Experimental neonatal respiratory failure induced by lysophosphatidylcholine: effect of surfactant treatment

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Lyso-PC is a catabolic product resulting from breakdown of lung volumes (24). Lysophosphatidylcholine (lyso-PC) line, lowering surface tension to near 0 mN/m at low tensions below that of water. High levels of lyso-PC at an air-water interface, reducing equilibrium surface concentrations to become reacylated in the recycling machine.

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Animal Experiments

Experiments were carried out on near-term newborn rabbits delivered by hysterotomy at a gestational age of 29.5 days (term, 31 days). At this stage of fetal development the lungs are mature, and adequate amounts of surfactant phospholipids have accumulated in the air spaces. The lungs are thus easily expanded at birth, retain air already after the first breath, and can be ventilated with a low transpulmonary pressure (21). Two different protocols were applied, as detailed below.

Protocol 1: Dose-response study. The animals were weighed and tracheotomized at birth and received via the tracheal cannula a 4 ml/kg of various concentrations of lyso-PC in saline (0–10 mg/ml; corresponding to a dose range of 0–40 mg/kg). These dose levels were chosen on the basis of data from Fornasier et al. (9) showing, under similar experimental conditions, biochemical evidence of lung injury (increased lactic dehydrogenase activity in bronchoalveolar lavage fluid) after administration of lyso-PC at a dose of either 16 or 40 mg/kg, particularly with the higher dose.

After the tracheotomy procedure, the animals were transferred to a system of body plethysmographs heated to 37°C. They were ventilated in parallel with a common ventilator system (Servo Ventilator 900 B, Siemens-Elema, Solna, Sweden) delivering 100% oxygen at a frequency of 40 breaths/min and an inspiration-to-expiration ratio of 1:1. Tidal volumes were recorded intermittently with a specially designed Fleisch tube connected to the plethysmograph box, a differential pressure transducer, an amplifier, and an integrator unit (EMT 32, EMT 31, and EMT 41, Siemens-Elema). Insufflation pressure was adjusted individually without limitation to generate tidal volumes of ~10 ml/kg (27). Electrocardiogram was recorded from needle electrodes at regular intervals. Animals were ventilated for 120 min and then killed by intracerebral injection of lidocaine (causing an immediate cardiac arrest). The abdomen was opened and the diaphragm examined for evidence of pneumothorax. Then the chest was opened and the blood sampled from the usually bulging right ventricle for determination of PCO₂ and pH.

Pressure-volume recordings. The lungs were allowed to collapse for 30–60 min after the blood-gas measurements and were then connected via the tracheal tube to a system for parallel pressure-volume recordings (8). Static lung volumes were recorded during stepwise 5-cmH₂O increments up to an insufflation pressure of 30 cmH₂O and during a corresponding deflation maneuver. One minute of stress relaxation was allowed at each pressure level. Volume measurements were corrected for compression in the system.

Histological examination. A catheter was tied in the pulmonary trunk, and the lungs were again expanded at a transpulmonary pressure of 30 cmH₂O for 1 min. The pressure was then lowered to 10 cmH₂O, which was maintained while the lungs were fixed by vascular perfusion with 4% formaldehyde via the pulmonary arteries at a pressure of 65 cmH₂O. The lungs were stored in the same fixative and embedded in paraffin for histological examination, with particular reference to the alveolar expansion pattern and the presence of airway epithelial necrosis, alveolar hyaline membranes, hemorrhage, and recruitment of inflammatory cells to the air spaces. Alveolar volume density was determined by conventional point counting using total parenchyma as reference volume. The histological examination was done on coded sections, i.e., without knowledge of the experimental conditions of individual animals.

Protocol 2: Timing of surfactant treatment, lung leakage of serum albumin, and surfactant inactivation. In a second series of animals, tracheotomized and ventilated as described above, we compared the effects of exogenous modified natural surfactant (Curosurf) administered via the tracheal tube at two different time points. In these experiments, we tested the hypothesis that an exogenous surfactant would be more effective when administered at birth than after a period of ventilation allowing leakage of plasma proteins into the air spaces.

The animals were allocated at random to four subgroups, receiving 1) lyso-PC (10 mg/kg) at birth without concomitant or subsequent treatment with surfactant; 2) lyso-PC (10 mg/kg) at birth together with the recommended clinical dose of Curosurf (200 mg/kg); 3) lyso-PC (10 mg/kg) at birth followed by treatment with Curosurf (200 mg/kg) after 30 min of mechanical ventilation; and 4) normal saline (4 ml/kg) via the airways at birth, without concomitant or subsequent surfactant treatment. All animals received at birth an intravenous injection of 10% human albumin (Sigma Chemical; dose 7 ml/kg) via a jugular vein exposed during the tracheotomy procedure. The human albumin served as a lung permeability marker (1) and was quantified by immunodiffusion (LC-Partigen plates, Behring, Marburg, Germany) in lung lavage fluid obtained at the end of the experiments. The lungs were washed via the tracheal cannula with normal saline. A volume of liquid, corresponding to 40 ml/kg body wt, was instilled and withdrawn twice, and this double lavage was repeated with fresh saline five times (total lavage volume 200 ml/kg; average recovery 93%, without differences among the groups). The volume of fluid recovered was recorded, and an aliquot was used for analysis of human albumin. The vascular-to-alveolar leakage of albumin was expressed as percentage of the injected amount of the marker.

