

Fibrinolysis-inhibitory capacity of clot-embedded surfactant is enhanced by SP-B and SP-C

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Markart, Philipp, Clemens Ruppert, Friedrich Grimminger, Werner Seeger, and Andreas Günther. Fibrinolysis-inhibitory capacity of clot-embedded surfactant is enhanced by SP-B and SP-C. *Am J Physiol Lung Cell Mol Physiol* 284: L69–L76, 2003. First published September 6, 2002; 10.1152/ajplung.00037.2002.—Incorporation of pulmonary surfactant into fibrin inhibits its plasminic degradation. In the present study we investigated the influence of surfactant proteins (SP)-A, SP-B, and SP-C on the fibrinolysis-inhibitory capacity of surfactant phospholipids. Plasmin-induced fibrinolysis was quantified by means of a ^{125}I -fibrin plate assay, and surfactant incorporation into polymerizing fibrin was analyzed by measuring the incorporation of ^3H -labeled L- α -dipalmitoylphosphatidylcholine into the insoluble clot material. Incorporation of a calf lung surfactant extract (Alveofact) and an organic extract of natural rabbit large surfactant aggregates (LSA) into a fibrin clot revealed a stronger inhibitory effect on plasminic cleavage of this clot than a synthetic phospholipid mixture (PLX) and unprocessed LSA. Reconstitution of PLX with SP-B and SP-C increased, whereas reconstitution with SP-A decreased, the fibrinolysis-inhibitory capacity of the phospholipids. The SP-B effect was paralleled by an increased incorporation of phospholipids into fibrin. We conclude that the inhibitory effect of surfactant incorporation into polymerizing fibrin on its susceptibility to plasminic cleavage is enhanced by SP-B and SP-C but reduced by SP-A. In the case of SP-B, increased phospholipid incorporation may underlie this finding.

hyaline membrane; coagulation; pulmonary surfactant; surfactant protein; acute respiratory distress syndrome; fibrosis

ALVEOLAR FIBRIN FORMATION is a histopathological hallmark of the acute respiratory distress syndrome and of various other acute or chronic lung diseases (3, 8, 10, 17–19, 24, 25, 30). Under inflammatory conditions, both alveolar macrophages and alveolar epithelial cells may produce and shed significant amounts of procoagulant activity, which can be almost exclusively attributed to the extrinsic pathway enzyme tissue factor and factor VII (12, 17–19, 25, 31). Moreover, the fibrinolytic activity of the alveolar compartment is markedly impaired due to depressed urokinase (u-PA) activities and increased activities of the plasminogen activator inhibitor (PAI-I) and α_2 -antiplasmin (4, 18,

19). Fibrinogen, entering the alveolar space because of increased endothelial and epithelial permeability, may thus rapidly be converted to fibrin.

Polymerizing fibrin was noted to cause a severe impairment of surfactant function by incorporating hydrophobic surfactant constituents such as phospholipids and surfactant protein (SP)-B (29). The surfactant inhibitory capacity of polymerizing fibrin surpasses that of fibrinogen, soluble fibrin monomer, and albumin by more than two orders of magnitude, thus rendering fibrin formation the most effective surfactant inhibitory mechanism hitherto described for plasma proteins. Therefore, alveolar fibrin may thus well contribute to the impairment of gas exchange and lung mechanics in acute lung injury. Moreover, delayed clearance of fibrin may provide a provisional matrix for subsequent invasion by fibroblasts (1, 2, 5, 9, 11, 38). Surfactant impairment due to protein leakage, alveolar collapse, persistent “fibrin gluing” of opposed alveolar septae, and fibroblast invasion have been suggested as important sequelae in the pathogenesis of lung fibrosis, a process previously termed “collapse induration” (8). Lysis of such surfactant-incorporating fibrin clots, on the other hand, was noted to result in the liberation of intact surfactant material and marked improvement in surface tension properties (14). Timely dissolution of alveolar fibrin may thus represent an important feature for recovery from acute lung injury and prevention of fibrotic events.

Our group reported that surfactant-rich fibrin clots are less susceptible to proteolysis by plasmin, trypsin, or elastase (14). The present study was performed to further characterize the influence of the surfactant proteins on the fibrinolysis-inhibitory capacity of clot-embedded surfactant. In essence, this was found to be further enhanced by the hydrophobic proteins SP-B and SP-C, but reduced by SP-A. Thus these surfactant proteins apparently exert a differential influence on the kinetics of fibrin turnover in the alveolar space.

MATERIALS AND METHODS

Calf lung surfactant extract (CLSE, Alveofact) was a generous gift from Dr. H. Weller (Thomae, Biberach, Germany).

