Influence of phosphatidylglycerol on the uptake of liposomes by alveolar cells and on lung function

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The alveolus is lined with a thin layer of lipids and proteins, called surfactant, and this surface active agent has an essential role in maintaining normal lung function. Pulmonary surfactant is produced by alveolar type II cells. By weight, ~90% of surfactant consists of lipids. Although the lipid composition varies in different species, its major component is phosphatidylcholine (70–80%), of which nearly 50% is saturated dipalmitoylphosphatidylcholine, which is the major surface tension-reducing component of surfactant. In addition, surfactant contains variable amounts of phosphatidylethanolcerol (PG; 7–18%), phosphatidylcholine also affects the uptake of surfactant by alveolar cells (1, 14, 15, 33). However, in vitro studies have demonstrated that, apart from these SPs, the different lipid components also affect the uptake (25, 27). Significant differences in lipid composition between adults and neonates have been described (2, 11–13). In addition, the use of exogenous surfactant is now widely accepted as a treatment of surfactant dysfunction in neonates and is under consideration as a therapy in adults (20). Considering the natural differences in surfactant liquid composition between adults and neonates, as well as the fact that neonates rely more on recycling of surfactant than adults (16, 21), the role of the single-lipid components on the uptake of surfactant by alveolar cells has to be elucidated.

PG is the second major component after phosphatidylcholine, and in vitro studies have demonstrated a significant effect of PG on the uptake (25, 27). However, extrapolation of the results of in vitro experiments on the uptake of liposomes to the in vivo situation is not always valid; our group has already demonstrated significant differences when comparing in vivo and in vitro uptake of surfactant-like liposomes (26). Therefore, the present study investigated the effect of different concentrations of PG incorporated in the liposome on the uptake in ventilated rats by using flow cytometry. In addition, the possible effects of different concentrations on lung mechanics were also explored.

MATERIALS AND METHODS

Ethical guidelines. This study was approved by the Institutional Animal Committee at the Erasmus MC Rotterdam. The studies were performed in 72 male Sprague-Dawley rats (IFFA CREDO, The Netherlands) with a body weight of 310 ± 10 g.

Materials. Dipalmitoylphosphatidylcholine (DPPC), egg phosphatidylcholine, PG, phosphatidylinositol, phosphatidylethanolamine, and cholesterol were purchased from Sigma (Zwijndrecht, The Netherlands). Head-group rhodamine-labeled phosphatidylethanolamine was obtained from Molecular Probes (Leiden, The Netherlands).

Liposome preparation. To prepare liposomes, the different lipids (Sigma) were mixed at the weight ratio indicated in Table 1. The mixture of lipids was dried under a stream of nitrogen gas. The liposomes were suspended in either PBS (in mM: 137 NaCl, 2.7 KCl, 8.1 Na2HPO4, 1.5 KH2PO4, pH 7.4) or solution A (in mM: 140 NaCl, 5 KCl, 2.5 phosphate buffer, 10 HEPES, 6 glucose, 2.0 CaCl2, and 1.3 MgSO4) to a concentration of 1.0 mg lipids/ml by using glass pearls and vortexing. Immediately before use, the liposome suspension was sonicated for 2 min on ice using an ultrasonic disintegrator (Branson Sonifier 250) to prepare small unilamellar liposomes (26). The size of the liposomes was determined by dynamic light scattering at 25°C with a Malvern 4700 system using a 25-mW He-Ne laser (NEC, Tokyo, Japan) and the auto measure version 3.2 software (Malvern, Tokyo, Japan). The size of the liposomes was determined by dynamic light scattering at 25°C with a Malvern 4700 system using a 25-mW He-Ne laser (NEC, Tokyo, Japan) and the auto measure version 3.2 software (Malvern, Tokyo, Japan).
Table 1. Composition of the fluorescent-labeled liposomes

<table>
<thead>
<tr>
<th>Lipid Composition</th>
<th>0% PG</th>
<th>8% PG (Normal)</th>
<th>20% PG</th>
<th>40% PG</th>
<th>60% PG</th>
<th>86% PG</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>61</td>
<td>55</td>
<td>46</td>
<td>32</td>
<td>17.4</td>
<td>0</td>
</tr>
<tr>
<td>PC</td>
<td>23</td>
<td>21</td>
<td>18</td>
<td>12</td>
<td>6.6</td>
<td>0</td>
</tr>
<tr>
<td>PI</td>
<td>0</td>
<td>8</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>86</td>
</tr>
<tr>
<td>PE (labeled)</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Values are the weight ratio of the individual lipids used for the composition of the different liposomes. DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

Malvern, UK). As a measure of particle-size distribution of the dispersion, the system reports a polydispersity index. This index ranges from 0.0 for a monodisperse up to 1.0 for an entirely polydisperse dispersion. After ultrasonification the liposome size ranged from 140 to 165 nm and the polydispersity index ranged from 0.2 to 0.35. No variations were observed in the sizes of the liposomes with different lipid composition.