In animals receiving surfactant, we also determined total phospholipids in the lavage fluid, using the method of Bartlett (3). In addition, surface properties of the crude lavage fluid were determined with pulsating bubble by using the method described above (7).

Pressure-volume properties were not recorded in these experiments, and since the lungs were lavaged, we made no efforts to evaluate alveolar expansion in histological sections.

Statistical Evaluation

Values are presented as means ± SD or as median and range when not normally distributed. Differences between groups were evaluated with ANOVA followed by the Newman-Keuls test. The χ² test was used for comparison of survival rates among groups and linear regression analysis for assessment of correlations between compliance, albumin leakage, and surface tension of lung lavage fluid. The limit level of statistical significance was defined as P < 0.05.

RESULTS

In Vitro Assessment of Surfactant Inactivation by Exposure to Lyso-PC

Surface properties of natural rabbit surfactant (10 mg/ml) mixed with various concentrations of lyso-PC are shown in Table 1. These data, based on measurements with pulsating bubble, show a significant elevation of minimum surface tension at concentrations of lyso-PC ≥ 2.5 mg/ml, indicating surfactant inactivation. There was a concomitant elevation of maximum surface tension at lyso-PC concentrations of 2.5 and 20 mg/ml. Corresponding data for Curosurf also shown in Table 1 demonstrate that this surfactant is more easily
inactivated, as reflected by elevated values for maximum and minimum surface tension already at a lyso-PC concentration of 1.25 mg/ml (P < 0.01 vs. natural rabbit surfactant).

Animal Experiments

Protocol 1. A survey of animals used for studies of lung compliance and lung morphology after exposure to various doses of lyso-PC is given in Table 2. There were no differences in body weight, survival rate, or final heart rate among the groups. PCO2 in heart blood was increased significantly in animals receiving the highest dose of lyso-PC (P < 0.05 vs. controls), and this was associated with a significant fall in pH (P < 0.01).

TIDAL VOLUME AND COMPLIANCE MEASUREMENTS. Mean tidal volume was kept between 10 and 11 ml/kg throughout the period of ventilation, without difference among the groups (data not shown). The maximum peak insufflation pressure used in this series of experiments was 37.5 cmH2O. Final values for lung-thorax compliance, recorded after 120 min in animals receiving different doses of lyso-PC without supplementary surfactant, are shown in Fig. 1. The fall in compliance after exposure to lyso-PC was clearly dose dependent, and statistically significant differences vs. the control animals were found for doses ≥10 mg/kg.

PRESSURE-VOLUME RECORDINGS. Static lung volumes at maximum insufflation pressure and at deflation pressure of 5 cmH2O in animals exposed to different doses of lyso-PC are shown in Fig. 2. At both pressure levels, lung volumes decreased with increasing doses of lyso-PC, and differences vs. the control group were statistically significant (P < 0.01) for lyso-PC doses ≥20 mg/kg.

HISTOLOGICAL OBSERVATIONS. Twenty-three of the twenty-four animals receiving lyso-PC at doses ≥5 mg/kg had histological evidence of lung injury, characterized by focal alveolar collapse (Fig. 3A), usually associated with necrosis and desquamation of airway epithelium (Fig. 4A), mild-to-moderate recruitment of granulocytes to the air spaces (Fig. 4B), and some interstitial and intra-alveolar hemorrhage (Fig. 4C). Alveolar hyaline membranes were observed in two animals receiving 10 and 20 mg/kg of lyso-PC and in four of the six animals receiving 40 mg/kg of lyso-PC (Fig. 4D). In one animal exposed to 20 mg/kg of lyso-PC, no histological abnormalities were found. One of the six animals receiving the lowest dose of lyso-PC, 2.5 mg/kg, had histological evidence of lung injury, similar to that in animals exposed to higher doses. In the remaining five animals in this group and in the five nonexposed control animals, the lungs were unremarkable (Fig. 3B). Animals receiving lyso-PC showed a dose-dependent decrease in alveolar volume density, statistically significant at doses ≥10 mg/kg (Table 3). As illustrated in Fig. 3A, animals exposed to larger doses of lyso-PC had an irregular alveolar expansion pattern, contrasting to the uniform expansion in controls.