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Purified human fibrinogen (>95% clottability, containing minor amounts of factor XIII) was kindly provided by Prof. N. Heimburger (Behringwerke, Marburg, Germany). Human plasmin (specific activity 8 U/mg) was purchased from Boehringer Mannheim (Mannheim, Germany). Bovine thrombin was from Behring (Behringwerke). L- α -Dipalmitoylphosphatidylcholine (DPPC) and phosphatidylglycerol (PG, derived from egg yolk) as well as *n*-octyl- β -D-glucopyranoside were received from Sigma (Munich, Germany). 125 I-labeled human fibrinogen (specific activity 200 μ Ci/mg fibrinogen, >90% clottability) and dipalmitoylphosphatidyl (*N*-methyl- 3 H)choline (specific activity 84.1 mCi/mg) were obtained from Amersham Buchler (Braunschweig, Germany). Immunoplates (Maxisorp) were purchased from Nunc (Wiesbaden, Germany). All other chemicals were from Merck (Darmstadt, Germany).

Determination of Phospholipid Concentration

Phospholipids were quantified by a colorimetric phosphorus assay as described by Rouser et al. (26). Aliquots of the samples were extracted according to Bligh and Dyer (6), and organic phases were taken for quantification. All measurements were performed in quadruple.

Isolation of Surfactant Proteins

Dimeric SP-B and SP-C were isolated from rabbit lavage (New Zealand rabbits, 2.5–2.8 kg body wt) by means of LH60-chromatography [(36); \geq 95% purity as assessed by SDS-PAGE according to Schagger and von Jagow (27)]. SP-A was obtained from rabbit lavage fluids on extraction with *n*-octyl- β -D-glucopyranoside according to van Iwaarden et al. (35), followed by application to mannose chromatography as described [(22); \geq 95% purity as assessed by SDS-PAGE (27)]. We showed the purified SP-A to be active by documenting induction of phospholipid aggregation using the assay system as described by Haagsman et al. (16).

Determination of Surfactant Protein Concentration

We determined the concentration of the purified surfactant proteins using a protein assay according to Bradford [SP-B, SP-C; (7)] or a commercially available bicinchoninic acid protein assay based on the Lowry principle [(23, 33); SP-A]. For determination of the filtrate recovery of SP-A, SP-B, and SP-C in the filter experiments as detailed below, a solid-phase adsorption ELISA (SP-B, SP-C) and a competitive ELISA protocol (SP-A) were used as described recently (20, 21, 28).

Preparation of Surfactant Mixtures

Alveofact was delivered as lyophilisate and was resuspended with 150 mM NaCl containing 3 mM Ca^{2+} by brief sonication (Bandelin Sonopuls, Berlin, Germany; 50 W, 25 kHz, 1 min). Phospholipid concentration of the stock solution was measured and was adjusted to a final concentration of 50 mg/ml. We achieved supplementation of Alveofact with SP-A by adding increasing amounts of recombinant SP-A in 150 mM NaCl containing 3 mM Ca^{2+} .

A synthetic phospholipid mixture (PLX) was prepared by dissolving DPPC and PG in chloroform-methanol (2:1 vol/vol) at a ratio of 7:3 (wt/wt). After lyophilization by a vacuum centrifuge (Univap, Munich, Germany), the lipids were resuspended in 150 mM NaCl containing 3 mM Ca^{2+} by brief sonication (Bandelin Sonopuls; 50 W, 25 kHz, 1 min). Supplementation of this synthetic PLX with the surfactant proteins was achieved either by adding the hydrophobic SP-B

and SP-C in chloroform-methanol to the DPPC/PG stock solution before lyophilization or by admixture of SP-A in saline after resuspension of the dried lipids with 150 mM NaCl containing 3 mM Ca^{2+} by brief sonication (50 W, 25 kHz, 1 min). The final phospholipid concentration of all preparations was adjusted to 50 mg/ml.

Bronchoalveolar lavages obtained from healthy New Zealand rabbits of either sex were cell depleted by centrifugation at 300 *g* (10 min, 4°C), and large surfactant aggregates (LSA) were isolated by high-speed centrifugation at 48,000 *g*. Aliquots of the 48,000 *g* pellet were SP-A depleted by chloroform-methanol extraction (6), and the organic phase was lyophilized and resuspended in 150 mM NaCl containing 3 mM Ca^{2+} (sonication as above). Both LSA preparations were analyzed for phospholipid content and adjusted to a final phospholipid concentration of 50 mg/ml. In addition, aliquots of the LSA stock solution were butanol extracted (32), and the aqueous phase was lyophilized and resuspended in the same volume of distilled water as initially taken for extraction. Corresponding volumes of this preparation compared with the native LSA were used in the fibrinolysis assay.

