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Biophysical Chemistry 104 (2003) 229–238

Biophysical Chemistry

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Impact of surface tension on the conversion rate of large to small surfactant aggregates

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Received 20 August 2002; received in revised form 15 November 2002; accepted 15 November 2002

Abstract

The extracellular alveolar surfactant can be separated into the highly surface active large surfactant aggregates (LA) and the less active small surfactant aggregates (SA). Conversion of LA to SA is encountered upon cyclic surface area changes and demands the presence of enzymatic activity. In the present study we investigated the influence of surface tension on the conversion of LA to SA. Bronchoalveolar lavage fluid (BALF) obtained from healthy rabbits was cycled for various time periods in absence or presence of increasing amounts of serum proteins or oleic acid. LA were isolated, quantified and the minimum surface tension (γ_{min}) of uncycled or cycled LA was assessed in absence or presence of increasing amounts of serum proteins or oleic acid. In additional experiments, already cycled LA with a γ_{min} of $\sim$20 mN/m were pooled, diluted to a similar PL concentration as original BALF and cycled a second time. Serum proteins and oleic acid dose-dependently: (i) increased the γ_{min} values of LA when added to the isolated LA; and (ii) decreased the LA to SA conversion when added to BALF. These events were directly correlated, suggesting inhibition of LA to SA conversion by increase in γ_{min} of the LA fraction. In line with this suggestion, already cycled LA displaying poor surface activity showed no further conversion in a second cycling maneuver, whereas LA being similarly prepared from uncycled BALF did. We conclude that LA to SA conversion is inversely correlated with the surface tension of the LA fraction. An increase in γ_{min} of the LA fraction during the cycling procedure blocks further LA to SA conversion and may thus represent a negative feedback mechanism.

Keywords: ARDS/ALI; Pulmonary surfactant; Phospholipid; In vitro cycling; Large surfactant aggregates; Surfactant convertase

1. Introduction

Large surfactant aggregates (LA) are the predominant surfactant fraction in the alveolar space, being composed of ultraheavy and heavy surfactant subfractions (lamellar bodies, tubular myelin and large multilamellar vesicles) and characterized by high surface activity [1–3]. The LA are assumed to represent the precursor fraction of the interfacial active surfactant layer and thus to feed this layer under cyclic surface area changes. In contrast, the light surfactant fraction, also referred to as small surfactant aggregates (SA), mainly consists of small unilamellar vesicles and displays only poor surface activity. SA probably represent the meta-

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PII: S0301-4622(02)00370-8

bolic end product of the interfacial surfactant film that may be recycled by alveolar type II cells, degraded by alveolar macrophages or transported across the mucociliar escalator [1–3]. Indeed, a transit from LA to SA can be observed in vivo [4] and under cyclic surface changes in vitro [1,2,5]. The molecular mechanisms underlying the conversion are not well understood, but strict dependency of the conversion from cyclic surface area changes is known. In addition, it was recently suggested that a serine-active carboxyl esterase termed convertase may be involved in the conversion process. Although the substrate and the putative cleavage site(s) are yet not precisely defined for this convertase [6–8], a dramatic loss of the hydrophobic apoprotein B (SP-B), recently observed to occur during cycling of the LA fraction, suggests that SP-B might represent one candidate [2,9].

In experimental lung injury [10] as well as in acute inflammatory (severe pneumonia, ARDS [9,11,12]) or chronic interstitial (IPF [13]) human lung diseases a markedly reduced percentage of the LA fraction is present throughout, with concomitantly increased SA concentrations. Again, the underlying reason for this surfactant subfraction imbalance is presently poorly understood. Decreased alveolar type II cell secretion of freshly synthesized or recycled surfactant material, accelerated conversion of LA to SA or degradation of LA due to inflammatory mediators including phospholipases or proteases are all offered as putative mechanisms. Notwithstanding these uncertainties it is well imaginable that, next to the changes in the surfactant composition and the inhibition of surfactant by plasma proteins, proteases and phospholipases, such an imbalance of the alveolar surfactant subfractions, may significantly contribute to the severe abnormalities in surface activity causing alveolar instability and impairment of gas exchange in lung inflammation [9,11].

