Pulmonary Surfactant Protein SP-C Counteracts the Deleterious Effects of Cholesterol on the Activity of Surfactant Films under Physiologically Relevant Compression-Expansion Dynamics

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ABSTRACT The presence of cholesterol is critical in defining a dynamic lateral structure in pulmonary surfactant membranes. However, an excess of cholesterol has been associated with impaired surface activity of surfactant. It has also been reported that surfactant protein SP-C interacts with cholesterol in lipid/protein interfacial films. In this study, we analyzed the effect of SP-C on the thermodynamic properties of phospholipid membranes containing cholesterol, and the ability of lipid/protein complexes containing cholesterol to form and respread interfacial films capable of producing very low surface tensions upon repetitive compression-expansion cycling. SP-C modulates the effect of cholesterol to reduce the enthalpy associated with the gel-to-liquid-crystalline melting transition in dipalmitoylphosphatidylcholine (DPPC) bilayers, as analyzed by differential scanning calorimetry. The presence of SP-C affects more subtly the effects of cholesterol on the thermotropic properties of ternary membranes, mimicking more closely the lipid composition of native surfactant, where SP-C facilitates the miscibility of the sterol. Incorporation of 1% or 2% SP-C (protein/phospholipid by weight) promotes almost instantaneous adsorption of suspensions of DPPC/palmitoyloleoylphospatidylcholine (POPC)/palmitoyloleoyl-phosphatidylglycerol (POPG) (50:25:15, w/w/w) into the air-liquid interface of a captive bubble, in both the absence and presence of cholesterol. However, cholesterol impairs the ability of SP-C-containing films to achieve very low surface tensions in bubbles subjected to compression-expansion cycling. Cholesterol also substantially impairs the ability of DPPC/POPC/POPG films containing 1% surfactant protein SP-B to mimic the interfacial behavior of native surfactant films, which are characterized by very low minimum surface tensions with only limited area change during compression and practically no compression-expansion hysteresis. However, the simultaneous presence of 2% SP-C practically restores the compression-expansion dynamics of cholesterol- and SP-B-containing films to the efficient behavior shown in the absence of cholesterol. This suggests that cooperation between the two proteins is required for lipid-protein films containing cholesterol to achieve optimal performance under physiologically relevant compression-expansion dynamics.

INTRODUCTION

Pulmonary surfactant is a complex mixture of lipids and proteins whose main function is to reduce the surface tension at the alveolar air-liquid interface of lungs to facilitate the work of breathing. A lack, deficiency, or inactivation of this essential material can cause severe respiratory disorders such as neonatal respiratory distress syndrome (NRDS) or acute respiratory distress syndrome (ARDS) (1). It is composed of ~90% lipids (mainly phospholipids) and 8–10% proteins; ~80% of surfactant by mass is composed of phosphatidylcholine (PC), half of which is dipalmitoylphosphatidylcholine (DPPC) (2). The acidic phospholipids phosphatidylglycerol (PG) and phosphatidylinositol (PI) account for 8–15% of the total surfactant phospholipid pool. Cholesterol is the major neutral lipid, accounting for up to 8–10% of surfactant by weight. The phospholipid fraction within lung surfactant, and particularly DPPC, is essentially responsible for the maximal surface tension reduction upon compression. However, the presence of proteins, particularly SP-B and SP-C, is absolutely required for interfacial adsorption, film stability, and respreading capacities (3,4).

SP-B and SP-C are small hydrophobic polypeptides that account for no more than 1–1.5% of total surfactant weight but play critical roles in the formation and stabilization of pulmonary surfactant films (5). In contrast to SP-B, surfactant protein SP-C is not absolutely required for survival. However, targeted deletion of the SP-C gene (SP-$C^{-/-}$) in mice leads to the development of severe progressive pneumonitis, depending on the genetic background (6,7). Experimental data suggest that SP-C would be required to maintain the association of surfactant complexes (the reservoir) with the interfacial film at the most compressed states, i.e., those reached at the end of expiration (3). The SP-C-promoted attachment would then facilitate the reinsertion of surface-active molecules from the reservoirs, with the probable critical participation of SP-B, during reexpansion (3,8–11).

Some authors have suggested the existence of SP-C/cholesterol complexes in membranes (10,12); however, both direct evidence and a model explaining the role of SP-C/cholesterol interactions in surfactant function are lacking. It has been proposed that cholesterol could contribute to prevent SP-C from aggregating, leading to a better miscibility of the protein into monolayers (12). On the other hand, a previous study suggested that concomitant changes in the concentration of cholesterol and SP-C may occur in

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the surfactant of heterothermic animals (13), and that the roles of these two molecules in surfactant may therefore be connected.

Our main goal in this study was thus to gain further knowledge about the occurrence and potential significance of interactions between cholesterol and SP-C. Herein, we report the thermodynamic characterization of model membranes containing cholesterol and SP-C and a functional study of these membranes. The results are discussed in terms of a combined role of both components in lung surfactant and the possible modulation of the effect of cholesterol in surfactant by SP-C.

EXPERIMENTAL PROCEDURES

Materials

DPPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3[phospho-rac-(1-glycerol)] (POPG), and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). Chloroform and methanol solvents, HPLC grade, were obtained from Scharlau (Barcelona, Spain). Native surfactant was purified from porcine lungs as described previously (14). Surfactant proteins SP-B and SP-C were isolated from minced porcine lungs as described elsewhere (15). Quantitation of proteins was achieved by amino acid analyses.

Reconstitution of lipid and lipid/protein samples

The standard lipid mixture used in this study as a surfactant model contained DPPC/POPC/POPG (50:25:15, w/w/w). This mixture simulates the proportion of saturated/unsaturated and zwitterionic/anionic phospholipids in surfactant. The mixture was modified by adding different proportions of cholesterol and surfactant proteins. Multilamellar suspensions were prepared by mixing protein and lipids in chloroform/methanol 2:1. Samples were dried overnight under vacuum and resuspended in 5 mM Tris/HCl buffer (pH 7) containing 150 mM NaCl, with incubation for 1 h at 51°C.