Intratracheal instillation of fluorescent liposomes. After induction of anesthesia with a mixture of nitrous oxide (66%), oxygen (33%), and isoflurane (1–2%), a sterile polyethylene catheter (0.8-mm outer diameter) was inserted into one of the carotid arteries. The animals were then tracheotomized, and a sterile metal cannula was inserted into the trachea.

After these surgical procedures, gaseous anesthesia was ended and replaced with an intraperitoneal injection of pentobarbital sodium (60 mg/ml, Nembutal, Algin, Maassluis, The Netherlands) at a dose of 30 mg/kg body wt every hour.

Muscle relaxation was induced and maintained by an hourly intramuscular injection of pancuronium bromide (2 mg/kg, Pavulon; Organon Technika, Boxtel, The Netherlands). The animals were then ventilated with a Servo ventilator 900 (Siemens-Elema, Solna, Sweden) set to pressure-control mode using a frequency of 30 breaths/min, an inspiratory-to-expiratory ratio of 1:2, a positive end-expiratory pressure of 2 cmH2O, a peak inspiratory pressure of 12 cmH2O, and inspired O2 fraction set to 1.

Before instillation of the labeled liposomes, positive end-expiratory pressure was increased to 6 cmH2O and peak inspiratory pressure was increased to 26 cmH2O. After disconnection from the ventilator, the liposomes were administered intratracheally as a bolus of 3 ml/kg followed by a bolus of air (12 ml/kg) directly into the endotracheal tube via a syringe, and the animals were immediately reconnected to the ventilator. Thirty minutes after instillation of the liposomes, positive end-expiratory pressure was reduced to 2 cmH2O and peak inspiratory pressure to 12 cmH2O.

Arterial blood gases were measured with conventional methods (ABL 555, Radiometer, Copenhagen, Denmark) at start of ventilation, immediately after instillation of the liposomes, and every 30 min thereafter. One hour after ventilation, the animals were killed by an overdose of pentobarbital sodium (60 mg/kg). Next, thorax and diaphragm were opened to (eliminate the influence of chest wall compliance and intra-abdominal pressure), and a static pressure-volume (P-V) curve from the lungs was recorded using conventional techniques (19).

Maximal compliance was defined as the steepest part of the P-V deflation curve and was determined for each animal. The Gruenwald index, which characterizes the surfactant system in situ (10), was calculated from the P-V curve, defined as \( \frac{2V_5 + V_{10}}{V_{max}} \), where \( V_5 \), \( V_{10} \), and \( V_{max} \) are the lung volumes at transpulmonary pressures of 5, 10, and 35 cmH2O, respectively, from the deflation limb. Thereafter, bronchoalveolar lavage (BAL) was performed with saline (30 ml/kg heated to 37°C) five times, and its percentage recovery was calculated. Cell debris was removed from the BAL by centrifugation at 400 g for 10 min. The protein concentration of the BAL fluid was determined using the Bradford method (Bio-Rad protein assay, Munich, Germany) with bovine serum albumin (Sigma, St. Louis, MO) as a standard (4).

Histological sampling. To study the presence of histological changes in lung morphology, 1 h after instillation of the liposomes, blood was removed from the lungs by perfusion of the pulmonary arteries with warm saline (37°C) supplemented with 20 IE heparin/ml. Next, the lungs were fixed as previously described (17). Briefly, after perfusion, the lungs were fixed with a solution consisting of 3.6% formaldehyde and 0.25% glutaraldehyde. Before fixation, the airway pressure was momentarily increased to 10 cmH2O and thereafter maintained at 10 cmH2O. Blocks of tissue were taken from the center of the upper and middle lobe and from the ventral and dorsal part of the lower lobe. The specimens were embedded in paraffin, and sectioned and stained with hematoxylin and eosin, and lung morphology was analyzed using light microscopy.

