

β-D-glucan promotes surfactant membrane fluidization and SP-A counteracts β-D-glucan effects

Given that βG did not compete for the air-liquid interface, we hypothesize that surfactant inhibition might be due to surfactant membrane perturbations [10,63]. Therefore, we next examined whether β-D-glucans could effectively affect the lipid order of LES membranes using the fluorescent probe DPH. Fig. 4A shows that the emission anisotropy of DPH incorporated in LES unilamellar vesicles decreased with increasing concentrations of βG, showing a saturation effect at a βG concentration of 8 nM. Given that the change in DPH anisotropy was not accompanied by changes in DPH fluorescence (data not shown), these results indicate that βG decreased the lipid order of LES membranes. Importantly, in the presence of 2.5 wt% SP-A, the βG-induced decrease in DPH anisotropy was reversed (Fig. 4A). SP-A alone does not influence the lipid order of LES membranes [42], suggesting that SP-A counteracts β-D-glucan fluidizing effects by binding to βG.

We also studied the effect of βG on the thermotropic properties of surfactant membranes. Fig. 4B shows that LES multilamellar vesicles exhibited an endothermic transition with a gel/liquid crystalline transition temperature (T_m) of 34.3 ± 0.1 ºC and a transition enthalpy (ΔH) of 3.1 ± 0.3 kcal/mol. Addition of increasing amounts of βG decreased both the T_m and the transition enthalpy of LES membranes in a dose-dependent manner. This indicates that βG decreases van der Waals interactions among adjacent surfactant lipid molecules. Fig. 4B also shows that SP-A counteracted βG effects on T_m ($T_{m\ \beta G}$ = 28.5 ± 0.1 ºC vs $T_{m\ \beta G+SP\text{-}A}$ = 33.6 ± 0.1 ºC, Bonferroni-corrected p-value < 0.001, n = 3) and the melting enthalpy of surfactant membranes ($\Delta H_{\beta G}$ = 1.6 ± 0.3 kcal/mol vs. $\Delta H_{\beta G+SP\text{-}A}$ = 2.7 ± 0.3 kcal/mol, Bonferroni-corrected p-value = 0.017, n = 3). As previously reported [42,43] SP-A alone did not influence the thermotropic behavior of LES (Fig. 4B), suggesting that SP-A neutralizes βG perturbing effects on surfactant membranes.

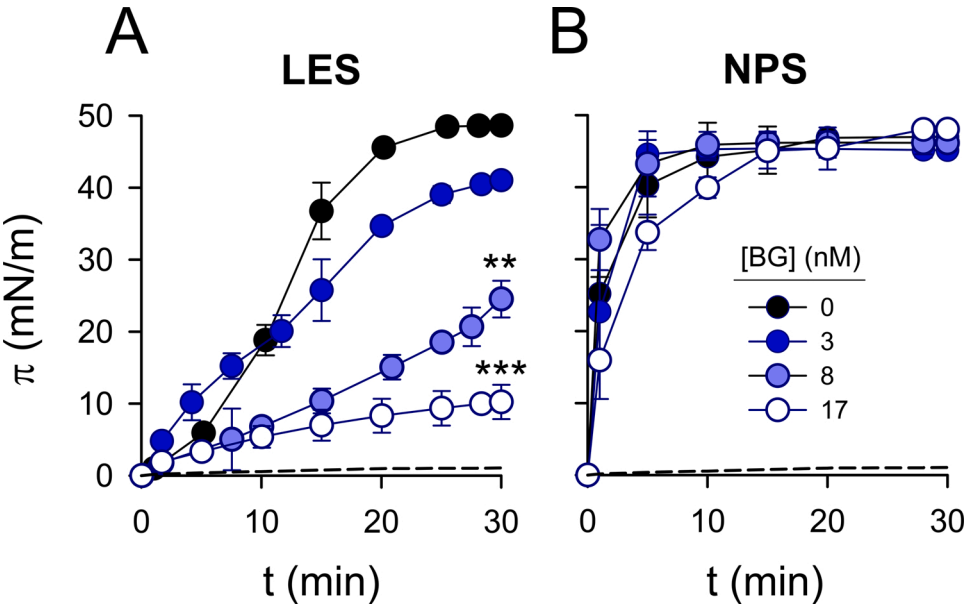

Fig. 1. Inhibitory effect of (1→3)(1→4)-β-D-glucan on (*A*) LES or (*B*) NPS. Surface adsorption was assayed at a phospholipid concentration of 150 μM in the absence and presence of increasing amounts of β-D-glucan. Pure βG (17 nM) did not adsorb onto the clean air/liquid interface (dashed line). Measurements were performed in 25 mM HEPES buffer, pH 7.0, containing 150 mM NaCl, and 0.5 mM $CaCl_2$.. Data are expressed as means ± SD of three experiments. A p-value < 0.0001 was obtained for overall one-way ANOVA (Bonferroni-corrected p-values: ** $p < 0.01$, *** $p < 0.001$ when compared with LES without βG treatment).