Differential scanning calorimetry

For differential scanning calorimetry (DSC), the excess heat capacity (*Cp*) of lipid or lipid/protein samples was measured in a VP-DSC differential scanning microcalorimeter (MicroCal, Northampton, MA). Two membrane models were used in these experiments. The first set of experiments measured DPPC or DPPC/SP-C multilamellar suspensions (50:1 or 10:1, w/w) at a final phospholipid concentration of 0.5 mg/mL. Other experiments were performed using DPPC/POPC/POPG or DPPC/POPC/POPG/SP-C multilamellar suspensions (50:25:15 or 50:25:15:2, w/w) at a phospholipid concentration of 2 mg/mL. The vesicles contained 0%, 3%, 5%, 10%, or 20% cholesterol for pure DPPC membranes, and 0%, 2%, or 5% cholesterol/total phospholipid (w/w) for the ternary membranes. Samples and reference cells were heated from 25°C to 85°C at 0.5°C/min. The calorimetric data were analyzed using ORIGIN software (MicroCal) after baseline correction and concentration normalization. The molar enthalpy of the phase transition, ΔH, was obtained from the area under the main DSC peak. The temperature of the transition, T_m, is reported as the temperature at which *Cp* is maximal.

Captive bubble surfactometry

We determined the surface activity of the model surfactant mixtures using a captive bubble surfactometer (CBS) (16), which allowed us to monitor the surface tension (γ) of surfactant films subjected to compression-expansion dynamics. We used a modification of a previously described procedure (17), which included the addition of sucrose (10% w/w) to the buffer in the chamber to increase its density so that the surfactant suspensions would float and remain in contact with the bubble upon injection. The temperature was maintained between 36.8°C and 37.1°C. To start each experiment, ~250 nL of lipid or lipid/protein suspension (10 mg/mL) were deposited close to the air-buffer interface of a small air bubble (~50 μL) by means of a thin capillary. The high density of the sucrose-containing buffer prevents dilution within the whole chamber of the small volume of aqueous surfactant suspension that is introduced, which then forms a thin layer of liquid sourrounding the bubble, from which the surfactant complexes can adsorb into the interface. In our opinion, this setup mimics reasonably well the actual geometry of the thin layer of concentrated surfactant at the alveolar surface in the lungs. The bubble was imaged with a video camera (TM 7 CN; Pulnix America Inc., Sunnyvale, CA) and recorded for later analysis. A 5 min period followed the introduction of surfactant suspensions into the chamber, during which the change in γ was monitored to follow film formation. The chamber was then sealed and the bubble was rapidly (1 s) expanded to a volume of 0.15 mL. Quasistatic cycling commenced 5 min after the bubble was expanded (to permit for postexpansion adsorption and film equilibration). The bubble size was first reduced and then enlarged in several steps, each of which included a change in volume and 5 s of delay. There was an intercycle delay of 0.5 min between each of the four quasistatic cycles, and a further 2 min delay between the quasistatic and dynamic cycles. In the latter, the bubble size was continuously varied for 20 cycles at a rate of 20 cycles/min. The bubble volume and area and surface tension were calculated from examination of the shape of the bubble using an axisymmetric model (18). We performed at least three independent experiments for each surfactant suspension, using at least two different batches of purified protein, and obtained qualitatively comparable results. The figures represent illustrative example experiments for each surfactant model.

RESULTS

We investigated the thermotropic behavior of DPPC bilayers in the absence or presence of SP-C and cholesterol to analyze the nature of the interaction between SP-C and lipids from a thermodynamic point of view. Fig. 1 shows the effect of the presence of increasing proportions of cholesterol on the thermotropic behavior of DPPC membranes, in either the absence or presence of SP-C 2% or 10% (protein/lipid, w/w). The phase transition of pure DPPC bilayers is abrupt, as demonstrated by the peak sharpness. The effects of a gradual increase of the concentration of cholesterol include the disappearance of the L_β-$P_{\beta'}$ pretransition peak, which occurs in DPPC at ~35°C; the broadening of the main transition at 41°C; and a remarkable reduction of the transition enthalpy, as revealed by a decrease in the area under the main calorimetric peak. In the presence of SP-C, the broadening of the transition is even more noticeable, even though the effects of both membrane components are not additive, as shown in Fig. 2 and in Table S1 of the Supporting Material.

The combined effect of cholesterol and the protein requires further analysis. Fig. 2*A* plots the enthalpy of the phase transition, calculated per mol of DPPC, as a function of the DPPC/cholesterol molar ratio, in the presence and absence of SP-C 2% or 10% (protein to phospholipid, w/w). The presence of cholesterol progressively reduces the enthalpy associated with transition in a linear fashion. In the presence of SP-C, the interaction follows the same linear trend at high proportions of cholesterol. However, at