The LA to SA conversion occurring during in vitro cycling of BALF was recently noted to be paralleled by a marked deterioration of surface activity of the remaining LA fraction [9]: the minimum surface tension (γ_{min}) after 5 min of the post-cycled LA was increased to $\sim$20 mN/m, in contrast to near zero of the LA fraction originating from non-cycled BALF. No further LA to SA conversion was observed for these post-cycled fractions, suggesting that the surface activity of this fraction might per se influence the conversion rate.

In the present study we, therefore, investigated the influence of the surface tension on surfactant subtype conversion in vitro. For this purpose, we employed plasma proteins and oleic acid for inhibition of surface activity, and we analyzed post-cycled BALF to obtain LA fractions with increased surface tension. In essence, LA to SA conversion was indeed found to directly correlate with the surface tension of the LA fraction.

2. Experimental

2.1. Materials

Oleic acid was purchased from Sigma (Munich, Germany). Pooled rabbit serum was prepared by withdrawal of 10-ml blood from the antecubital vein from 20 healthy rabbits. Serum was obtained after complete coagulation by centrifugation at $4500 \times g$ for 10 min. Fifteen milliliters-Falcon blue cups (#2096) were delivered by Becton Dickinson (Meylan, Cedex, France). A bicinchoninic acid-based protein assay (BCA assay) was purchased from Pierce.

2.2. Preparation of a rabbit BALF pool ($BALF_{rab}$)

Rabbits of either sex were killed by an intravenous application of a lethal dose of pentobarbital/ketamine. A catheter was immediately placed into the trachea and the lungs were lavaged three times with 50-ml saline. After filtration through sterile gauze and sedimentation of cells ($300 \times g$, 15 min, 4 °C), supernatants were pooled and aliquoted in 5-ml fractions in polypropylene tubes while being continuously stirred. The aliquots were then stored at −85 °C until further processing. The phospholipid content was approximately 81 μg/ml.

2.3. Addition of serum proteins and oleic acid to BALF and LA

To assess the influence of serum proteins or oleic acid on the conversion kinetics, increasing

amounts of pooled rabbit serum (protein/PL = 1:1–16:1; w/w) or of oleic acid (oleic acid/PL = 1:8–1:1; w/w) were admixed to the pooled rabbit BALF prior to cycling. The protein content of the pooled rabbit serum was determined using a commercially available bicinchoninic acid protein assay based on the Lowry principle [14,15]. To examine the influence of serum proteins or oleic acid on surface activity these agents were recombined with uncycled LA (2 mg/ml PL) from rabbit BALF at the ratios outlined above.

2.4. In vitro cycling of rabbit BALF

Aliquots of the pooled rabbit BALF in the presence or absence of serum proteins or oleic acid, were fixed on a rotating disk in a 37 °C incubator. As previously described [5,9], surface area cycling was then performed at a rate of 32×/min for 120 or 240 min, respectively, with approximately nine-fold surface area changes. In a second set of experiments, LA obtained from cycled or uncycled rabbit BALF were isolated by high speed centrifugation as detailed below, resuspended in 150 mM NaCl/3 mM $CaCl_2$, adjusted to a PL concentration of 80 μg/ml and re-cycled for another 240 min.

2.5. Surfactant subtype separation and LA quantification

Separation of LA from SA was performed by high speed centrifugation (48 000×*g*, 4 °C, 1 h) using a Sorvall centrifuge (DuPont, Bad Homburg, Germany; SS34 rotor). The LA-containing pellets were resuspended in saline with 3 mM $CaCl_2$. The relative LA content was assessed by relating the fraction of pelleted PL to the total amount of PL provided.

2.6. Analysis of phospholipid content

Original BALF or isolated LA fractions were extracted according to Bligh and Dyer [16] and organic phases were taken for quantification. Phospholipids were quantified by means of a colorimetric phosphorus assay as described by Rouser [17].