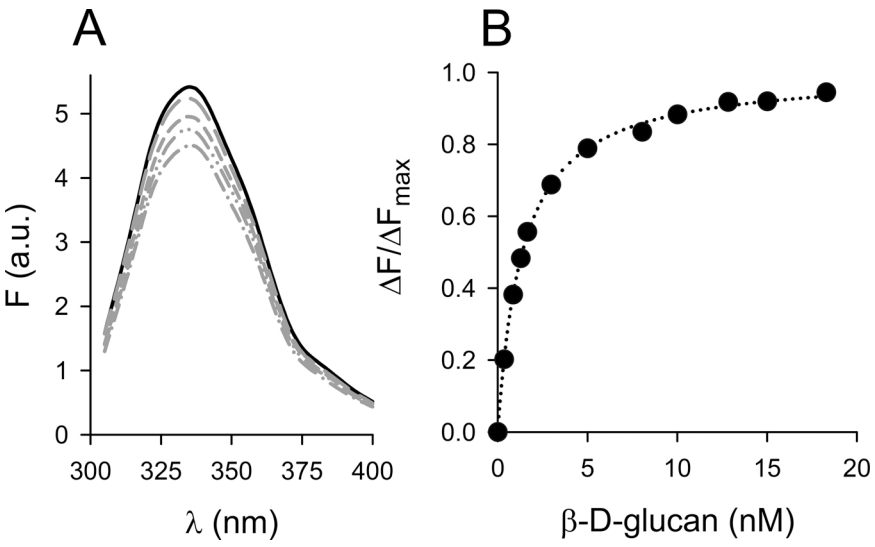

Fig. 2. Binding of SP-A to β-D-glucan at 25 °C in buffer A. (*A*) Emission spectra of SP-A on excitation at 295 nm, in the absence and presence of different concentrations of β-D-glucan: (——) 0 nM, (— —) 2 nM, (————) 7 nM, (—·—) 13 nM, and (—··—) 17 nM. (***B***) Changes in tryptophan fluorescence emission intensity of SP-A (15 nM) at 338 nm on addition of increasing amounts of β-D-glucan. Results are means ± SD of 3 experiments. The apparent K_d value and Hill coefficient for SP-A binding to βG, calculated from the saturation curve by nonlinear least-squares, were 1.5 ± 0.1 nM and 1.00 ± 0.1, respectively.

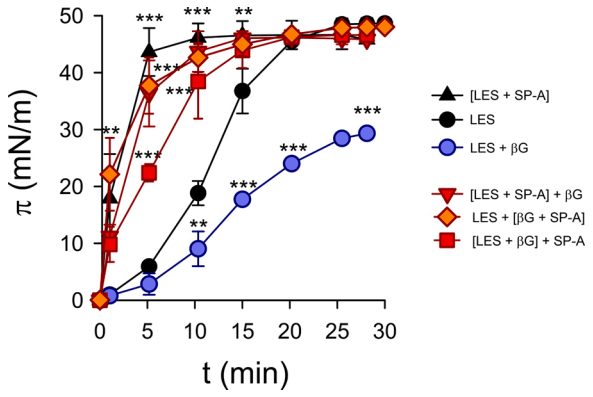

Fig. 3. Protective effect of SP-A against surfactant inhibition induced by βG. The interfacial adsorption kinetics of LES were performed with 150 μM phospholipid (112 μg/ml) in the presence or absence of 8 nM βG and with or without 2.8 μg/ml SP-A (2.5 wt% SP-A relative to surfactant lipids). To evaluate whether the binding of SP-A to LES prevents βG inhibition, LES was pre-incubated with or without SP-A for 10 min, and then allowed to interact, with or without βG, for another 10 min (prevention: [LES + SP-A] + βG). To evaluate whether the binding of βG to LES membranes can be reversed by adding SP-A, LES was allowed to interact first with βG for 10 min and then with/without SP-A for 10 min (reversion: [LES + βG] + SP-A). To evaluate whether the binding of SP-A to βG blocks βG interfacial adsorption inhibition, SP-A and βG were allowed to interact for 10 min and then were mixed with LES for 10 min (blockage: LES + [βG + SP-A]. In all experiments, the samples were injected into the subphase to measure the change in surface pressure as a function of time. Results are means ± SD of three independent experiments. ANOVA followed by Bonferroni multiple comparison test was used. ** $p < 0.01$, *** $p < 0.001$ when compared with LES without SP-A and βG.

β-D-glucan causes withdrawal of lipid molecules from surfactant membranes

To determine whether the fluidizing effect of βG on surfactant membranes is due to glucan incorporation, we recorded the compression isotherms of LES monolayers in the absence and presence of increasing amounts of βG. Fig. 5A shows that compression isotherms of surfactant films collapsed at $\pi \geq 60$ mN/m and displayed a plateau at $\pi \sim 45$–50 mN/m, which corresponds to the monolayer-to-multilayer transition region [10,63]. This squeeze-out plateau is associated with the reversible removal of lipids and proteins from the monolayer, which are unable to sustain high surface pressures, by forming a surfactant reservoir [10,63]. Injection of βG into the subphase slightly shifted the compression isotherm to lower relative areas (Fig. 5A), indicating that βG did not incorporate into LES monolayers, but squeezed out lipid molecules from the interfacial film. In addition, βG decreased the maximum surface pressure reached by the LES monolayer in a dose-dependent manner (Fig. 5A).