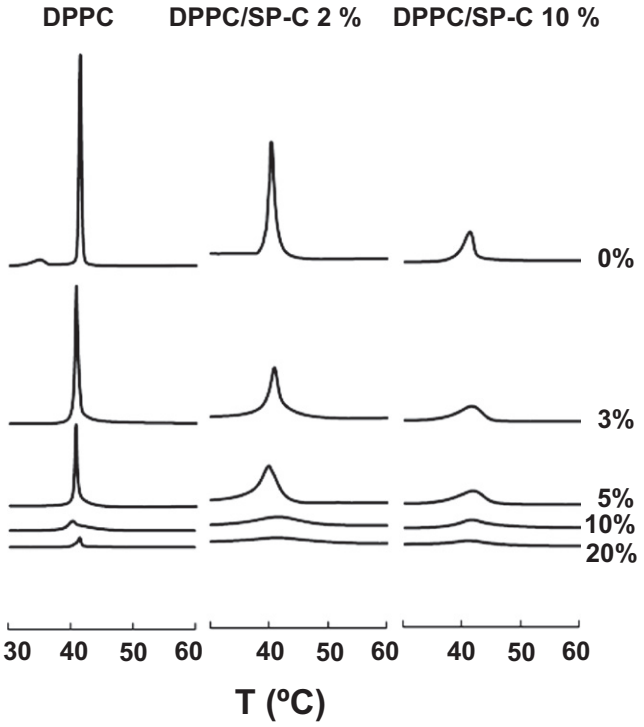

FIGURE 1 Thermotropic behavior of DPPC bilayers in the presence of cholesterol and SP-C. DSC thermograms of DPPC multilamellar suspensions were obtained in the absence (*left*) or presence of 2% (*center*) or 10% (right) protein/phospholipid by weight of porcine SP-C containing the indicated proportions of cholesterol (w/w with respect to phospholipid).

lower concentrations of cholesterol, the sterol does not seem to significantly reduce the enthalpy associated with phase transition in the lipid/protein membranes. The amount of cholesterol above which the enthalpy of the lipid complexes is again reduced depends on the proportion of SP-C present. Cholesterol proportions higher than 10% (mol/mol) with respect to phospholipid are required to reduce the calorimetric enthalpy in membranes containing 2% SP-C, whereas only proportions of cholesterol higher than 20% substantially reduce the enthalpy of membranes containing 10% SP-C. The concentration of cholesterol needed to significantly affect the thermodynamics of the system is thus higher in the presence of a higher concentration of protein, which may indicate a direct interaction between cholesterol and SP-C. The additivity of the effects of cholesterol and protein is further analyzed in the contour plot that shows the proportions of cholesterol and SP-C that produce equivalent enthalpy values once they are incorporated into the DPPC membranes (Fig. 2*B*). If the combined action of cholesterol and SP-C is merely additive, the lines in this plot should be diagonal lines connecting the two axes. If the combined effect is not additive (e.g., one of the components buffers the effect of the other), the lines should be parallel to one of the axes. Fig. 2*B* illustrates how the presence of increasing amounts of cholesterol and DPPC produces nonadditive effects, consistent with partial counteraction between SP-C and cholesterol with respect to their mutual effect on the transition enthalpy.

We also characterized the thermotropic behavior of ternary DPPC/POPC/POPG bilayers in the absence or presence of SP-C and/or cholesterol. These bilayers mimic the ratio of zwitterionic/anionic and saturated/unsaturated phospholipids found in native surfactant. The effect of increasing proportions of cholesterol on DPPC/POPC/POPG membranes in the absence or presence of SP-C 2% (protein/lipid, w/w) is shown in Fig. S1. Remarkably, the thermograms of membranes containing 5% cholesterol with respect to phospholipid (w/w) are very similar to those of native porcine surfactant (19). The thermotropic transition that occurs in natural surfactant membranes has been interpreted as a liquid-ordered to liquid-disordered phase transition, with a reduced enthalpy compared to that of a typical gel-to-liquid crystalline transition (19). The effects of increasing proportions of cholesterol on the ternary membranes are not as dramatic as in pure DPPC membranes because the transition of ternary membranes is already broadened in the absence of cholesterol, but they include a further broadening of the main transition, occurring now around 30°C, and a reduction of the transition enthalpy (see Table S2). In the presence of SP-C, the transition is even more broadened, but, as occurs in DPPC membranes, the effects of SP-C and cholesterol

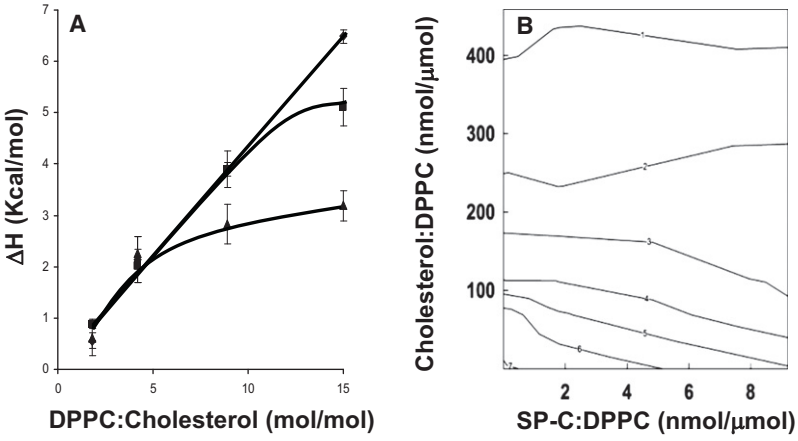

FIGURE 2 Dependence of the phase transition enthalpy of DPPC bilayers on cholesterol content in the absence or presence of SP-C. (*A*) The enthalpy associated with the main phase transition of DPPC bilayers, determined from the area under the main calorimetric peak in thermograms of Fig. 1, is plotted against the DPPC/cholesterol molar ratio in the absence (diamonds) or presence of SP-C 2% (*squares*) or 10% (*triangles*) by weight with respect to phospholipids. (*B*) Contour plot of values of equivalent enthalpy determined from the calorimetric behavior of DPPC bilayers containing variable amounts of cholesterol and/or SP-C. Lines connect the combined proportions of cholesterol and SP-C that produce the similar enthalpy value indicated in each line once they are incorporated into DPPC suspensions.