2.7. Assessment of surface activity

Surface activity of the LA alone or enriched with increasing amounts of serum proteins and oleic acid, respectively, was measured at a constant PL concentration of 2 mg/ml by means of a pulsating bubble surfactometer as previously described [11]. The technique provided readings of the surface tension after 12 s of film adsorption (γ_{ads}, static measurement) and the surface tension at a minimum bubble radius after 5 min of film oscillation ($_{\gamma min}$, dynamic measurement). In brief, LA or LA protein or LA oleic acid mixtures were incubated for 30 min at 37 °C after brief sonication. After filling the sample chamber with 30 μl of the sample, it was immediately transferred to the pulsating bubble surfactometer. Adsorption measurements were performed by machine-driven generation of the bubble at minimum bubble size (radius 0.4 mm) and maintenance of it at this size for 12 s. Next, pulsation was started with a rate of 20 cycles/min. The bubble radius varied then between a minimum size of 0.4 mm and a maximum size of 0.55 mm and minimum and maximum surface tension were recorded over 5 min (the γ_{min} value given here refers to the minimum surface tension value obtained after 5 min of pulsation). For calculation of the mean ± S.D. four independent experiments were conducted.

2.8. Synthetic substrate esterase assays

To investigate the influence of oleic acid and serum proteins on the esterase and amidase activity of porcine liver carboxyl esterase, two assays were employed according to Heymann and Mentlein [18]. The esterase activity was characterized using *p*-nitrophenylacetate as substrate (5×10^{-4} M); this substrate is split into 4-nitrophenol and acetic acid by the esterase activity. In these experiments, the porcine liver esterase was used at a concentration of 0.06 U/ml (2.8 μg/ml, 4×10^{-8} M). The absorbance kinetics at $\lambda = 405$ nm in the absence or presence of oleic acid (oleic acid/esterase = 1:8–1:1, w/w) or serum proteins (serum proteins/esterase = 1:1–16:1, w/w), respectively, was recorded at 25 °C, pH 7.5, for 3 min in 10-s intervals. The amidase activity of the porcine liver

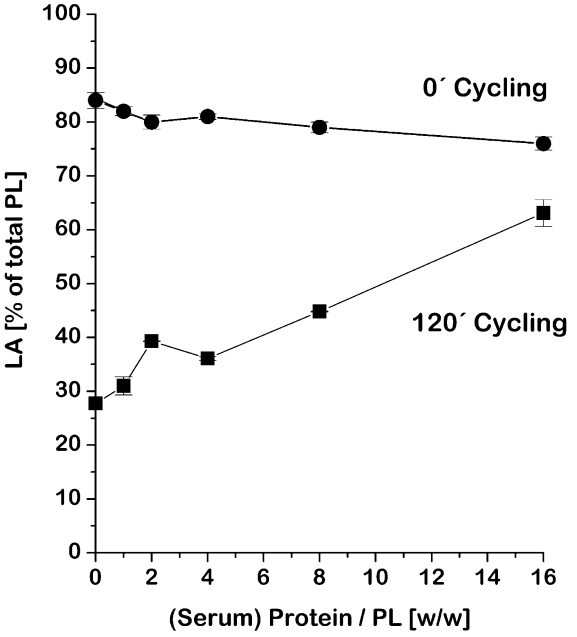

Fig. 1. Influence of increasing amounts of serum proteins on the LA to SA conversion rate occurring upon cycling. Increasing amounts of serum proteins (protein/PL = 1:1–16:1; w/w) were added to rabbit BALF. The content of LA was determined after sham incubation (0 min of cycling) and after and 120 min of cycling, and is given in % of total BALF. Means ± S.D. of four independent experiments are given.

esterase (used at 1 U/ml = 28 μg/ml, 4×10^{-7} M) was demonstrated using butanilicaine as substrate (8×10^{-3} M), which is split into 2-chloro-6-methylaniline and *N*-butyl-glycine. The absorbance kinetics at $\lambda = 285$ nm in the absence or presence of oleic acid (oleic acid/esterase = 1:8–1:1, w/w) or serum proteins (serum proteins/esterase = 1:1–16:1, w/w), respectively, was determined at 25 °C, pH 7.5 for 3 min in 10-s intervals. The enzymatic activity was calculated from the slope of these readings employing a serially diluted standard curve and related to the activity in the absence of oleic acid or serum proteins (100% control).