Statistical analysis. Differences in blood-gas values over time and differences between the groups that received liposomes with a different composition were analyzed using a repeated-measurement ANOVA followed by a Bonferroni post hoc test. Differences in mean fluorescence per cell and percentage of cells involved in the uptake (gated cells) were analyzed using ANOVA followed by a Bonferroni post hoc test, as were differences in maximal compliance. Gruenwald index, maximal volume at 35 cmH2O, protein concentration in the BAL, and number of macrophages retrieved by BAL. Differences were considered significant at \( P < 0.05 \). Values are expressed as means ± SD.
RESULTS

Influence of the PG content of fluorescent-labeled liposomes on the uptake. As reported previously (25), incorporation of a higher concentration of PG in the labeled liposomes increases the uptake of these liposomes by alveolar macrophages and alveolar type II cells in vitro. To determine whether these effects of PG on the uptake are also present in vivo, ventilated rats were intratracheally instilled with fluorescent-labeled liposomes that contained various concentrations of PG. One hour after instillation of these liposomes, the animals were killed and alveolar cells were isolated. The uptake of liposomes by alveolar type II cells decreased significantly when the amount of PG was increased to 20% compared with the uptake of normal liposomes, which contained only 8% PG. However, a further increase of the amount of PG did not have a significant effect on the uptake of these liposomes by alveolar type II cells compared with the normal liposomes; however, the uptake tends to increase compared with the uptake of 20% PG liposomes (Fig. 1A). When the liposomes are resuspended in solution A containing cofactors known to affect the uptake (e.g., calcium and magnesium), no significant differences are observed compared with the same liposomes resuspended in PBS. The number of alveolar type II cells involved in the uptake is unaffected by the amount of PG within the liposome compared with the normal liposomes (Fig. 1B).

As surfactant-like liposomes are taken up by both alveolar type II cells and alveolar macrophages, the effects of the concentration of PG on the uptake was also studied in alveolar macrophages. An increase in the amount of PG within the liposomes results in an increased uptake, with an optimum at 40% PG, where the increase is significantly higher compared with normal liposomes containing 8% PG. When the PG concentration is raised to 60%, no significant differences compared with normal liposomes are observed; however, a further increase of the amount of PG within the liposomes (86%) results in an absence of alveolar macrophages in the BAL and thus no cell-associated fluorescence can be determined using 86% PG liposomes (Fig. 1C). Increasing the amount of PG tends to increase the amount of macrophages taking part in the uptake with an apparent optimum at 20%, although the increase of alveolar macrophages is not significantly different compared with normal liposomes. Higher PG concentrations result in a decrease in the number of cells involved in the uptake, accumulating at a PG concentration of 86% in the total absence of alveolar macrophages in the lung lavage.

However, by suspending the liposomes in solution A instead of PBS, as was done in the previous experiment, an increase in the amount of PG within the liposomes results in an enhanced uptake for all concentrations with a possible optimum at 20% PG compared with normal liposomes (8% PG). Also, the number of alveolar macrophages involved in the uptake depends on the PG concentration. An apparent optimum is observed at 20%; increasing the concentration of PG results in a gradual decrease in the number of cells that internalize liposomes (Fig. 1D).

Alveolar macrophages and protein concentration in BAL. Because the presence of large amounts of PG (i.e., 86%) in the liposomes leads to an absence of alveolar macrophages in the BAL, we wanted to know at which concentration of PG this effect occurs. When the amount of PG is increased to as much as 20%, the number of alveolar macrophages decreases significantly; this decrease is also observed for liposomes with 40 and 60% PG incorporated.

However, when the liposomes are suspended in solution A, normal amounts of alveolar macrophages are found in BAL of animals instilled with the different liposomes (Fig. 2A). In addition, the influx of protein in the lungs increases when the PG concentration in the liposomes is increased for liposomes suspended in PBS, whereas no effect on the protein content of
the BAL is observed when liposomes are suspended in solution A (Fig. 2B).

Histological changes with 86% PG liposomes. As mentioned above, no alveolar macrophages could be retrieved by BAL when the liposomes were suspended in PBS. To study whether the PG concentration in the liposomes also influences lung histology, histological samples of the lungs of animals instilled with either normal or 86% PG liposomes were obtained and analyzed using light microscopy. The samples were acquired from animals with intratracheally instilled normal (8% PG) liposomes and from animals that had received 86% PG liposomes. The histological samples from the latter animals showed patchy interstitial infiltrate causing severe wall thickening, indicating early diffuse alveolar damage (Fig. 3).