Analysis of the compression isotherms in terms of the compressibility modulus (Fig. 5B) shows that, in the 25–45 mN/m surface pressure range, pure LES films showed a broad peak with a maximum C_S^{-1} value of ~ 145 mN/m, characteristic of the tilted condensed (TC) phase (100–200 mN/m) [17], followed by a pronounced minimum at a surface pressure of 45 mN/m, where the monolayer-to-multilayer transition occurs [19]. The interaction of βG with LES monolayers decreased the maximum C_S^{-1}value and shifted the dip to lower surface pressures in a dose-dependent manner (Fig. 5B). This indicates that βG would fluidize LES monolayers and destabilize surfactant films.

Given that Fig. 5A shows that βG did not incorporate into LES monolayers but squeezed out lipid molecules from the interfacial film and Fig. 5B shows that βG seems to fluidize LES monolayers, we hypothesize that βG must extract lipids from the monolayer. To corroborate this hypothesis, we studied the relaxation kinetics of LES films in the absence and presence of βG at a constant surface pressure of 47 mN/m (Fig. 5C), where the monolayer-to-multilayer transition occurs. Pure LES monolayers exhibited a stable behavior with minimal area loss during the relaxation period (Fig. 5C). However, injection of increasing amounts of βG into the subphase significantly increased the relaxation rate of surfactant monolayers in a dose-dependent manner (Fig. 5C). To determine whether the area loss of surfactant films induced by βG was due to desorption of lipid molecules or increased nucleation and growth of three-dimensional aggregates, we analyzed the variance of -log(A/A_0) for LES films with the square root of time in the absence and presence of βG (Fig. 5D). According to the nucleation and growth model of monolayer collapse proposed by Smith and Berg [51], if the monolayer molecular loss is due to desorption of lipid molecules into the subphase, a linear relationship between -log(A/A_0) and the square root of time would be obtained. The absence of a linear relationship would indicate that the area loss during relaxation would be caused by the formation of three-dimensional aggregates. The desorption process was biphasic. The first step would correspond to desorption of phospholipids present in the fluid-like phase (liquid expanded phase, LE), whereas the second would correspond to desorption of lipids present in the more insoluble, DPPC-enriched, solid-like TC domains. Accordingly, the desorption rates of the second step were lower than those obtained for the first step (Table 1-Suppl Mat). Fig. 5E shows the relaxation kinetics of LES films

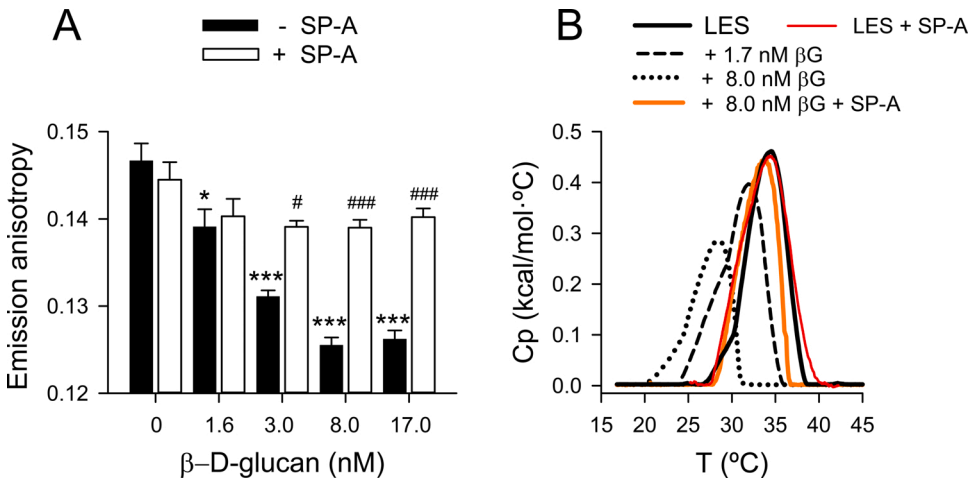

Fig. 4. SP-A inhibits βG fluidizing effect on LES membranes. (*A*) The emission anisotropy of DPH was measured with excitation at 360 nm and emission at 430 nm. All measurements were performed at 25 °C in buffer A, at SP-A and phospholipid concentrations of 38 nM and 1.3 mM respectively. Data shown are means ± SD of three independent measurements. A *p*-value < 0.0001 was obtained for overall one-way ANOVA (Bonferroni-corrected *p*-values: * $p < 0.05$, *** $p < 0.001$ when compared LES without βG with samples treated with βG; #$p < 0.05$; ###$p < 0.001$, when groups with and without SP-A are compared). (*B*) LES (1 mg/ml) MLVs were mixed with different amounts of βG and allowed to interact for 10 min before the DSC heating scans were recorded. Mixing 8 nM βG with SP-A (2.5% by weight with respect to the phospholipids) (38 nM) before addition to LES MLVs abrogated the effect of βG on the thermotropic behavior of LES membranes. Addition of 2.5 wt% SP-A to LES did not influence the thermotropic behavior of these membranes. Calorimetric scans were performed at a scanning rate of 0.5 °C/min. Data shown are means of thermograms of three independent experiments.

compressed at 30 mN/m, which resembles the estimated lateral pressure of lipid bilayers [32]. βG exerted a similar destabilizing effect on the relaxation kinetics of these monolayers. The desorption process was also biphasic (Fig. 5 F) with the first step being faster than the second one (Table 1-Suppl Mat).