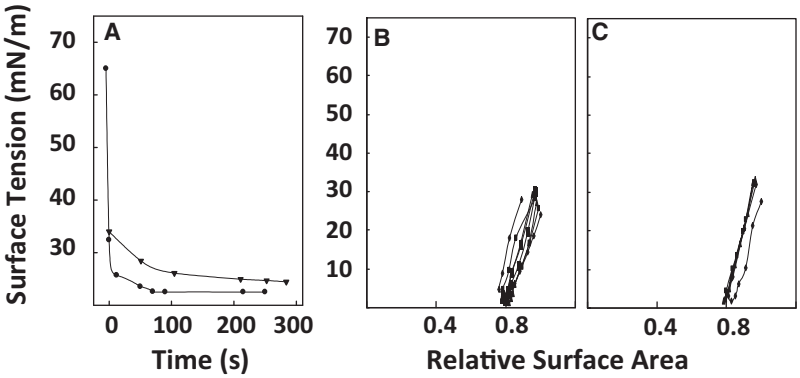

FIGURE 3 Functional behavior of native porcine surfactant in a CBS. (*A*) Time course for initial (●) and postexpansion (▲) adsorption of native surfactant (250 nL, 10 mg/mL phospholipid) deposited at the captive bubble interface. (*B*) Cycles 1 (◆), 2 (■), 3 (▲), and 4 (×) are represented. (*C*) Compression-expansion isotherms under dynamic cycling. Cycles 1 (◆), 10 (■), and 20 (▲) are represented.

do not seem to be additive. Of interest, the presence of SP-C seems to facilitate the homogeneous mixing of cholesterol. In the absence of the protein, introduction of 2% cholesterol yields a complex thermogram, including a secondary peak at ~22°C overlapping with the main transition, which exhibits its maximum heat capacity at 29.5°C. This is likely an indication of the coexistence of two segregated membrane regions with presumably different cholesterol contents. The secondary component practically disappears in the presence of SP-C, indicating that cholesterol distributes more homogeneously in SP-C-containing bilayers.

To characterize a possible modulation of the surfactant function by the interaction between cholesterol and SP-C, we evaluated the activity of samples containing different concentrations of cholesterol and SP-C in a CBS. As a reference, Fig. 3 shows the behavior of native pulmonary surfactant, as purified from porcine lungs. Native surfactant adsorbed to the interface extraordinarily fast (Fig. 3 *A*), producing equilibrium surface tensions close to 22 mN/m within the first minute. Compression of such adsorbed surfactant films under quasistatic conditions produced surface tensions lower than 1 mN/m with only a 20% area reduction, whereas surface tension hardly increased above 30 mN/m upon bubble expansion (Fig. 3 *B*). Furthermore, the compression and expansion moieties of the cycling isotherms showed almost no hysteresis, indicative of the extraordinary stability of the native surfactant film during cycling dynamics. This remarkable behavior is even more pronounced when the films are subjected to rapid, physiological-like compression-expansion dynamics at 20 cycles/min (Fig. 3 *C*). The ability of surfactant to produce very low surface tensions with only limited area change during compression, the relatively low maximal surface tensions upon expansion, and the little hysteresis are considered essential requirements of a functional lung surfactant.

Fig. 4 shows the time course for the reduction of surface tension upon adsorption of model membranes consisting of the mixture DPPC/POPC/POPG (50:25:15, w/w/w), in the absence or presence of cholesterol 5% or 10% (w/w) and SP-C 1% or 2% (protein/phospholipid, w/w). In the absence of protein, adsorption of pure lipid samples was limited, which reduced surface tension to values of ~45 mN/m, and further impaired in the presence of cholesterol. Expansion of these films resulted in almost no further change in surface tension. In contrast, all the samples containing SP-C reduced surface tension to values of ~22 mN/m. The minimum surface tensions reached were lowest in the absence of cholesterol and in the presence of the highest concentration of protein. Film expansion, in the presence of SP-C, led to the adsorption of additional surfactant material associated with the surface. Lower decreases in surface tension occurred at cholesterol 5%, reflecting a lower efficiency in the insertion of new material from reservoirs. However, increasing the concentration of cholesterol from 5% to 10% produced a slight improvement in the surface tension reduction, possibly due to a direct effect of the sterol on the membrane fluidity or the segregation of membrane phases (20).

Figs. 5 and 6 respectively show the quasistatic and dynamic compression-expansion isotherms of films formed from suspensions of DPPC/POPC/POPG (50:25:15, w/w/w) containing SP-C 1% or 2%, in the absence or in the presence of 5% or 10% cholesterol. In the presence of 1% SP-C, the films were not able to reach sufficiently low minimum surface tensions under quasistatic compression, in either the presence or absence of cholesterol, and the presence of increasing proportions of cholesterol produced progressively higher minimal tensions. All the isotherms presented conspicuous plateaus at ~22–23 mN/m, which were previously interpreted as the result of compression-driven, three-dimensional transitions (13). In the absence of cholesterol and in the presence of 1% SP-C, quasistatic compression of DPPC/POPC/POPG films allowed progression to minimum tensions on the order of 13–15 mN/m, which are still higher than those required to properly stabilize the interface. Maximal surface tensions, reached at the end of expansion, were also high, suggesting that the excluded material is not respreading into the interface efficiently. Inclusion of progressively higher proportions of cholesterol further impairs the ability of the films to progress toward very low surface tensions. Of interest, increasing proportions of cholesterol also reduce the maximal surface tensions achieved along