Lung mechanics after instillation of fluorescent-labeled liposomes with different concentrations of PG. During the experiment, blood-gas values were determined to study the effects of the liposomes on arterial oxygenation. Instillation of 86% PG liposomes results in a significant decrease over time, and after 1 h the arterial blood-gas values approximate international criteria for acute respiratory failure (3).

This dramatic effect of PG liposomes is concentration-dependent, as increasing the amount of PG within the liposomes leads to a concomitant decrease in arterial oxygenation (Fig. 4). However, when the liposomes are resuspended in solution A, no significant deterioration in alveolar oxygenation is observed.

To determine whether this dramatic decrease in gas exchange is caused by a diminished surfactant function, static P-V curves were recorded at the end of the experiment. The Gruenwald index, as a measure of lung stability (10), demonstrates significantly lower values for all liposomes with a PG content higher than 8% suspended in PBS (Fig. 5A). When the liposomes are resuspended in solution A, no significant decrease is present, except for the liposomes containing 60% PG (Fig. 5A).

These results are corroborated by the changes in maximal compliance; instillation of liposomes with a PG concentration higher than the physiological concentration of 8% and suspended in PBS results in a significant decrease of the maximal compliance (Fig. 5B). However, when the liposomes are resuspended in solution A, only for 60 and 86% PG liposomes are significant changes observed. The maximal lung volume at 35 cmH2O is not affected by the amount of PG within the liposomes nor by the solution in which the liposomes are resuspended (Fig. 5C).

DISCUSSION

Pulmonary surfactant is essential for normal physiological lung function. To maintain a low variable surface tension, surfactant forms a lipid monolayer, lining the alveolo-capillary membrane. As a result of normal breathing, the radius of the alveolus increases and decreases, causing lipids to be squeezed...
out of the lipid monolayer during expiration. In addition, the surfactant in the alveolus is turned over from active to inactive surfactant. In other words, the amount of surface-active surfactant within the alveolus would decrease if the alveolar type II would not compensate the loss by secreting pulmonary surfactant. Because de novo synthesis of surfactant has been suggested to be insufficient to compensate for the constant "loss" of active surfactant, alveolar type II cells rely on recycling inactivated surfactant (16, 21). To ensure recycling, the inactivated surfactant is taken up from the alveolar space by alveolar type II cells, and inactive surfactant is also cleared by alveolar macrophages (16).

This process of uptake or reuptake of surfactant lipids is thought to be essential in the surfactant metabolism. Both alveolar type II cells as well as alveolar macrophages have been demonstrated to take up surfactant, although their relative contribution to the clearance remains under discussion because the interpretation of the results of previous studies is somewhat contradictory (22, 28, 29). However, most of the studies have focused on the uptake of surfactant's main component, DPPC, or on the effects of the SPs on the uptake of DPPC. Both SP-A and SP-C have been shown to enhance the uptake of liposomes by alveolar cells (1, 14, 15, 31, 33, 38). Other than SPs and DPPC, the other individual surfactant lipids can also influence the uptake; Quintero and Wright (27) and Poelma et al. (25) demonstrated the enhanced uptake of PG-containing liposomes by isolated alveolar macrophages and alveolar type II cells. However, these studies were performed in vitro, and significant differences between in vivo and in vitro experiments have been reported (26).

Because surfactant therapy is nowadays more common in clinical practice, and studies on surfactant therapy mainly focus on the addition of SPs (35–37), very little information is available on the influence of the individual surfactant lipids on uptake or lung function.