2.9. Statistics

All data are given as mean ± S.D. Linear regression analysis was performed by a computer-based calculation program (MiroCal Origin), the Pearson's correlation coefficient (R) is given.

3. Results

The results for serum proteins are shown in Figs. 1–3 and for oleic acid are shown in Figs. 4–6. Addition of increasing amounts of serum proteins (protein/PL = 1:1–16:1; w/w) or oleic acid (oleic acid/PL = 1:8–1:1; w/w) to BALF did not affect the relative large surfactant aggregate (LA) content (~80% of total PL) in the absence of cycling (serum proteins Fig. 1; oleic acid Fig. 4). In contrast, in vitro cycling in the presence of serum proteins or oleic acid resulted in a dose-dependent retardation of the conversion of LA to SA. LA content, which was decreased to ~25%

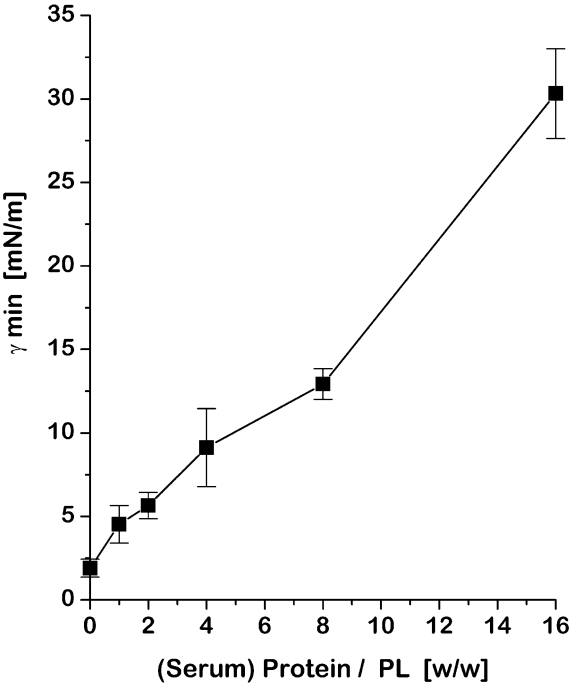

Fig. 2. Influence of increasing amounts of serum proteins on the minimum surface tension (γ_{min}) of LA in absence of cycling. Increasing amounts of serum proteins (at the same ratios as depicted in Fig. 1) were added to LA from native rabbit BALF (2 mg/ml PL) and γ_{min} was assessed in a pulsating bubble surfactometer (surface tension read after 5 min of film oscillation). Means ± S.D. of four independent experiments each are given.

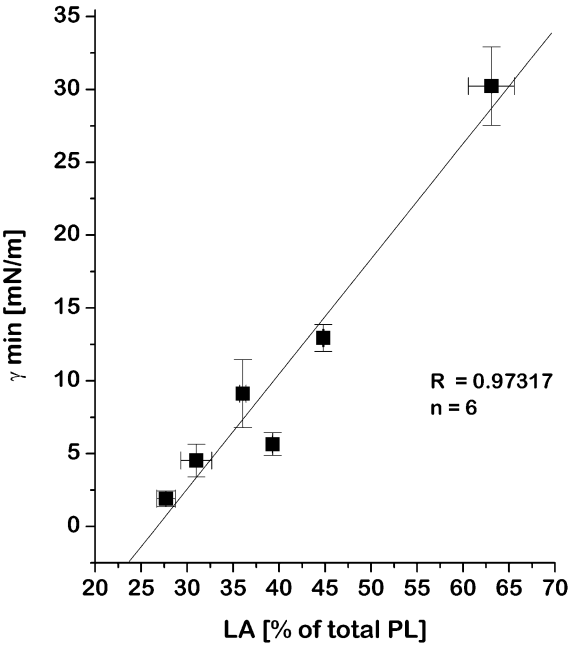

Fig. 3. Correlation between minimum surface tension of rabbit LA and relative content of LA (percentage of total phospholipids) after 120 min of in vitro cycling in presence of increasing amounts of serum proteins (protein/PL = 1:1–16:1; w/w). Data correspond to those in Figs. 1 and 2.

of total PL at the end of the 120-min cycling maneuver in the absence of proteins and oleic acid, was raised to ~60% at a protein/PL ratio of 16:1 (w/w) and to ~70% at a oleic/PL ratio of 1:1 (w/w).