Fibrin Plate Assay

To address the influence of surfactant on fibrinolysis in dependence of the surfactant protein composition, we used a 125 I-fibrin plate assay as previously described (34). All components were dissolved in 150 mM NaCl containing 3 mM Ca^{2+} . First, unlabeled (5 mg/ml) and labeled fibrinogen were mixed with various natural and synthetic, surfactant protein-based surfactant preparations (0–30 mg/ml) and were introduced into the wells at a volume of 30 μ l. All concentrations refer to this volume. Complete clotting, ascertained by preceding control experiments, was achieved by incubation with thrombin predissolved in 20 μ l of buffer fluid (10 mU/ml, 37°C, 1.5 h). The plates were then spun at 1,485 *g* for 10 min (Univapo 150H centrifuge) for further consolidation of the clot material. Next, we carefully layered 200 μ l of saline/ Ca^{2+} or plasmin (1 U/ml) onto the surface of the clot and incubated it while gently vortexing (100 \times /min) the plates at 25°C. One hundred twenty minutes after the onset of protease or sham incubation, 150 μ l of the supernatant were aspirated, and counts were measured in a Canberra Packard γ -counter. We corrected the liberation of radiolabel in the presence of plasmin for baseline release of 125 I activity detected in the parallel control experiments with saline incubation. This baseline release of tracer persistently ranged <10% of total radioactivity. All data of plasmin-induced liberation of radiolabel from the insoluble clot are given in percent of total activity applied to each well.

The following protocols were employed.

Influence of a bovine surfactant extract (CLSE) vs. a synthetic, surfactant protein-free PLX on fibrinolysis. Increasing amounts of CLSE (Alveofact) or PLX (0–30 mg/ml) were admixed to the fibrinogen before clotting.

Influence of the native vs. the organic or aqueous fraction of rabbit LSA on fibrinolysis (native LSA, lipophilic and aqueous compounds). Increasing amounts (0–20 mg/ml) of native LSA or lipophilic or aqueous compounds of LSA were prepared as detailed in *Preparation of Surfactant Mixtures* and admixed to the fibrinogen before clotting.

Impact of surfactant proteins on the inhibition of fibrinolysis by a synthetic PLX. PLX (20 mg/ml), supplemented with increasing amounts (0.02–0.4 mg/ml, corresponding to 0.1–2% wt/wt, related to the lipids) of natural, dimeric rabbit SP-B, natural rabbit SP-C, and natural rabbit SP-A were added to the fibrinogen before clotting.

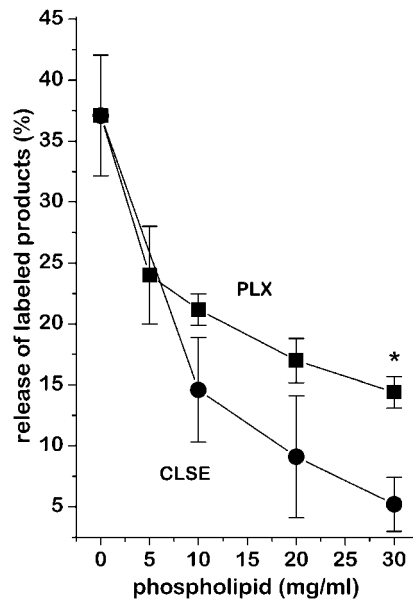


Fig. 1. Effect of calf lung surfactant extract (CLSE, Alveofact) and phospholipid mixture (PLX) on plasmin-induced fibrinolysis. Increasing amounts of CLSE and PLX were added to ^{125}I -labeled fibrinogen (5 mg/ml) before clotting with thrombin (10 mU/ml). Fibrinolysis was induced by addition of plasmin (1 U/ml), and release of radiolabeled fibrinolysis products were measured after 120 min and are given as a percentage of the initially provided radioactivity. Means \pm SE of 4 independent experiments each are given. *Statistically significant diversity between the 2 dose-effect curves for the highest concentration, $P < 0.05$.

Impact of SP-A on the inhibition of fibrinolysis by a bovine surfactant extract (CLSE). CLSE (Alveofact, 20 mg/ml supplemented with increasing amounts (0.1–0.2 mg/ml, corresponding to 0.5–1% wt/wt related to the lipids) of natural rabbit SP-A was added to the fibrinogen before clotting.

Impact of isolated surfactant proteins on the fibrinolysis in the absence of surfactant phospholipids. Natural rabbit SP-A, SP-B, and SP-C (0.2 mg/ml each) were added without any surfactant phospholipids to the fibrinogen before clotting.