Therefore, the present study investigated the effects of the second major component of surfactant, PG, on the uptake in ventilated rats, as well as the effects on endogenous surfactant function by monitoring arterial oxygenation and determining lung mechanics (protein in lavages fluid, Gruenwald index). The results of the present study show that incorporation of PG does influence the uptake of surfactant-like liposomes by alveolar cells, although the effects on both cell types differ. Only at a 20% PG concentration is a significant decrease in the uptake of liposomes by alveolar type II cells seen, whereas at the other PG concentrations no effects were observed. For alveolar macrophages, the influence of intratracheal instillation of PG-containing liposomes is more dramatic, in particular, on the number of alveolar macrophages obtained from the BAL after instillation of PG-containing liposomes. Even the incorporation of 20% PG, which is approximately twice that of the normal PG concentration (8%), within the liposomes resulted in a decreased number of macrophages collected from the BAL. Histological samples of the lungs of animals that were instilled with liposomes containing 86% PG revealed complete absence of alveolar macrophages in the lung. The cells might
The relation between the decrease/absence of alveolar macrophages in the BAL fluid and the deterioration in surfactant function (i.e., a worsening arterial oxygenation, increased amounts of proteins in the BAL, and a decreased Gruenwald index) might be explained by at least two options. First, the presence of a variety of cytokines, inflammatory mediators and proteolytic enzymes (23) produced by stimulated alveolar macrophages, some of which have been suggested to be related to respiratory failure (5, 7, 9, 24, 32), might relate the absence of alveolar macrophages to the deteriorated surfactant function. Next, the increased PG content of the liposomes leads to overstimulation of the alveolar macrophage and consequently to bursting of the macrophage, releasing its contents into the alveolar space. However, after this expulsion of the contents of macrophages has taken place, these substances are removed very rapidly and can hardly be measured. However, damage to the surfactant monolayer has already occurred, and a vicious circle is initiated. Protein concentration will rise in the alveolar space and result in a dose-dependent inactivation/inhibition of surfactant (18): surfactant is rate-limiting for the transfer of protein over the alveolar capillary membrane, loss of surfactant function will result in more protein influx, which, in turn, will inactivate/inhibit even more surfactant (17, 18, 30). Further evidence that the surfactant system is damaged and that no additional fibrosis is present comes from the maximal volume data, which are similar in both groups (Fig. 5C). This demonstrates that, when sufficiently high pressures are applied, collapsed alveoli can still be recruited.

Despite these severe effects of PG on the surfactant metabolism, more interestingly the adverse consequences of increasing amounts of PG can be avoided. When the liposomes are suspended in solution A rather than in PBS, the amount of alveolar macrophages is not decreased in contrast to the similar liposomes resuspended in PBS, except for liposomes containing 60% PG. In addition, when the amount of PG is increased and the liposomes are resuspended in solution A, a significant increase in uptake by alveolar macrophages is observed, showing a maximum uptake at a PG concentration of 20%. This increased uptake of liposomes with a high content of PG incorporated and suspended in solution A is similar to earlier results obtained by studying the influence of PG on the uptake of liposomes suspended in solution A by alveolar macrophages in vitro (25). In the present study, resuspending the liposomes in solution A has almost no influence on the uptake of these liposomes by alveolar type II cells, whereas in vitro the uptake of liposomes by alveolar type II cells is stimulated by an increase in the amount of PG incorporated in these liposomes (25). Although both of these studies were performed using the same technique, the effects of a high PG concentration on the uptake by alveolar type II cells is different. It can thus be concluded that, other than the presence of the substances in solution A, in both the present study and the previous one (25), additional environmental factors affect the uptake of surfactant-like liposomes by alveolar type II cells in vivo. In a previous study (25), our laboratory demonstrated a common pathway for the uptake of phospholipids that has a higher affinity for negatively charged phospholipids. For the sake of argument, we hypothesized the presence of a phospholipid receptor that is not equally distributed over alveolar macrophages and alveolar type II cells.
In the present study, the protection obtained by suspending the liposomes in solution A may be explained in several ways. Obviously, the presence of divalent cations (Mg, Ca) in solution A might “neutralize” the negative charge of PG. Another explanation could be that the presence of components necessary for alveolar macrophages and normal lung function in solution A (e.g., Ca, Mg, and glucose) replenishes the dilution of these factors in vivo caused by the intratracheal instillation of 1 ml of liposome suspension. Because the volume of the liquid phase is estimated to be ~200 μl in the rat, instillation of 1 ml will lead to a sixfold dilution of the intra-alveolar solutes.

In summary, the present study shows that varying the PG concentration in intratracheally instilled liposomes may have serious consequences for alveolar macrophages as well as for lung functioning. A practical implication may be that not only the presence of the hydrophobic SPs are important for surfactant therapy but also that the PG concentration in the applied surfactant may play an important role in surfactant therapy. Therefore, further studies are needed to elucidate the complex mechanism(s) that underlies these results not only to improve our knowledge of surfactant homeostasis but also to achieve improved therapeutic interventions.

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REFERENCES