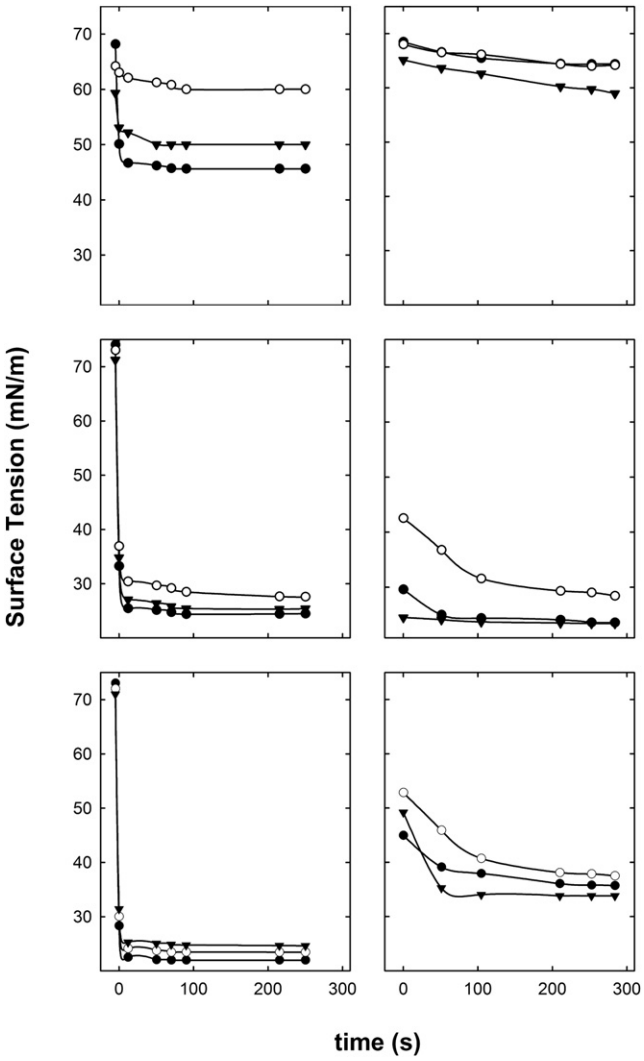

FIGURE 4 Effect of SP-C and cholesterol on the interfacial adsorption of phospholipids at a bubble air-liquid interface. (*Left panels*) Surface tension versus time adsorption isotherms were obtained after spreading 0.5 μL of a suspension 10 mg/mL of the mixture DPPC/POPC/POPG (50:25:15:1, w/w/w) in the absence (*upper panels*) or presence of SP-C 1 or 2% (w/w) (*central and lower panels*, respectively), at the surface of a 50 μL air bubble formed into a subphase of 5 mM Tris, 150 mM NaCl, pH 7.0, containing sucrose (10% w/w). (*Right panels*) Interfacial adsorption leading to a decrease in surface tension was monitored upon expansion of the bubble from 0.05 to a volume of 0.15 mL. The samples assayed contained 0% (•), 5% (○), or 10% (▼) cholesterol/phospholipid by weight.

the different quasistatic compression-expansion cycles. In the absence of cholesterol and in the presence of SP-C 2%, the films were able to reach close to zero surface tension upon compression. However, in the presence of cholesterol 5% or 10%, the films only reduced surface tensions to 5 and 20 mN/m, respectively, under quasistatic compression.

When subjected to dynamic compression-expansion cycling, all the films containing 1% or 2% SP-C were able to reach very low tensions, in either the absence or presence of cholesterol. However, the changes in relative surface area that were required were considerable (see Table S3) and showed large compression-expansion hysteresis, reflecting the low stability of compressed phases. A further increase in the concentration of SP-C, up to 3% or 5% by weight with respect to phospholipids, did not reestablish the original behavior of these films in the absence of cholesterol (see Fig. S2).

We repeated our experiments in the presence of 1% SP-B purified from porcine lungs. Membranes containing SP-B or both SP-B and SP-C were functionally tested in the CBS in the absence or presence of cholesterol 5%. Fig. 7 shows the adsorption kinetics from SP-B-containing membranes during the initial or postexpansion adsorption. In the absence of cholesterol, SP-B promoted a very rapid adsorption of lipids into the interface, to equilibrium tension values of ~22–23 mN/m, with or without SP-C. The introduction of cholesterol significantly impaired the ability of lipid-protein complexes containing SP-B to rapidly achieve low surface tensions. The simultaneous presence of SP-C, however, substantially restored the ability of cholesterol- and SP-B-containing films to produce tensions below 30 mN/m. The quasistatic and dynamic compression-expansion isotherms of the SP-B/lipid films in the absence or presence of SP-C and cholesterol are shown in Figs. 8 and 9, respectively. The performance of these films in the absence of SP-C and cholesterol was surprisingly similar to that of whole native surfactant. Minimum surface tensions were reached very efficiently, with only an ~20% area change, and the system expanded to maximal tensions lower than 30 mN/m with almost no hysteresis. The presence of cholesterol dramatically impaired the performance of SP-B-containing films under both quasistatic and dynamic compression-expansion cycling. Compressions on the order of 60% of the initial area were required to reach the minimal tension in these films, which was higher than the minimal tension reached in the absence of cholesterol. Cholesterol also produced much higher maximal surface tensions and introduced a considerable hysteresis into the compression-expansion cycles. Given these results, one might conclude that the presence of cholesterol makes the films much less efficient in terms of reaching low surface tensions during compression. However, the simultaneous inclusion of 2% SP-C prevented most of the deleterious effects caused by 5% cholesterol in the compression-expansion dynamics of SP-B containing films. Films containing both SP-B and SP-C and cholesterol were able to produce under compression as low surface tension as films containing SP-B in the absence of cholesterol, with similarly limited compression and reduced hysteresis.