When added to isolated LA (2 mg/ml PL) from native rabbit BALF at the same ratios outlined above, both serum proteins and oleic acid were noted to inhibit the surface activity in a dose-dependent manner (serum proteins Fig. 2; oleic acid Fig. 5). In detail, the minimum surface tension after 5 min of film oscillation (γmin), which was near zero in the absence of proteins/oleic acid, increased to ~30 mN/m at a protein/PL ratio of 16:1 (w/w) and to ~20 mN/m at an oleic acid/PL ratio of 1:1 (w/w). Accordingly, a direct correlation between the decrease in the LA to SA conversion rate and the increase in surface tension of the LA fraction was noted for both the serum proteins (Fig. 3) and the oleic acid (Fig. 6).

When isolating the LA fraction from cycled or uncycled rabbit BALF it was found that excellent surface activity (γ_{min} ~4 mN/m) was encountered for the LA from the uncycled source (Table 1), whereas the γmin of the LA obtained from cycled rabbit BALF was increased to ~20 mN/m. In vitro cycling of these LA preparations showed that the LA preparation from uncycled BALF presented with a similar LA to SA conversion rate as native rabbit BALF, whereas the LA preparation originating from pre-cycled BALF was poorly if at all converted into the LA subtype (Table 1).

To exclude any possible influence of oleic acid or serum proteins on the putatively involved esterase we incubated porcine liver carboxylesterase, an enzyme showing high homology with the surfactant convertase that may induce surfactant conversion in vitro, with increasing amounts of oleic

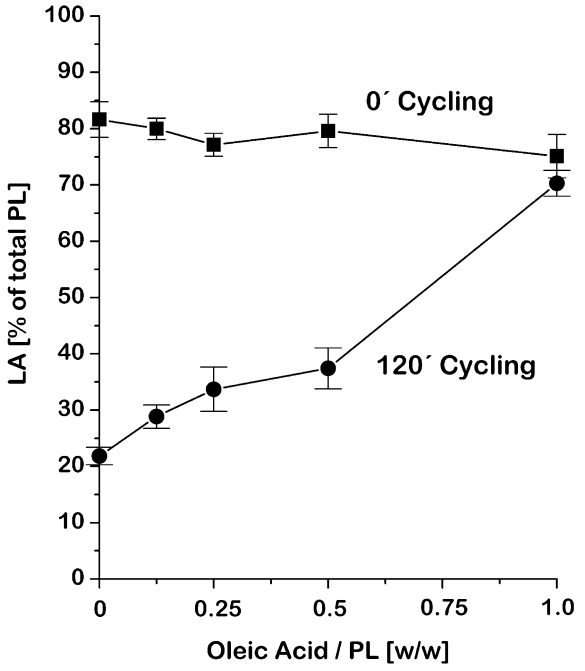

Fig. 4. Influence of increasing amounts of oleic acid on the LA to SA conversion rate occurring upon cycling. Increasing amounts of oleic acid (oleic acid/PL = 1:8–1:1; w/w) were added to rabbit BALF. The content of LA was determined after sham incubation (0 min of cycling) and after 120 min of cycling, and is given in % of total BALF. Means ± S.D. of four independent experiments are given.