Protocol 2. A survey of the four groups of animals used for this part of the study is given in Table 2.
were no differences in body weight, survival rate, and final heart rate among the groups. Values for P_{CO_2} were increased in animals exposed to lyso-PC without receiving surfactant or treated with surfactant after 30 min (P < 0.05 vs. controls).

**TIDAL VOLUME AND COMPLIANCE MEASUREMENTS.** Mean tidal volumes ranged between 10 and 11 ml/kg throughout the period of ventilation, without difference among the groups (data not shown). The maximum peak insufflation pressure used in this arm of our study was 33 cmH_2O. Values for lung-thorax compliance at different time intervals in the four groups of animals are shown in Fig. 5. As in animals studied according to protocol 1, exposure to lyso-PC led to a significant reduction in compliance. This difference was established already at the first time point (15 min). Administration of surfactant together with lyso-PC prevented this fall in compliance. The difference between animals receiving only lyso-PC and those receiving lyso-PC plus surfactant at birth was statistically significant at all intervals (P < 0.01–0.05), except at 45 and 90 min. Treatment with surfactant 30 min after the onset of ventilation had no effect on lung compliance, which remained, throughout the course of the experiment, at the same low level as in animals exposed to lyso-PC without receiving surfactant.

**VASCULAR-TO-ALVEOLAR ALBUMIN LEAKAGE.** In animals exposed to lyso-PC without receiving surfactant, the average leakage of human albumin into the air spaces was ~8%. Administration of surfactant at birth reduced this leakage to ~1%. Treatment with surfactant 30 min after onset of ventilation also reduced the albumin leakage, but only to a level of ~4%. Lung leakage of albumin in animals not receiving lyso-PC was negligible (Fig. 6).

**PHOSPHOLIPID CONTENT AND DYNAMIC SURFACE PROPERTIES OF LUNG LAVAGE FLUID.** The phospholipid content of lung lavage fluid was slightly lower in animals treated with surfactant after 30 min than in those receiving the
same dose at birth (1.32 ± 0.80 vs. 1.86 ± 0.83 mg/ml; not significant). Minimum surface tension after 5 min of cyclic film compression in the pulsating bubble system was about twice as high in animals receiving surfactant after 30 min as in those treated with surfactant at birth (22 ± 2.4 vs. 9.9 ± 6.6 mN/m; P < 0.001). This was associated with a significant increase also in maximum surface tension (47 ± 3.0 vs. 39 ± 3.5 mN/m; P < 0.01).

**CORRELATIONS AMONG COMPLIANCE, ALBUMIN LEAKAGE, AND DYNAMIC SURFACE PROPERTIES.** In the material as a whole, there was a strong inverse correlation between lung-thorax compliance and albumin leakage (r = −20.63; P < 0.001). In the two groups of animals receiving surfactant, compliance was inversely correlated with both minimum and maximum surface tension during cyclic compression (r = −0.56; P < 0.05, and r = −0.67; P < 0.01, respectively).

**DISCUSSION**

As outlined above, increased levels of lyso-PC in the air spaces can interfere with lung function by several pathophysiological mechanisms, including destabilization of the alveolar film of surfactant phospholipids, direct toxic action on the lung epithelium with increased lung permeability, and further inactivation of surfactant by plasma proteins leaking into the air.

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**Table 3.** Alveolar volume density in animals exposed to various doses of lyso-PC (protocol 1)

<table>
<thead>
<tr>
<th>Dose of Lyso-PC, mg/kg</th>
<th>n</th>
<th>Vv</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>0.73 ± 0.08</td>
</tr>
<tr>
<td>2.5</td>
<td>6</td>
<td>0.73 ± 0.06</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>0.64 ± 0.06</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>0.60 ± 0.07*</td>
</tr>
<tr>
<td>20</td>
<td>6</td>
<td>0.61 ± 0.08*</td>
</tr>
<tr>
<td>40</td>
<td>6</td>
<td>0.55 ± 0.07†</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = no. of rabbits. Vv, volume density.

*P < 0.05 vs. 0 mg/kg and P < 0.05 vs. 2.5 mg/kg lyso-PC; †P < 0.01 vs. 0 mg/kg and P < 0.01 vs. 2.5 mg/kg lyso-PC.
spaces from areas of epithelial injury (18). High levels of lyso-PC in the surfactant also increased the sensitivity to inactivation by fibrinogen and albumin (5).