To determine whether βG was capable of extracting DPPC molecules from surfactant monolayers, we evaluated the effect of 0.6 nM βG on the relaxation kinetics of pure DPPC and mixed DPPC/POPC monolayers at 30 mN/m, since the miscibility between DPPC and POPC (unsaturated PC abundant in lung surfactant) [39,41,42] might facilitate βG-induced DPPC molecular loss from surfactant membranes. Fig. 6A shows that βG destabilized both films, increasing their relaxation rate (Fig. 6A). This effect was greater on mixed DPPC/POPC films, which show LE/TC phase separation at 30 mN/m [39], than on pure DPPC monolayers, which show solid TC domains occupying most of the monolayer at 30 mN/m [8,10] (Fig. 6A). The desorption rate values obtained for DPPC/POPC 7:3 (w/w) and DPPC films upon addition of βG were similar to those determined for the first and second desorption steps of surfactant films in the presence of βG, respectively (Table 1-Suppl. Mat).

To further characterize the effects of βG on DPPC films mixed with POPC, we analyzed the compression isotherms of DPPC/POPC 7:3 (w/w) monolayers in the absence and presence of βG (Fig. 6B). We found that the interaction of βG with the interfacial monolayer shifted the compression isotherms of DPPC/POPC films to lower surface areas, in agreement with βG induction of lipid molecular loss from mixed DPPC/POPC films observed in Fig. 6A. Fig. 6C shows that DPPC/POPC monolayers exhibited a compressibility modulus value of ~ 67 mN/m at surface pressures ranging from 14 to 41 mN/m. This value, which is intermediate between those of pure LE (12.5–50 mN/m) and pure TC (100–200 mN/m) phases [17], correlated with the lateral lipid order of mixed DPPC/POPC monolayers estimated by molecular dynamic simulations [35]. Injection of βG into the subphase did not alter the maximum compressibility modulus value but, at surface pressures below 26 mN/m, decreased the compressibility modulus to values characteristic of the liquid expanded phase (Fig. 6C) indicating that βG triggers withdrawal of DPPC molecules from mixed DPPC/POPC films since monolayer decrease in DPPC molecules would decrease compressibility modulus.

β-D-glucan inhibits the ability of lung surfactant to reduce surface tension and respread

We then evaluated the effect of βG on the ability of lung surfactant monolayers to effectively decrease surface tension upon film compression and respread upon film expansion. We recorded repeated compression-expansion isotherms of LES films spread in excess conditions in the absence and presence of βG, and/or SP-A.

During dynamic cycling, surfactant films reached high surface pressures (> 60 mN/m) when compressed past collapse, and they displayed large hysteresis loops (Fig. 7A). These loops, which are common to most lung surfactants, reflect that part of the material ejected from the film upon compression deficiently re-enters the interfacial monolayer upon expansion. This squeeze-out, which enriches the monolayer in DPPC by eliminating part of the unsaturated phospholipid species, results in the displacement of the subsequent compression cycles to the left [34] and decreases the minimal attainable surface pressure.

Fig. 7B and C show that βG's inhibitory effect on the ability of pulmonary surfactant to sustain very low values of surface tension upon compresion was persistent and did not reverse during cycling. Unlike pure LES films, the shape of the compression isotherms of LES+ βG films after the first compression-expansion cycle changed very little as cycling proceeded, and showed almost no hysteresis.

Fig. 7D shows that addition of SP-A to surfactant monolayers helped to refine the monolayer during cycling as previously described [48]. This is shown by the larger displacement of the second and subsequent compression cycles to the left, which decreased the hysteresis area and the lengths of the squeeze-out plateaus. As a result, lower compression ratios were needed to achieve the collapse pressure. Importantly, pre-incubation of SP-A with βG partially abrogated the inhibitory effect of the glucan, restoring the hysteresis of the dynamic cycles (Fig. 7E) and the capability of surfactant monolayers to attain surface pressures above 50 mN/m (Fig. 7E and F).

Discussion

Pulmonary mycoses impair lung function, at least in part, by disrupting the pulmonary surfactant system [2,21,26,47,58,60]. Fungi-induced surfactant dysfunction has been related to the host inflammatory response and the leakage of edema fluid into the lung [60]. Plasma proteins and cell membrane lipids, present in the lung because of the inflammatory injury, may inhibit surfactant by competitive