Filter Experiments

Phospholipid and surfactant protein incorporation into fibrin was analyzed by means of filter experiments as recently described (29). For phospholipid incorporation, ^3H -labeled DPPC was mixed with 2 mg/ml CLSE (Alveofact) or PLX, followed by the addition to increasing concentrations (0–4 mg/ml) of nonlabeled fibrinogen in 150 mM NaCl containing 3 mM Ca^{2+} . In separate experiments, PLX was supplemented with increasing amounts of the purified surfactant proteins (0–2% wt/wt related to the lipids) before the addition of the tracer. After all mixtures were stirred rigorously and incubated for 1 h at 37°C, 10 mU/ml thrombin were added and further incubated for 1.5 h at 37°C. The aqueous phase was then separated from the insoluble clot material by centrifugation through a nylon gauze (pore size 150 μm , 170 g, 5 min). In alternative experiments, the DPPC label was omitted. Instead, ^{125}I -labeled fibrinogen (200 $\mu\text{Ci}/\text{mg}$ fibrinogen) was added to the fibrinogen preparation to assess the influence of surfactant protein supplementation of PLX on the fibrin formation. ^3H and ^{125}I counts were determined in the filtrate and compared with the total activity provided in each individual experiment.

The incorporation of SP-A, SP-B, and SP-C into fibrin was similarly assessed either in absence of any phospholipids or

in presence of 2 mg/ml PLX. We used 0.02 mg/ml of surfactant protein (1% wt/wt related to lipids) throughout. SP-A, SP-B, and SP-C recoveries in the filtrates were determined by means of ELISA technique.

Statistics

All data are given as mean \pm SE. The Mann-Whitney U -test was performed to assess the level of significance between the various experimental groups, which is indicated throughout the figures (+ or $*P < 0.05$, ++ or $**P < 0.01$, and +++ or $***P < 0.001$).

RESULTS

As could be anticipated from the previous studies, incorporation of a natural CLSE (Alveofact) into a fibrin matrix caused a dose-dependent inhibition of plasmin-elicited fibrinolysis (Fig. 1). This held true also for the surfactant protein-free synthetic PLX (Fig. 1), but PLX was clearly less effective than CLSE. At the highest dosage used (30 mg/ml), CLSE reduced the enzymatic release of split products from 37.1% (control, absence of surfactant) to 5.2%, whereas the corresponding data was 14.4% for PLX.

Compared with PLX, LSA exerted similar inhibitory capacity with a reduced enzymatic release of split products to 46% (LSA) and 43% (PLX) of control at a phospholipid concentration of 20 mg/ml (Fig. 2). More

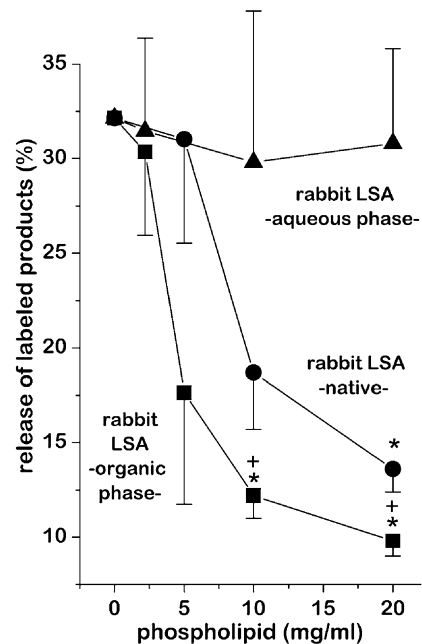


Fig. 2. Effect of the native vs. the organic or aqueous fraction of rabbit large surfactant aggregates (LSA) on plasmin-induced fibrinolysis. Increasing amounts of LSA or its organic or aqueous phase extract were added to ^{125}I -labeled fibrinogen (5 mg/ml) before clotting with thrombin (10 mU/ml). Fibrinolysis was induced by addition of plasmin (1 U/ml), and release of radiolabeled fibrinolysis products was measured after 120 min, given as a percentage of the initially provided radioactivity. Each point reflects the mean \pm SE of 4 independent experiments. *Statistically significant diversity between the native or the organic fraction and the aqueous fraction of LSA, $P < 0.05$; + significant diversity between the organic and the native fraction of LSA, $P < 0.05$.