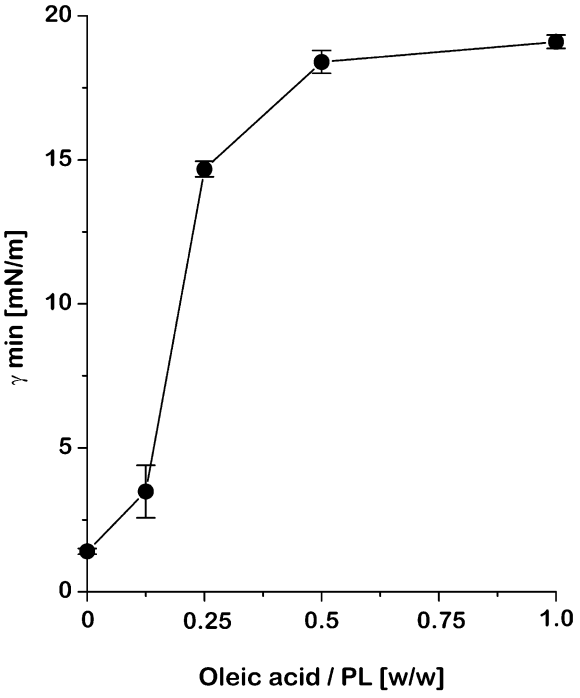

Fig. 5. Influence of increasing amounts of oleic acid on the minimum surface tension (γ_{min}) of LA in absence of cycling. Increasing amounts of oleic acid (at the same ratios as depicted in Fig. 4) were added to LA from native rabbit BALF (2 mg/ml PL) and γ_{min} was assessed in a pulsating bubble surfactometer (surface tension read after 5 min of film oscillation). Means ± S.D. of four independent experiments each are given.

acid and serum proteins, respectively, and determined the remaining activity. As obvious from Table 2, no deterioration of the enzymatic activity, neither that of esterase nor that of amidase, was demonstrated for serum proteins. In case of oleic acid, a slight inhibition of the amidase but not the esterase activity was observed at high oleic acid/esterase ratios.

4. Discussion

It has recently been shown that in vitro cycling of BALF originating from healthy lungs results in a time-dependent decrease of the LA in favor of the SA fraction [1,2,5] and that this LA to SA conversion is paralleled by a loss in surface activity of the remaining LA fraction [9]. In the present study we focused on the influence of the surface tension on the conversion kinetics and forwarded evidence that the surface tension of the LA fraction has major impact on the extent of subtype conversion. Three lines of evidence underscore this conclusion: firstly, addition of serum proteins, known as potent inhibitors of surfactant function increasing its minimum surface tension [19,20], effected both an increase in γ_{min} of the LA fraction and a decrease of the LA to SA conversion rate. Secondly, the same held true for admixture of oleic acid, another compound known to severely affect surface tension lowering properties, capable of substantially increasing γ_{min} values: this agent similarly decreased the LA to SA conversion rate. With both interventions, a strict correlation between the increase in surface tension of the LA fraction and the decrease in conversion rate was observed. And thirdly, we found that isolated and pooled LA from cycled BALF displayed highly

Table 1
Influence of cycling on the conversion rate of LA isolated from pre-cycled and uncycled rabbit BALF

	LA from pre-cycled BALF		LA from uncycled BALF	
	LA-content	γ_{min} (mN/m)	LA-content	γ_{min} (mN/m)
0 min cycling	98.3 ± 2.8%	20.3 ± 1.1	97.7 ± 1.4%	3.6 ± 0.7
240 min cycling	86.7 ± 4.6%	19.0 ± 2.0	29.9 ± 2.1%	18.8 ± 1.4

Rabbit BALF (~81 μg/ml PL; LA content ~80% of total PL) was cycled for 240 min, or sham-cycling (no rotation) was performed. The LA fractions were then isolated, resuspended in 150 mM NaCl/3mM $CaCl_2$ and adjusted to 81 μg/ml PL, resulting in a relative LA content of this material of near 100%. Cycling for (another) 240 min was then performed, and the LA content is given in relation to total PL before and after this cycling maneuver. In addition, the minimum surface tension after 5 min of film oscillation (γ_{min}) is given for the different LA fractions (assessed at a PL concentration of 2 mg/ml). Values are means ± S.D. of three independent experiments.