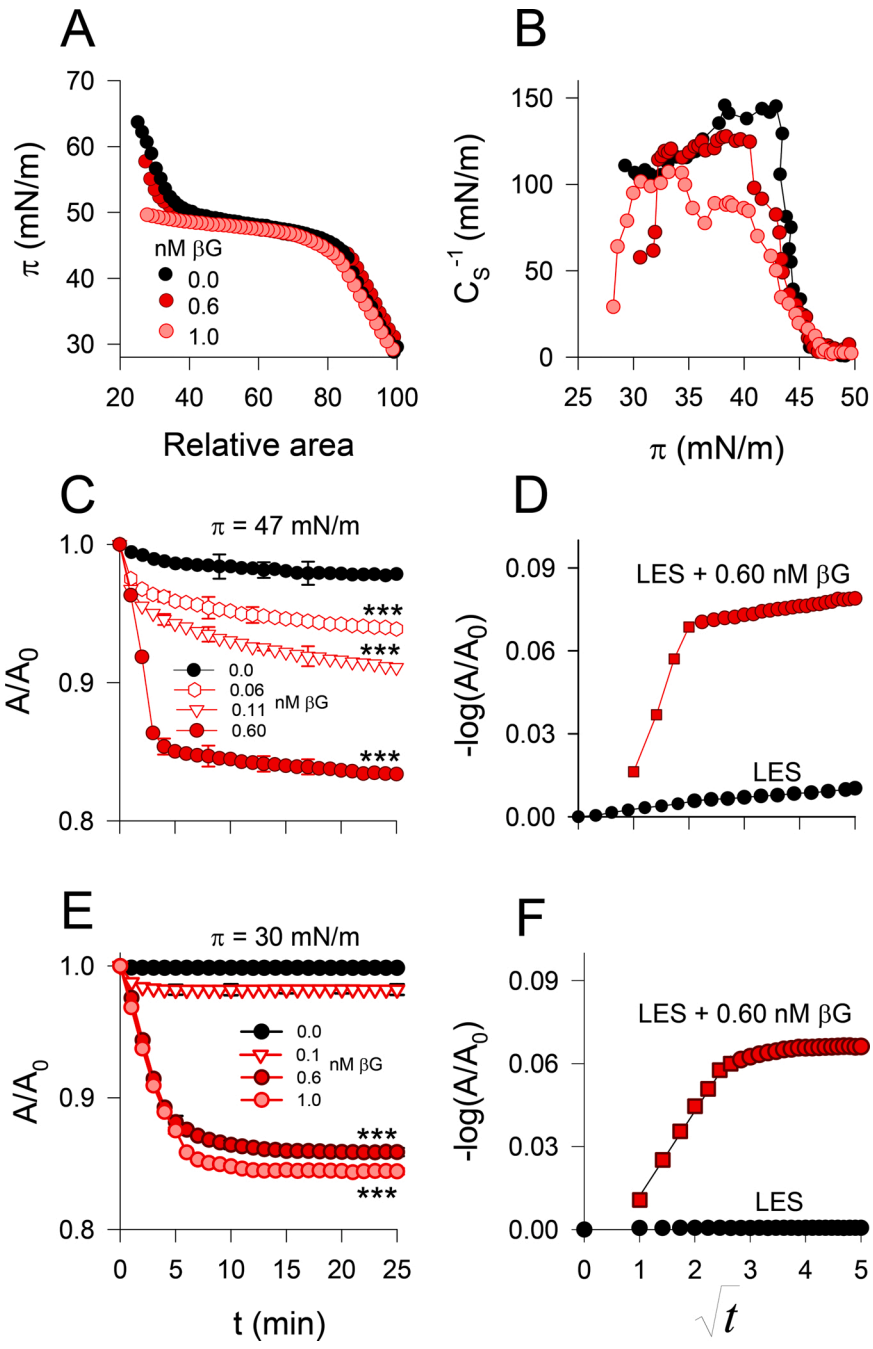

Fig. 5. βG withdraws phospholipid molecules from surfactant films. (*A*) Effect of βG on the compression isotherm of porcine LES spread in excess conditions. The monolayers were compressed at 50 cm^2/min on a subphase containing buffer A. (*B*) Compressibility modulus (Cs^{-1})-surface pressure dependencies for LES monolayers in the absence (grey symbols) and presence of increasing amounts of βG (red symbols). (*C*) Relaxation kinetics of monolayers of porcine LES (15 μg), in the absence and presence of increasing amounts of βG, compressed to a surface pressure of 47 mN/m. A and A_0 are the trough surface areas at a given time t and t = 0, respectively. (*D*) Relaxation data of porcine LES, with and without βG, at a surface pressure of 47 mN/m expressed as –log (A/A_0) vs. $\sqrt{t}$. (*E*) Effect of βG on the relaxation kinetics of LES films compressed to a surface pressure of 30 mN/m. (***F***) Relaxation data of porcine LES, with and without βG, at a surface pressure of 30 mN/m expressed as –log (A/A_0) vs. $\sqrt{t}$. Data shown are means ± SD of five independent measurements, performed at 25.0 ± 0.1 °C. A *p*-value < 0.0001 was obtained for overall one-way ANOVA (Bonferroni-corrected *p*-values: *** $p < 0.001$ when compared with LES without βG).

adsorption [10,62] or by mixing with surfactant components, altering surfactant structure [10,43,63,8]. Here we show that (1→3)(1→4)β-D-glucan (βG), a structural component of the cell wall of pathogenic fungi, inhibits the biophysical function of surfactant preparations lacking SP-A by provoking fluidization of surfactant bilayers and monolayers. Our results suggest that βG must extract significant amounts of DPPC and other saturated molecular species of PC from surfactant bilayers and monolayers.