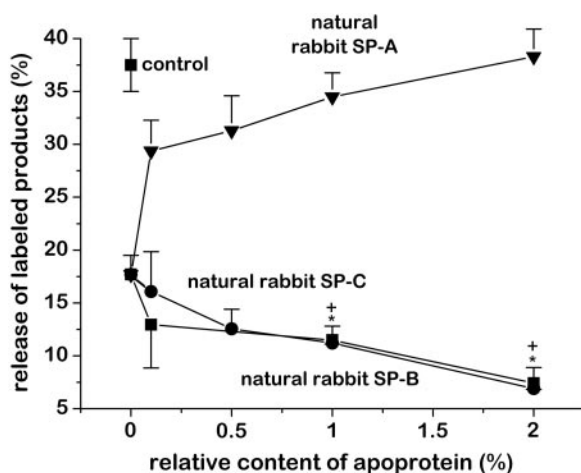


Fig. 3. Effect of surfactant proteins on the inhibition of fibrinolysis by PLX. PLX (20 mg/ml) supplemented with increasing amounts of the different surfactant proteins (% wt/wt related to lipids) were added to ^{125}I -labeled fibrinogen (5 mg/ml) before clotting with thrombin (10 mU/ml). Fibrinolysis was induced by addition of plasmin (1 U/ml), and release of radiolabeled fibrinolysis products were measured after 120 min, given as a percentage of the initially provided radioactivity. Control was performed in absence of surfactant. Each point reflects mean \pm SE of 4 independent experiments. *Statistically significant diversity between 1 and 2% of surfactant protein (SP)-B or SP-C and the corresponding concentrations of natural SP-A, $P < 0.05$; +statistically significant diversity between the highest relative concentrations of SP-B and SP-C and values at 0%, $P < 0.05$.

prominent inhibitory capacity was noted for the organic phase of LSA, which approximated that of CLSE (38 and 41% of control at a phospholipid concentration of 10 mg/ml, respectively). In contrast, no inhibitory capacity was noted for corresponding amounts of aqueous LSA constituents.

Combining PLX with increasing amounts of hydrophilic vs. lipophilic surfactant proteins exerted opposite effects on the fibrinolysis inhibition using this synthetic PLX (Fig. 3). Although the addition of natural rabbit SP-A (0.2–2%, wt/wt related to lipid) almost completely antagonized the inhibitory effect of 20 mg/ml PLX on fibrinolysis, the supplementation of PLX with corresponding concentrations of natural rabbit SP-B or SP-C resulted in a pronounced augmentation of the fibrinolysis inhibitory properties. In this respect, SP-C and SP-B were similarly effective. Moreover, natural rabbit SP-A (0.5–1%, wt/wt related to lipid) was capable of reversing the inhibitory effect of CLSE on plasmin-induced fibrinolysis (see Fig. 4). In contrast, the addition of the surfactant proteins in the absence of phospholipids (0.2 mg/ml, corresponding to 1% in Fig. 3) did not exert any influence on the cleavage kinetics (control, 36.6%; +SP-B, 36.9%; +SP-C, 39.3%; +SP-A, 37.8%)

As assessed by filter studies, addition of neither 2 mg/ml PLX alone nor PLX supplemented with SP-A, SP-B, or SP-C (1% each) exerted any effect on the clot formation (control, 95.1%; PLX, 96.5%; PLX plus SP-A, 95.7%; PLX plus SP-B, 91.7%; PLX plus SP-C, 93.3%). Approximately 95% of the ^{125}I -label remained within

the insoluble clot material, suggesting complete coagulation and excluding significant dissociation between label and fibrinogen. When assessing the phospholipid incorporation using ^3H -labeled DPPC, we observed marked differences between PLX and CLSE. As depicted in Fig. 5, generation of 4 mg/ml fibrin in the presence of 2 mg/ml surfactant resulted in $\sim 45\%$ incorporation of ^3H -labeled DPPC in the case of PLX but $\sim 70\%$ incorporation in the case of CLSE. Reconstitution of PLX with natural rabbit SP-B, SP-C, or SP-A exerted markedly different effects on the incorporation rate of this synthetic phospholipid mixture (Fig. 6). Although SP-A and SP-C displayed only minor efficacy, an admixture of SP-B dose dependently increased the incorporation rate of PLX. In the presence of 2% SP-B, the percentage of PLX incorporated into the arising clot increased to $\sim 70\%$ compared with $\sim 35\%$ in the absence of SP-B, thus approaching incorporation rates observed for CLSE (containing $1.7 \pm 0.3\%$ SP-B). Accordingly, an increased phospholipid incorporation and enhanced potency for fibrinolysis inhibition were noted to be closely correlated for PLX with varying SP-B content (Fig. 7). In line with this finding, we observed that SP-B itself was substantially incorporated into polymerizing fibrin in both the presence and absence of phospholipids, whereas neither SP-A or SP-C showed a significant degree of binding to fibrin columns (Fig. 8).