Our present data from pulsating bubble measurements confirm that admixture of lyso-PC inactivates surfactant in a dose-dependent manner. In natural surfactant suspended at a concentration of 10 mg/ml, nearly complete inactivation with a mean minimum surface tension $\geq 15$ mN/m was observed with concentrations of lyso-PC $\geq 2.5$ mg/ml. We also found that the resistance to inactivation was higher for "complete" natural surfactant than for Curosurf. This could be attributed, at least to some extent, to a difference in their content of surfactant-specific proteins. In particular, SP-A increases resistance to inactivation (6), and this water-soluble protein is absent in Curosurf and all other surfactants prepared by extraction of lung tissue or lung lavage fluid with organic solvents.

Lyso-PC probably adsorbs to an air-liquid interface in competition with other surfactant lipids, fluidizing the surface film (11) and increasing the degree of surface compression required to reduce surface tension to very low values. This was reflected in our in vitro experiments by increased minimum surface tension during 50% surface compression in a pulsating bubble system. The concomitant increase in maximum surface tension indicates interference with adsorption and spreading kinetics, leading to ineffective reentry of surfactant molecules in the surface film during surface expansion. The changes in lung mechanics and morphology documented in near-term newborn rabbits after instillation of lyso-PC into the airways are also, at least in part, explained by a direct interference with surfactant function, especially as the fall in compliance was established already at the first (15-min) recording after administration of lyso-PC.

The effect of lyso-PC on lung-thorax compliance was clearly dose dependent and implied a decrease from mature level in control animals to a level corresponding to that of immature, surfactant-deficient fetuses delivered at a gestational age of 27 days (27) in animals receiving a dose of lyso-PC $\geq 10$ mg/kg. The dose of exogenous lyso-PC must be viewed in relation to the estimated pool size of endogenous alveolar surfactant phospholipids in a near-term newborn rabbit, $\sim 20$ mg/kg (26). Maximal effect was thus obtained when the dose of exogenous lyso-PC amounted to about one-third of the total pool of endogenous and exogenous surfactant in the alveolar spaces. Similar data were recently reported by Fornasier et al. (9), who evaluated potential toxic effects of lyso-PC in exogenous surfactant subjected to thermal decomposition using a rabbit
model analogous to that applied in the present experiments. In this experimental model, surfactant function may also become disturbed by exposure to membrane lipids from degenerating epithelial cells. Damage to airway epithelium could be a direct toxic effect of lyso-PC (2, 19, 20, 28) but could, in principle, be also caused by mechanical disruption secondary to iterated collapse and reexpansion of destabilized peripheral lung units (17). Our studies document a substantial vascular-to-alveolar leak of albumin in animals exposed to 10 mg/kg of lyso-PC. Most animals receiving lyso-PC at doses \( \geq 5 \) mg/kg had histological evidence of lung injury, including recruitment of inflammatory cells to the air spaces and, in some cases, also hyaline membranes, suggesting that lung permeability was disturbed by mechanisms involving necrosis of airway epithelium.

Upgrading the pool of alveolar surfactant by giving 200 mg/kg of Curosurf at birth counterbalanced the effect of lyso-PC on lung compliance and prevented to a large extent the leakage of albumin into the air spaces. This illustrates that noxious effects of lyso-PC not only depend on the dose but also on the amount of "normal" surfactant present in the air spaces. Interestingly, this protective effect of exogenous surfactant was only obtained when the treatment was given at birth; administration of the same dose of Curosurf to animals ventilated for 30 min after receiving lyso-PC did not improve compliance to any significant degree and caused only a moderate reduction in the vascular-to-alveolar leakage of albumin. A substantial part of this leak probably occurred during the first 30 min of the experiment, before surfactant was given, and the plasma proteins accumulating in the air spaces during these 30 min may have inactivated the subsequently administered exogenous surfactant. Such inactivation was, indeed, documented in lung lavage samples obtained after 2 h of ventilation. Both minimum and maximum surface tension, measured with a pulsating bubble, were significantly higher in animals receiving Curosurf after 30 min than in those treated at birth. We are aware of the fact that the concentration of surfactant phospholipids in the lavage fluid was slightly higher in animals treated at birth, maybe due to less permeation of surfactant components from the airways to the lung interstitium, but this difference was relatively small and can hardly explain the large difference in dynamic surface tension documented during cyclic film compression in the pulsating bubble system.

Although inactivation of surfactant probably occurs in the late-treated animals, variation in the distribution of the exogenous material may also have influenced the results. Studies on immature newborn lambs have revealed that exogenous surfactant is distributed more uniformly and has a more long-standing effect in animals treated prophylactically at birth than in those receiving surfactant after development of respiratory failure in the neonatal period (14, 15). However, possible differences in the distribution of surfactant were not analyzed in the present study. We conclude that lyso-PC at doses \( \geq 10 \) mg/kg has a significant impact on neonatal lung function by inactivating surfactant and increasing lung permeability, that these toxic effects of lyso-PC can be counterbalanced by exogenous surfactant, and that timing of surfactant administration may be essential for the therapeutic response.

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