The inhibitory effect of βG is opposed to that of dextran, a complex branched α-glucan that promotes surfactant aggregation, increasing the resistance of exogenous surfactants to functional inhibition by competitive adsorption [31]. Considering the ability of glucose to form hydrogen bonds with the phosphate head groups of DPPC molecules [55], it is possible that the absence of lateral chains in the structure of βG would allow the interaction of the glucose monomers with surfactant lipids, whereas the lateral chains of dextran would hamper such interaction. In this regard, it has been shown that β-cyclodextrins (cyclic oligosaccharides consisting of α-(1→4)-D-glucopyranose units) extract phosphocholine-containing phospholipids like DPPC [38] and sphingomyelin [23], probably by establishing hydrogen bonds with the phosphodiester group [38]. The βG-promoted withdrawal of surfactant phospholipids is consistent with the decrease in phospholipid content determined in bronchoalveolar lavage of humans [21,26] and rats [28,49] with *Pneumocystis* pneumonia. It is also consistent with the finding that the major lipid component of *P. carinii* isolated from rat lungs contained a large amount of firmly attached surfactant DPPC [36].

Our results indicate that the βG-promoted desorption of phosphocholine-containing phospholipids inhibited the adsorption, respreading, and surface-tension lowering capabilities of pulmonary surfactant. The fluidization of surfactant membranes would hinder their

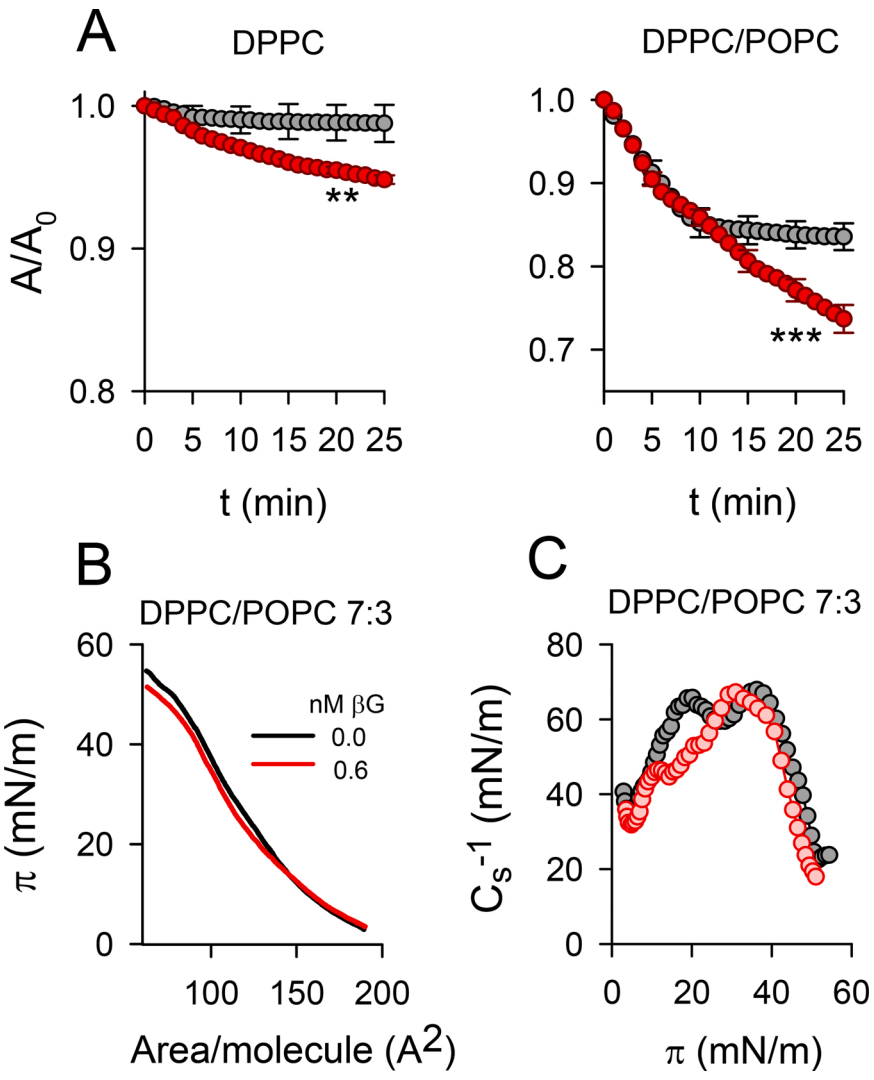

Fig. 6. βG extracts saturated and unsaturated phospholipids. (*A*) Relaxation kinetics of DPPC and DPPC/POPC 7:3 (w/w) monolayers, in the absence and presence of βG, compressed to a surface pressure of 30 mN/m. *A* and A_0 are the trough surface areas at a given time t and t = 0, respectively. A *p*-value < 0.0001 was obtained for overall one-way ANOVA (Bonferroni-corrected *p*-values: ** $p < 0.01$ and *** $p < 0.001$ when compared with the phospholipid mixtures without βG). (*B*) Surface pressure (π)-area isotherms of DPPC/POPC 7:3 (w/w) mixed monolayers in the absence and presence of 0.6 nM βG. The monolayers were compressed at 50 cm^2/min on a subphase containing buffer A. (*C*) Analysis of the compression isotherms of DPPC/POPC (7:3, w/w) films in the absence (•) and presence (•) of 0.6 nM βG, in terms of the dependence of the monolayer compressibility modulus with the surface pressure. Measurements were performed at 25.0 ± 0.1 °C. Data shown are means ± SD of five independent measurements.