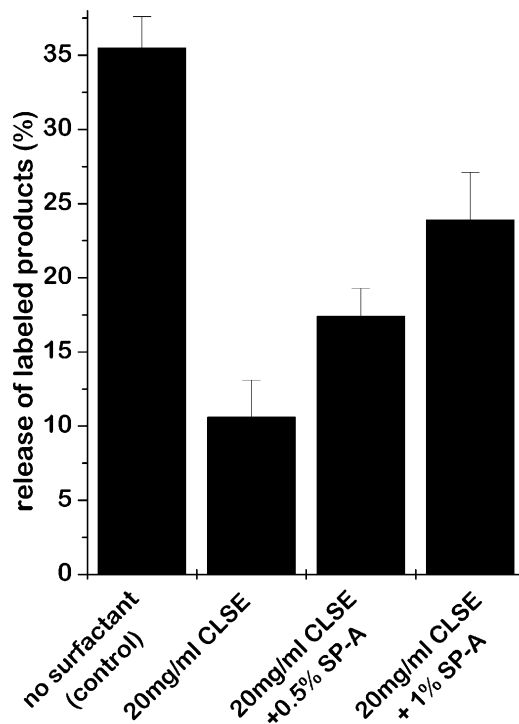


Fig. 4. Effect of SP-A admixture on the fibrinolysis inhibition of CLSE. CLSE (Alveofact, 20 mg/ml), in the absence or presence of 0.5 and 1% natural rabbit SP-A, respectively, was mixed with 4 mg/ml ^{125}I -labeled fibrinogen, and clotting was achieved by incubation with thrombin. Tracer release induced by subsequent incubation with plasmin for 120 min is given as a percentage of total radioactivity. Control was performed in absence of surfactant proteins. Values are means \pm SE of 4 independent experiments.

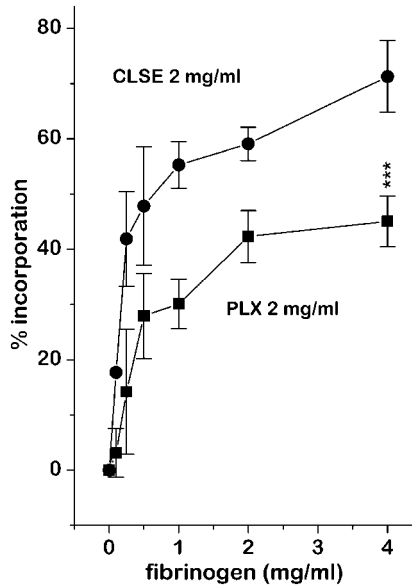


Fig. 5. Incorporation of CLSE (Alveofact) and PLX into polymerizing fibrin. ³H-labeled L- α -dipalmitoylphosphatidylcholine (DPPC) was admixed to CLSE or PLX (2 mg/ml) followed by the addition of increasing amounts of fibrinogen. After coagulation with thrombin, the percentage of label associated with the insoluble clot material was assessed by filter passage. Means \pm SE of 4 independent experiments each are given. ***Statistically significant diversity between the highest concentration of CLSE or PLX, $P < 0.001$.

DISCUSSION

In accordance with previous studies, surfactant incorporation into polymerizing fibrin dose dependently inhibited the susceptibility of this clot to subsequent

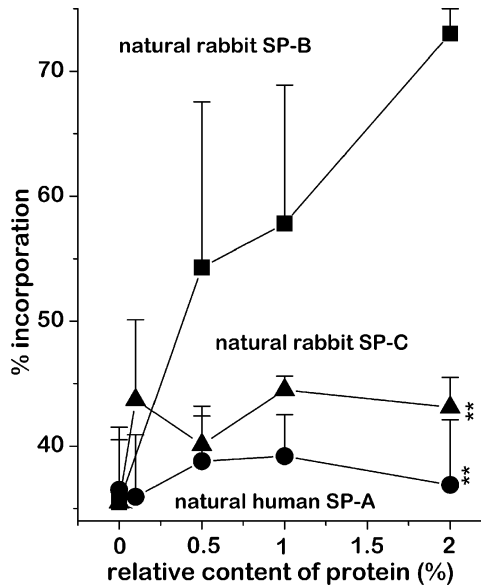


Fig. 6. Effect of surfactant proteins on phospholipid incorporation into polymerizing fibrin. ³H-labeled DPPC was admixed to 2 mg/ml PLX supplemented with different quantities of surfactant proteins (0–2% wt/wt related to the lipids), followed by the addition of 4 mg/ml fibrinogen. After coagulation with thrombin, the clot-associated label was assessed by filter passage. Each point reflects mean \pm SE of 4 independent experiments. **Statistically significant diversity between the highest relative concentration of SP-B and SP-A, $P < 0.01$.