DISCUSSION

Even though cholesterol accounts for the major fraction of neutral lipids in lung surfactant, its role in this material remains unknown. Cholesterol has been reported to

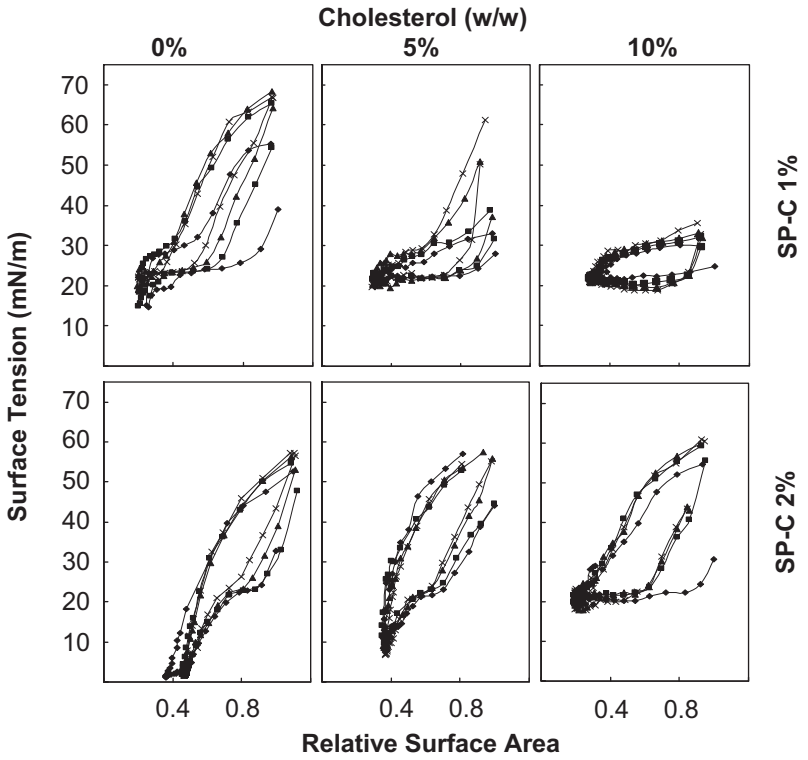

FIGURE 5 Effect of SP-C and cholesterol on the quasistatic compression-expansion isotherms of phospholipid films in a CBS. Quasistatic compression-expansion isotherms of films adsorbed from DPPC/POPC/POPG (50:25:15, w/w/w) suspensions containing 1% (*upper panels*) or 2% (*lower panels*) SP-C and 0% (*left*), 5% (*central*), or 10% (*right*) cholesterol/phospholipid by weight. Plotted are isotherms obtained from the first (◆), second (■), third (▲), and fourth (×) sequential compression and expansion cycles.

modulate the fluidity of lipid mono- and bilayers (10), and increase their stability and impermeability to water (21,22). Several studies have reported that the ability of different surfactant models to reach very low surface tensions is markedly impaired in the presence of cholesterol (12,23,24). Furthermore, the impaired surface activity of surfactant obtained from patients of ARDS has been attributed in part to an elevated content of cholesterol (25).

The physiological relevance of cholesterol in maintaining the structure of pulmonary surfactant membranes was proved

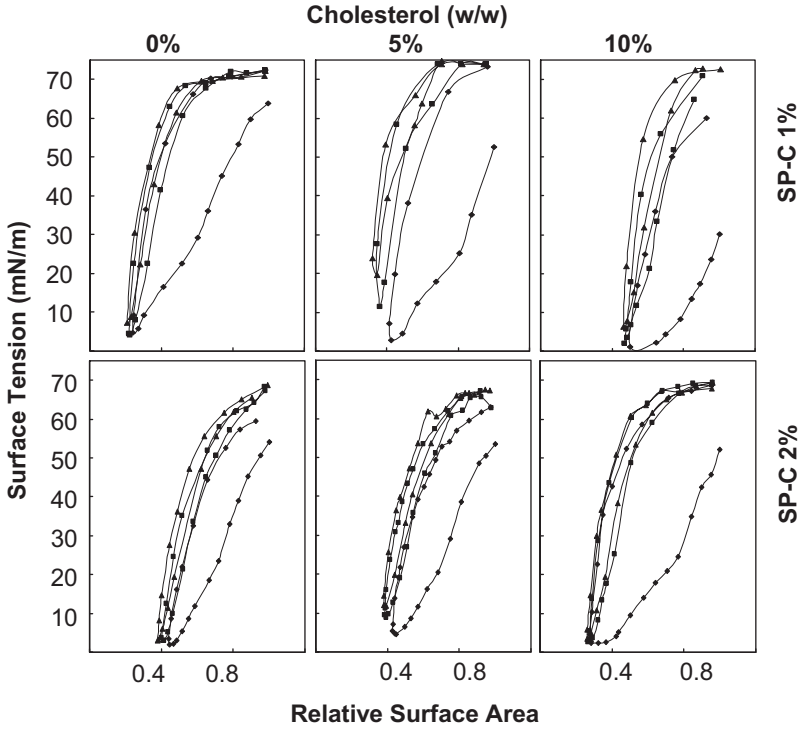

FIGURE 6 Effect of SP-C and cholesterol on the dynamic compression-expansion isotherms of phospholipid films in a CBS. Dynamic compression-expansion isotherms, obtained at 20 cycles/min, of films adsorbed from DPPC/POPC/POPG (50:25:15, w/w/w) suspensions containing 1% (*upper panels*) or 2% (*lower panels*) SP-C and 0% (*left*), 5% (*central*), or 10% (*right*) cholesterol/phospholipid by weight. Plotted are isotherms obtained from the first (◆), 10th (■), and 20th (▲) sequential compression and expansion cycles.