interfacial adsorption to the air/liquid interface. Adsorption of surfactant aggregates to the air-liquid interface is analogous to the fusion of two vesicles [14,63]. Since a reduction in the bending modulus suppresses bilayer fusion [4], it is possible that the βG-mediated fluidization of surfactant bilayers contributes to suppress fusion of the aggregates with the interface. Consistently, surfactant membranes with increased fluidity induced by C-reactive protein [43] or misbalanced lipid composition [63] fail in their ability to rapidly adsorb onto an air–water interface [10,63].

βG also fluidizes LES monolayers and destabilizes surfactant films, promoting their collapse at lower surface pressures. The inhibitory effect of βG on the ability of pulmonary surfactant to sustain very low values of surface tension upon compression was persistent and did not change during cycling. It is possible that the βG-induced removal of saturated PC molecules and subsequent fluidization of surfactant monolayers may result in softer monolayers that would collapse in-plane by shearing instead of out-of-plane [37]. This would prevent the loss of material from the surfactant films during dynamic cycling and would hinder the monolayer-to-multilayer transition and subsequent DPPC enrichment of the interfacial film. The βG-induced fluidization of surfactant monolayers would also alter the molecular interactions that are required for an efficient respreading [20,57]. As a result, both the respreading and the surface tension-lowering capabilities of pulmonary surfactant are significantly affected by βG. We are aware that our studies on the dynamic compression and expansion of the surfactant film using a Langmuir trough instead of a surfactometer have several drawbacks. The main limitation is the relatively slow speed of dynamic cycles using a Langmuir trough compared with the physiological respiratory rate. However, despite this important drawback, our results clearly show that βG inhibits respreading and surface tension-lowering activity of surfactant preparations containing SP-B and SP-C.

Deciphering potential mechanisms to counteract surfactant inhibition is important for the development of new therapies for lung immaturity and infectious and inflammatory lung diseases. Here we show that SP-A, a versatile recognition protein present in the alveolar fluid, binds to β-D-glucans with high affinity and blocks βG inhibitory effects on lung surfactant. The K_d values for the interaction of SP-A with β-D-glucans (K_d = 1.5 ± 0.1 nM for (1→3)(1→4)β-D-glucan, 1.1 ± 0.2 nM for (1→3)(1→6)-β-D-glucan, and 20 ± 2 pM for (1→3)-β-D-glucan phosphate) were similar to that determined for the interaction of (1→3)-β-D-glucan phosphate with the CD5 lymphocyte surface receptor (3.7 ± 0.2 nM) [56]. They are also in the range of the binding affinities determined for the interaction of different β-D-glucans with Dectin-1, the primary pattern recognition receptor for glucans [1]. By binding to βG, SP-A, on the one hand, withdraws free βG molecules, hampering their interaction with surfactant membranes and the subsequent surfactant inactivation. On the other hand, SP-A reverses the inhibitory effects of membrane-bound βG. It is noteworthy that SP-A binding to βG is calcium-dependent. Considering the ability of calcium ions to bind glucose molecules [54] and that SP-A has different calcium binding sites