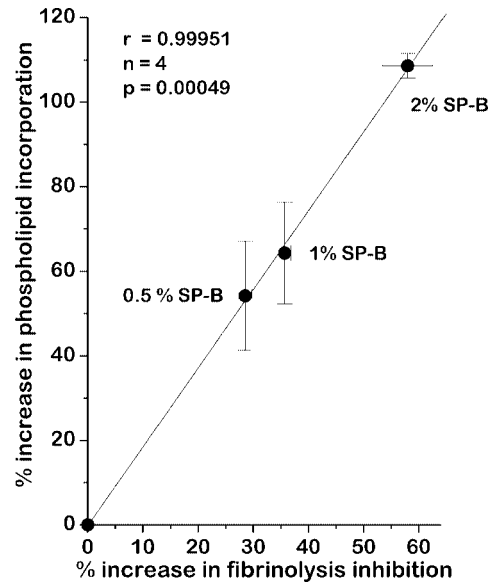


Fig. 7. Correlation between the increase in phospholipid incorporation and the increase in fibrinolysis inhibition in dependence of SP-B. PLX mixtures with different SP-B concentrations were employed. Data correspond to those in Figs. 3 and 6. Each point reflects mean \pm SE of 4 independent experiments.

fibrinolysis by plasmin. We noted a major impact of the surfactant proteins employed in concentrations as encountered under in vivo conditions. The hydrophobic surfactant proteins SP-B and SP-C augmented, but

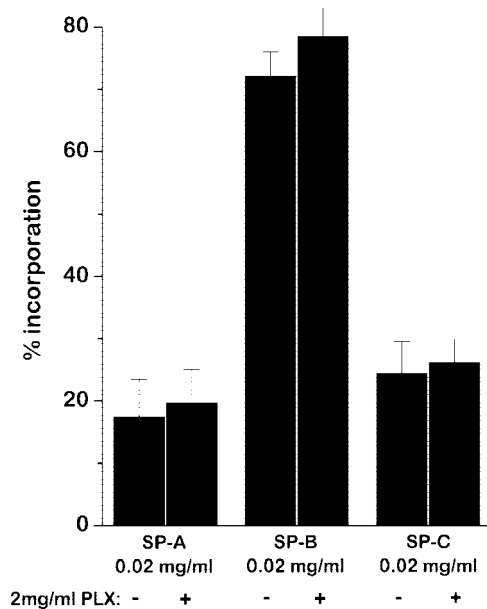


Fig. 8. Incorporation of SP-A, SP-B, or SP-C into fibrin in absence or presence of PLX. Unlabeled fibrinogen was mixed with 0.02 mg/ml of SP-A, SP-B, or SP-C in the absence of any phospholipids or in the presence of 2 mg/ml PLX. Clotting was achieved through incubation with thrombin. The aqueous phase was then separated from the insoluble clot material by centrifugation through a nylon gauze; SP-A, SP-B, and SP-C recoveries in the filtrates were determined by means of ELISA technique, and the percentage of incorporated surfactant proteins was calculated from this data. Values are means \pm SE of 4 independent experiments.

SP-A reduced, the fibrinolysis-inhibitory capacity of the surfactant lipids, as 1) the natural CLSE (Alveofact) containing both SP-C and SP-B was more effective than the surfactant protein-free PLX, 2) the organic solvent extract of rabbit lavage LSA was more effective than the native LSA containing all surfactant proteins, 3) the inhibitory capacity of PLX was dose dependently enhanced on reconstitution with SP-B or SP-C but reduced on reconstitution with SP-A, and 4) the inhibitory capacity of CLSE was reduced on reconstitution with SP-A.

As demonstrated in our previous study (14), the fibrinolysis-inhibitory capacity of surfactant phospholipids is linked to their effective incorporation into a growing fibrin clot: when administered after termination of the clot generation, this effect of the surfactant lipids is fully lost. Hydrophobic-hydrophobic interactions between the phospholipids and lipophilic clusters well known to exist within the fibrin matrix have been suggested as underlying mechanism (14). Interposition of phospholipids between the fibrin fibers, possibly with membranous tapering of single fibrin strands, is assumed to hamper the access of plasmin to its binding sites within the fibrin molecule and thereby retarding its enzymatic cleavage. This view was also supported by the finding that the three-dimensional structure and the physiological properties of the surfactant lipid-rich fibrin differ greatly from those of normal fibrin (15). A direct interference of surfactant phospholipids with plasmin as an alternative mechanism was excluded as plasmin lysis of preformed fibrin was not affected by these lipids (14).

Several mechanisms may underlie the current observation that the fibrinolysis-inhibitory capacity of surfactant lipids is markedly enhanced by SP-B and SP-C, but reduced by SP-A.