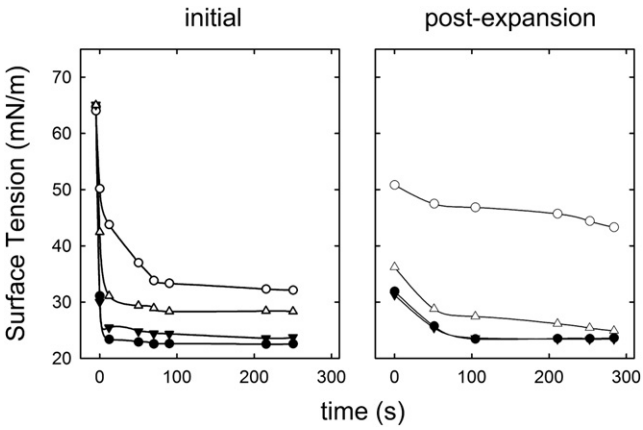

FIGURE 7 Effect of SP-C and cholesterol on the interfacial adsorption of phospholipid suspensions containing SP-B. (*Left panel*) The surface tension versus time adsorption isotherms was obtained after spreading 0.5 μL of a suspension of 10 mg/mL of the mixture DPPC/POPC/POPG (50:25:15:1, w/w/w) containing 1% SP-B (protein/phospholipid by weight) in the absence (*closed symbols*) or presence (*open symbols*) of 5% cholesterol/phospholipid (w/w), in the absence (*circles*) or presence (*triangles*) of 2% SP-C (protein/phospholipid, w/w; *central and lower panels*, respectively), and at the surface of a 50 μL air bubble formed into a subphase 5 mM Tris, 150 mM NaCl, pH 7.0, containing sucrose (10% w/w). (*Right panel*) Interfacial adsorption was further monitored in the same bubbles upon expansion of the bubble from 0.05 to a volume of 0.15 mL.

by Bernardino de la Serna et al. (20), who reported the coexistence of fluid-ordered and fluid-disordered-like phases promoted by cholesterol at physiological temperatures. This structure was dramatically affected by the extraction of cholesterol. The presence of cholesterol also produces a dynamic effect on the order, mobility, and lateral diffusion of surfactant phospholipids (19). Cholesterol has also been shown to introduce a dynamic contribution in the structure of surfactant films. Native surfactant films or films formed from the whole surfactant hydrophobic fraction, including cholesterol, exhibit complex compression-driven lateral transitions, including segregation and remixing of phases, that are profoundly altered when cholesterol is removed (26–28). Recent studies suggest that physiological levels of cholesterol (up to 10% by weight with respect to phospholipids) may modulate surfactant function (29–31). The beneficial function of physiological cholesterol levels in surfactant has been clearly established for heterothermic animals, in which the concentration of cholesterol varies rapidly in response to body temperature changes (32). The experimental data presented here strongly suggest an interaction between cholesterol-enriched phases and SP-C, and a functional role of this interaction in modulating the properties of the surfactant membranes and films. We cannot unequivocally establish at this point whether there is a direct molecular interaction between SP-C and cholesterol or whether SP-C has a preferential interaction with cholesterol-containing membrane phases, in which other lipids such as DPPC and PG may also play a role.

Model membranes containing DPPC/POPC/POPG and SP-C reached low minimum surface tensions but were not able to maintain a stable configuration, as evidenced by the large hysteresis and the failure to lower the maximum surface tension. The presence of cholesterol within these membranes lowered the efficiency in the insertion of new material from reservoirs, and also prevented the films from reaching minimum surface tensions along subsequent compression-expansion cycles, presumably due to the failure of the compressed films to sustain low tensions without deformation. The mechanical stability of surfactant films subjected to compression is usually associated with the segregation in the surfactant layers of DPPC-enriched domains, which are in a gel-like state at physiological temperatures. The presence of cholesterol increases the intrinsic fluidity of these ordered domains, which are then in a liquid-ordered state, with a smaller value of associated melting enthalpy and a lower mechanical stability.

The paradoxical effect of cholesterol on the interfacial performance of pulmonary surfactant films was previously investigated with the use of bovine lipid extract surfactant (BLES), a clinical surfactant produced by depletion of neutral lipids from organic extracts of bovine bronchoalveolar

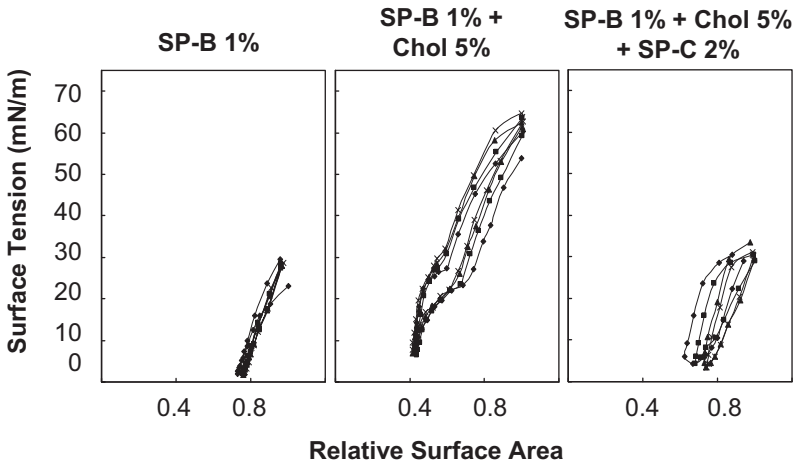

FIGURE 8 Effect of SP-C and cholesterol on the quasi-static compression-expansion isotherms of phospholipid films containing SP-B. Quasistatic compression-expansion isotherms of films adsorbed from DPPC/POPC/POPG (50:25:15, w/w/w) suspensions containing 1% SP-B in the absence of cholesterol and SP-C (*left panel*), in the absence of SP-C but in the presence of 5% cholesterol/phospholipid by weight (*central panel*), or in the presence of both cholesterol 5% and SP-C 2% (protein/phospholipid by weight). Plotted are isotherms obtained from the first (◆), second (■), third (▲), and fourth (×) sequential compression and expansion cycles.