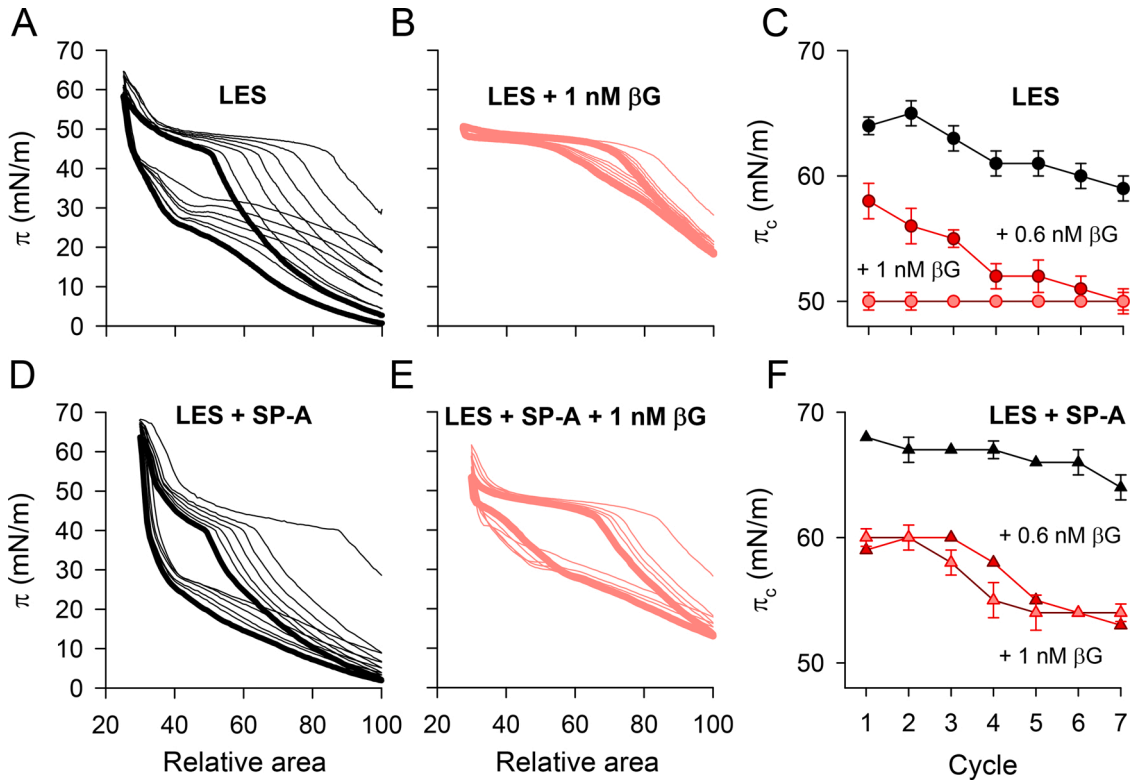

Fig. 7. Dynamic cycling surfactant films with and without SP-A, in the absence and presence of βG. (*A*) Representative π-A cycling isotherms of LES films spread in excess conditions. Seven π–A compression–expansion cycling isotherms were recorded for LES films on top of a buffer A subphase. (*B*) Effect of βG on π-A cycling isotherms of porcine LES. (*C*) Effect of βG on the collapse pressure (π_c) of LES films. (*D*) Effect of SP-A on the dynamic cycling of LES films. (*E*) Compression-expansion isotherms of LES films in the presence of βG and SP-A. (*F*) Collapse pressure as a function of the number of cycles for LES films in the presence of SP-A, with and without βG. All measurements were performed at 25.0 ± 0.1 °C and a compression rate of 89 cm^2/min. The last compression–expansion cycles are marked with thicker lines. In C and F, data shown are means ± SD of five independent measurements.

[24], it is possible that this cation could form bridges between SP-A and glucose monomers of βG, facilitating their interaction.

SP-A has an important role in overcoming surfactant adsorption inactivation by serum proteins [62,63], and in scavenging inhibitors that incorporate into surfactant membranes [8,43]. Our results indicate that, by binding to βG, SP-A sequesters βG, preventing βG interaction with surfactant membranes and reducing βG's effects on them.

SP-A also seems to prevent the removal of DPPC and other phospholipid molecules from the surfactant monolayer by βG, allowing squeeze-out of surfactant material from the interfacial film and formation of the surfactant reservoir. SP-A partially abrogated the inhibitory effect of βG on surface tension-lowering capabilities of pulmonary surfactant, and surfactant membranes containing SP-A are more resistant to inactivation. These results are consistent with the fact that replacement surfactants containing 5 wt% SP-A are superior to SP-A-depleted surfactants, showing an enhanced therapeutic effect on dynamic and static lung compliance in ventilated premature newborn rabbits [61] and improved physical and therapeutic properties [53].

Conclusions

We have demonstrated that the direct interaction between lung surfactant preparations containing SP-B and SP-C and the fungi component β-glucans causes surfactant dysfunction. The presence of β-glucans at concentrations closer to those found in the lungs of patients with invasive fungal infections [30,52] hampers surfactant respreading and its capability to decrease surface tension to very low values upon film compression. Our results suggest that the mechanism of β-glucan inhibition consists in the βG-promoted extraction of surfactant material, rendering surfactant monolayers and bilayers more fluid. We have also demonstrated that SP-A strongly binds to βG, with an affinity similar to those determined for dectin-1. Consequently, SP-A increases the resistance of pulmonary surfactant to βG-induced inhibition, preventing the interaction of βG with surfactant and counteracting β-glucan fluidizing effects on surfactant bilayers and monolayers. The increased resistance of SP-A-containing surfactant membranes to βG inactivation supports the need to supplement replacement surfactants with recombinant SP-A for the treatment of respiratory diseases.

CRediT authorship contribution statement

Olga Cañadas: conceived, designed, and performed experiments, collected, analyzed, and interpreted data, and wrote the manuscript.

Alejandra Sáenz: conceived, designed, and performed experiments, collected, analyzed, and interpreted data.

Alba de Lorenzo: performed research and contributed to experimental design.

Cristina Casals: funded and designed the research, organized the project and analyses, interpreted all data, and wrote the manuscript.

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Author contribution

Conceptualization, **all authors**. Investigation, **O.C., A.S., and A.de L.**; Methodology, **O.C., A.S., and A.de L.**; Formal analysis, **O.C., A.S.,**

and C.C.; Validation, **O.C., A.S., and C.C.**; Writing—original draft preparation, **O.C., A.S., and C.C.**; Visualization, **O.C., A.S., and C.C.**; Writing—review and editing, **O.C. and C.C.**; Supervision, **C.C.**; Project administration, **C.C** Funding acquisition for this study, **C.C.** All authors read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfb.2021.112237.

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