Direct Influence of Surfactant Proteins on Fibrinolysis or Fibrin Clot Formation

When tested in the absence of surfactant phospholipids, none of the surfactant proteins exerted any effect on the plasminic cleavage of fibrin. This is in line with the previous observation that surfactant proteins do not interfere with the cleavage of fibrinogen by plasmin *in vitro* (13). Moreover, the percentage of fibrin(ogen) incorporated into the growing fibrin clot was not affected by the surfactant proteins either in the absence or presence of surfactant phospholipids, with numbers consistently ranging >90%. Thus there is no evidence that the surfactant proteins per se exert a direct influence on the process of fibrin clot formation or the plasmin-induced fibrinolysis.

Influence of Surfactant Proteins on Phospholipid Incorporation

The inhibition of fibrinolysis is known to be dependent on the dose of surfactant present during the process of fibrin polymerization (29). Thus any alteration of the extent of surfactant incorporation into the growing fibrin clots could influence the resistance of this clot to plasminic cleavage. SP-B was, indeed, found to greatly enhance the

percentage of phospholipid incorporation, from ~35% in the absence, to ~75% in the presence, of 2% SP-B under the given experimental conditions. Accordingly, a close correlation between the increase in phospholipid incorporation and the resistance to plasminic cleavage was noted for the SP-B-enriched PLX. It is in line with this observation that, both in the presence and absence of any phospholipids, SP-B itself was largely incorporated (>70%) into the growing clot material. Although not well established at the present time, SP-B might thus facilitate phospholipid incorporation into polymerizing fibrin by its binding to both surfactant phospholipids (well established) and distinct moieties within the fibrin lattice, thereby resulting in enhanced "anchoring" of the phospholipids in the fibrin clot and increasing the resistance to plasminic cleavage.

Alteration of the Macromolecular Phospholipid Arrangement

In contrast to SP-B, SP-A and SP-C exerted no significant influence on the extent of phospholipid incorporation into the polymerizing fibrin; nevertheless, SP-A markedly enhanced, and SP-C decreased, its susceptibility to plasminic lysis and organically extracted LSA, which differs from native LSA mostly by the absence of SP-A and which showed markedly elevated fibrinolysis-inhibitory capacity. Alteration of the macromolecular phospholipid arrangement within the fibrin lattice offers the most feasible explanation for this observation. Because SP-A promotes lipid mixing, reconstitution of PLX with SP-A results in the formation of large multilamellar aggregates, whereas reconstitution with either SP-B or SP-C yields discoidal particles often associated with each other in vertical columns like stacked coins or in flat sheets of subunits arranged in a loose hexagonal array (37). If this also holds true for the architecture of phospholipids within the aqueous solvent channels of the fibrin matrix, a highly complex phospholipid arrangement due to SP-A might result in a rather nonhomogenous distribution of the phospholipid material, and a more membranous or discoidal arrangement of phospholipids due to SP-C might effect a more continuous tapering of the fibrin fibers, with disparate impact on the access of plasmin to its fibrin binding sites. SP-B might induce similar changes on the phospholipid architecture as SP-C but may be of minor importance in terms of fibrinolysis inhibition compared with the SP-B-induced increase in phospholipid incorporation rate. It remains currently open whether addition of SP-A to clinically used organic surfactant extracts may yield reduced inhibition of fibrinolysis on incorporation of these therapeutic surfactants into fibrin polymers formed *in vivo* under conditions of acute lung injury.

Conclusions

We found that the hydrophobic surfactant proteins SP-B and SP-C further decreased, whereas SP-A increased, the susceptibility of a fibrin clot, generated in the presence of surfactant phospholipids, to plasminic

cleavage. The most likely explanation for this observation is a differential impact of these surfactant proteins on the phospholipid arrangement within the fibrin matrix, thereby further inhibiting (SP-B, SP-C) or facilitating (SP-A) the access of the protease to its binding and cleavage sites on the fibrin strands. In addition, SP-B was found to augment the percentage of phospholipids incorporated into a growing fibrin clot, further adding to the resistance of the surfactant-phospholipid-rich clot to plasminic cleavage. In view of the profound disturbances of the alveolar hemostatic balance under conditions of acute and chronic inflammatory lung disease, including fibrinogen leakage and changes in alveolar surfactant protein concentrations, it is conceivable that such changes could have a major influence on the kinetics of fibrin turnover in the alveolar space. The differential impact of hydrophobic vs. hydrophilic surfactant proteins may influence therapeutic approaches with surfactant replacement, when the aim is to achieve timely dissolution of fibrin within the alveolar compartment to prevent loss of chronically atelectatic areas.

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