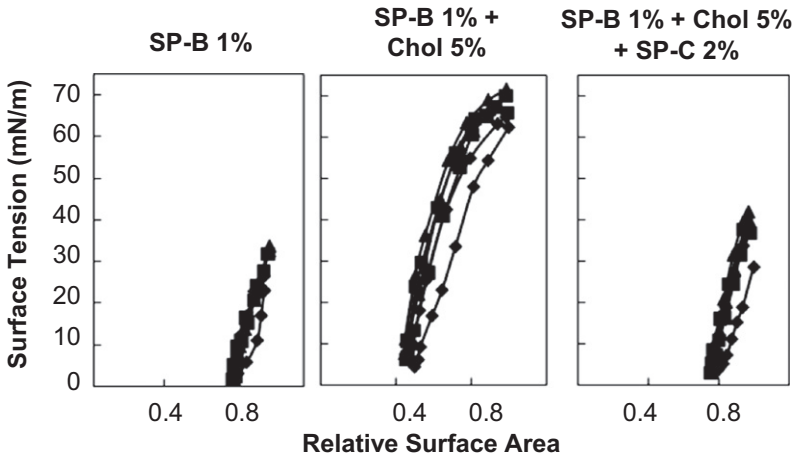

FIGURE 9 Effect of SP-C and cholesterol on the dynamic compression-expansion isotherms of phospholipid films containing SP-B. Dynamic compression-expansion isotherms, obtained at 20 cycles/min, of films adsorbed from DPPC/POPC/POPG (50:25:15, w/w/w) suspensions containing 1% SP-B in the absence of cholesterol and SP-C (*left panel*), in the absence of SP-C but in the presence of 5% cholesterol/phospholipid by weight (*central panel*), or in the presence of both cholesterol 5% and SP-C 2% (protein/phospholipid by weight). Plotted are isotherms obtained from the first (◆), 10th (■), and 20th (▲) sequential compression and expansion cycles.

lavages (30). BLES can accept up to 10% of cholesterol while maintaining the ability to reach very low surface tensions. Inclusion of cholesterol proportions on the order of 20%, however, significantly impaired the performance of BLES films. Our data suggest that the presence of SP-C in BLES extracts could be responsible for preventing the deleterious effects of cholesterol. An exacerbated amount of cholesterol, however, might not be properly buffered by the limited amount of SP-C and could progressively introduce a destabilizing effect.

We have demonstrated that a simple lipid mixture mimicking the actual proportions of DPPC and anionic phospholipid species in surfactant is able to reproduce the interfacial behavior of native surfactant as long as SP-B is present. If physiological proportions of cholesterol are also introduced, an efficient performance is observed only in the simultaneous presence of physiological-like SP-C proportions. Our calorimetric data suggest that this beneficial action of SP-C does not proceed through protein-promoted segregation of cholesterol from the surface-active lipid phases, because there is no reversion of the effect of cholesterol on the thermotropic properties of membranes in the presence of the protein. Rather, we propose that the interaction of SP-C and/or attached SP-C-containing membranes with cholesterol-enriched phases improves their mechanical performance without necessarily altering the lateral distribution of lipid species. Cholesterol and SP-C could therefore cooperate to provide surfactant with relatively dynamic properties without the caveat of losing mechanical stability at the highly compressed states. In a previous study, Gunasekara et al. (30) were able to partly restore the proper performance of BLES containing 20% cholesterol by incorporating additional amounts of DPPC. This finding indicates that it is probably the excessively fluid/dynamic character of cholesterol-containing surfactant phases that prevents them from reaching stable compressed states. Further addition of DPPC could shift the biophysical properties of certain regions of the films from a rather fluid liquid-ordered organization into a less fluid, gel-like type of organization that is mechanically more stable. However, restoration of the films' stability by the addition of higher proportions of DPPC would be achieved at the cost of a substantial decrease in dynamics and a marked impairment of the rheological properties of lipid/protein suspensions. This is a major problem in the context of the design of efficient clinical surfactants. In most cases, the surfactants currently used in respiratory medicine have an altered composition, particularly with respect to the content of SP-B and SP-C (33,34). For proper performance, these preparations require supplementation with additional amounts of DPPC and/or palmitic acid. We propose that the requirement of these spurious lipids is related to an inappropriate balance of lipid and protein species. As an undesired consequence, these clinical surfactants have higher viscosities than native surfactant, which may impair their ability to distribute efficiently toward the distal airways (35). In the presence of adequate proportions of SP-C, a surfactant containing limited amounts of DPPC and cholesterol could still sustain the lowest surface tensions at maximal compression while preserving a highly dynamic character. Our results provide additional support for the conclusion recently reached by Almlen et al. (36), that both SP-B and SP-C are required for optimal function.

It is known that cholesterol increases the fluidity of ordered phases of membranes enriched in saturated phospholipid species. This increase in fluidity would have a functionally contradictory effect on lung surfactant performance. Beneficially, it could favor structural transitions that facilitate the formation of surfactant surface reservoirs or accelerate respreading of compressed structures. In contrast, it would decrease the stability of the most compressed films. Although SP-B is the essential protein that permits surfactant to reach the lowest tensions, an association between SP-C and SP-C-containing membranes and relatively fluid DPPC/cholesterol-enriched domains could help improve the mechanical stability of surfactants and impede their out-of-plane relaxation at the end of expiration. This model would justify the intrinsic instability of surfactant from SP-C knockout mice, which may contribute to their chronic

respiratory pathologies (6,7). It is conceivable that the levels of SP-C and cholesterol in surfactant are coordinated in response to different physiological requirements by molecular mechanisms as yet unknown, as was previously pointed out in a study of heterothermic animals (17).

In conclusion, this work provides additional evidence of the existence of an interaction between cholesterol-enriched phases and SP-C, and confirms that physiological concentrations of cholesterol do not have any detrimental effect on lung surfactant with a proper lipid and protein composition. In the light of these results, it is clear that the inclusion of a proper and balanced proportion of cholesterol, in conjunction with the appropriate amounts of SP-C, should be considered in the future development of optimized clinical surfactants.

SUPPORTING MATERIAL

Three tables and two figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)01440-4.

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