Table 2
Influence of oleic acid and serum proteins on the enzymatic activity of a liver carboxylesterase

Concentration oleic acid/esterase (w/w)	Oleic acid		Concentration serum proteins/esterase (w/w)	Serum proteins	
	Esterase activity [%]	Amidase activity [%]		Esterase activity [%]	Amidase activity [%]
Control	100	100	Control	100	100
1:8	96.6±1.1	90.2±1.0	1:1	98.6±3.9	101.3±1.0
1:4	96.0±2.4	86.5±2.5	2:1	104.8±0.1	105.0±0.3
1:2	99.4±1.0	82.6±0.6	4:1	103.4±1.1	112.0±0.4
1:1	95.7±1.4	62.8±3.7	8:1	109.4±1.0	114.2±1.5
			16:1	109.5±2.9	113.9±1.6

Porcine liver carboxylesterase was incubated with increasing amounts of oleic acid and serum proteins, respectively. The enzymatic activity was determined using the synthetic substrates *p*-nitrophenylacetate (esterase activity) and butanilicaine (amidase activity), respectively. The activity was calculated from a standard curve and related to the activity of the control (without addition of oleic acid or serum proteins) and is given in%. Values are means±S.D. of three independent experiments.

impaired surface tension reducing properties with γ_{min} values approaching 20 mN/m, and no further conversion into SA was noted when subjecting this LA fraction to a second cycling maneuver. In contrast, LA prepared exactly the same way from uncycled rabbit BALF with near zero γ_{min} values, readily converted into SA under these conditions. All these findings are compatible with the suggestion that the surface tension itself exerts a major influence on the enzymatically driven process of LA to SA conversion.

Alternatively, the inhibitory effect of serum proteins and of oleic acid could be explained by a more direct interaction between these inhibitors and the convertase, with the increase in the minimum surface tension representing a completely independent aspect. However, the close correlation between the increase in γ_{min} and the decrease in conversion kinetics observed for both agents clearly suggests a dependency of both phenomena. Furthermore, in a set of additional experiments, we addressed the activity of liver esterase, an enzyme showing close sequence homology to the lung convertase [21], in synthetic substrate assays (substrates *p*-nitrophenylacetate and butanilicaine [18]). In these assays, the admixture of serum proteins or of oleic acid, employed in the same dose range used in the present studies, did not markedly impair the enzymatic activity of liver esterase with the exception of a an inhibition of the amidase but not the esterase activity of the liver esterase at the highest oleic acid concentration used, thus rendering any direct effect of the presently used inhibitors on the convertase activity less likely.

Which molecular mechanisms may underly the surfactant subtype conversion and, as suggested by the present study, its dependency on surface tension?

1. The LA to SA conversion indeed seems to be an enzymatically driven process, being mediated by the recently discovered serine-active carboxylesterase entitled convertase. This view is supported by the fact that some serine active enzyme inhibitors are capable of inhibiting the conversion kinetics without impairing surface activity [22]. In line with this reasoning, BALF depletion of convertase by pre-treatment with diisopropylfluoride (DFP) is resistant to LA-to-SA conversion by in vitro cycling [6]. Furthermore, reconstitution of synthetic phospholipid mixtures with the surfactant apoproteins SP-A, SP-B and SP-C and with the above-mentioned liver carboxylesterase results in LA-to-SA conversion under conditions of cycling, whereas the same surfactant preparation will not undergo conversion into any lighter types of surfactant fraction in the absence of esterase (unpublished own observations). Finally, convertase has been found in lamellar bodies and is co-secreted into the alveolar space and is then contained in the LA fraction [6]. In view of the recently noted dramatic loss in the SP-B antigen signal from

the LA fraction undergoing cycling [9], this apoprotein offers as a possible substrate of the convertase, however, additional experiments are needed to verify this hypothesis.

2. The observation that cycling with substantial relative surface area changes represents a prerequisite for any LA to SA conversion already suggests that some steric conditions at the air–water interface must be met for the enzyme to gain access to its substrate and to be capable of cleavage. This view is further supported by the present finding that not only surface area changes, but also very low minimum surface tension values are mandatory for the conversion process to take place. A closely packed (highly compressed) interfacial surfactant film, being reflected by very low surface tension values, may thus result in optimum presentation of the substrate to the convertase. This may also include the hydrophobic apoproteins, intimately linked with the compressed interfacial surfactant film. Loss of these specific features of the highly compressed surfactant film, as occurring in the presence of the surfactant-inhibitory agents (serum proteins, oleic acid), may thus block the convertase activity without interfering with the enzyme itself. An increase in surface tension might, therefore, be considered as an effective negative feedback inhibition of surfactant subtype conversion, downregulating impaired conversion kinetics once the surface activity of the LA is beginning to rise due to the action of the convertase itself or due to other mechanisms. Although it was not a primary goal of this study to investigate this negative feedback mechanism, the existence of such a mechanism might be of physiological importance by maintaining low surface tension in the lung and thereby preventing lung dysfunction. However, further studies are needed to elucidate this issue.

It has to be kept in mind that the nine-fold surface area changes used in the in vitro cycling studies are far beyond the surface area changes that occur in the alveolar space under physiological conditions (1.5–2-fold). In acute lung injury (ALI), and even more in the acute respiratory distress syndrome (ARDS), however, the alveolar gas exchange area is greatly reduced due to extensive atelectasis formation, resulting in overinflation of the remaining ventilated lung regions. Surface area changes far exceeding those in normal lungs and approximating those being used in our in vitro conversion assay might thus occur under these conditions and might, by the accelerated LA to SA conversion, contribute to the impairment of surfactant acitivity [23,24]. In line with such reasoning, it has recently been shown that low tidal volume ventilation of these patients, as compared to the conventional tidal volume, is advantageous in view of ARDS severity and outcome [25].

In addition, a pronounced plasma protein leakage is encountered in ARDS, leading to $\geq$10-fold increase in the alveolar protein concentration. In view of our results, one might speculate that such plasma protein leakage protects the endogenous

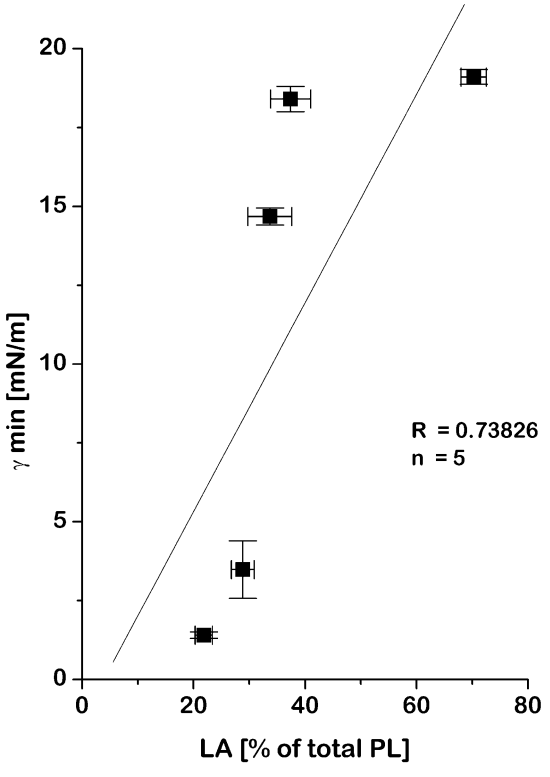

Fig. 6. Correlation between minimum surface tension of rabbit LA and relative content of LA after 120 min of in vitro cycling in presence of increasing amounts of oleic acid. Data correspond to those in Figs. 4 and 5.

LA pool from further LA to SA conversion. Inhibition of surface activity of LA from ARDS patients by leaking plasma proteins was, indeed, recently demonstrated [11]. Nevertheless, a markedly reduced LA content is noted under conditions of ARDS [9,11], suggesting that further mechanisms such as decreased alveolar type II cell secretion of freshly synthesized or recycled surfactant material or accelerated degradation of LA due to the presence of inflammatory mediators including phospholipases or proteases may contribute to the decline of the LA fraction under conditions of ARDS.

5. Conclusions

In conclusion, the present study suggests that the surface tension reached during film compression (γ_{min}) has major impact on the LA to SA conversion by the surfactant convertase. In view of the conversion-associated loss of surface activity of the remaining LA fraction, this might represent a negative feedback inhibitory mechanism of surfactant subtype conversion, relevant both under physiological and pathological conditions.

Acknowledgments

This study was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 547 'Kardiopulmonales Gefäßsystem' and GU 405/3-1). Portions of this investigation were performed in partial requirement for the thesis of Carolin Pucker and Kerstin Seybold.

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