Metabolism of phosphatidylglycerol by alveolar macrophages in vitro

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Quintero, Omar A., and Jo Rae Wright. Metabolism of phosphatidylglycerol by alveolar macrophages in vitro. Am J Physiol Lung Cell Mol Physiol 279: L399–L407, 2000.—In whole animal studies, it has been shown that turnover of surfactant dipalmitoylphosphatidylglycerol (DPPG) is faster than that of dipalmitoylphosphatidylcholine (DPPC). The goal of this investigation was to characterize the metabolism of DPPG by alveolar macrophages and to determine whether they contribute to the faster alveolar clearance of DPPG. Isolated rat alveolar macrophages were incubated with liposomes colabeled with \(^{3}H\)DPPG and \(^{14}C\)DPPC. Macrophages internalized both lipids in a time- and temperature-dependent manner. The uptake of both lipids was increased by surfactant protein (SP) A and by adherence of the macrophages to plastic slides. The isotope ratio of DPPC to DPPG internalized by macrophages in suspension in the absence of SP-A was significantly lower than the isotope ratio in liposomes, suggesting that macrophages preferentially internalize DPPG when SP-A is absent. Phospholipase activity in macrophage homogenate was higher toward \(sn\)-2-labeled DPPG than toward \(sn\)-2-labeled DPPC. These studies show that alveolar macrophages play an important role in catabolizing surfactant lipids and may be partially responsible for the relatively faster clearance of DPPG from the lung.

PULMONARY SURFACTANT is a mixture of lipids and proteins produced by the type II pneumocyte (20). The role of pulmonary surfactant is primarily to reduce surface tension at the air-liquid interface, although certain surfactant components have also been shown to play a role in host defense. SP-B and SP-C are small hydrophobic proteins that assist in the adsorption of the phospholipids to the air-liquid interface (24).

Surfactant lipid and protein are packaged into large secretory vesicles, or lamellar bodies, that are secreted by the type II cells at a rate of \(\sim 93 \, \mu g \, DSPC/g \, lung^{-1} \cdot h^{-1}\) (37). After secretion, the surfactant is thought to pass through an intermediate state known as tubular myelin before adsorption to the monolayer at the air-liquid interface. The mechanism of transformation from lamellar body to tubular myelin to monolayer is poorly understood. In addition, tubular myelin is not a prerequisite for surface film formation or normal lung function. SP-A-deficient mice lack tubular myelin because SP-A along with SP-B, PG, and phosphatidylcholine (PC) is an essential component of its structure. These mice have only minimal deficiencies in surface tension-reducing functions of surfactant (22).

Surfactant is continuously secreted and degraded. Clearance can occur one of four ways: surfactant can be internalized by the type II cell and degraded, internalized by the type II cell and recycled, internalized by the alveolar macrophage and degraded, or cleared by the mucociliary pathway (20).

Experimental evidence from in vitro experiments supports a role for SP-A in surfactant homeostasis. SP-A binds dipalmitoylphosphatidylcholine (DPPC) but not DPPG (23). SP-A has been shown to stimulate the association or internalization of surfactant-like liposomes by both type II cells and alveolar macrophages in vitro (34, 35). It has also been shown that SP-A inhibits phospholipase \(A_{2}\) (PLA\(_{2}\)) activity in isolated lamellar bodies and lung homogenate (8). The importance of this role in vivo remains unclear because SP-A-deficient mice have only minimal alterations in their pool size and surfactant turnover rates (22). It remains to be determined whether other compensatory mechanisms prevail in the absence of SP-A or whether SP-A is truly unimportant in regulating surfactant turnover.

Most of the uptake and degradation studies to date have focused on PC, although some studies have examined the effects of modifying the pools of surfactant lipids.
lipid precursors on the composition of pulmonary surfactant. For example, a decrease in serum cholesterol resulted in a decrease in surfactant disaturated phospholipids (5), whereas an increase in dietary inositol resulted in decreased surfactant PG and increased surfactant phosphatidinositol (7). Jacobs et al. (16) showed that in 3-day-old rabbits both DPPG and dipalmitoylphosphatidylethanolamine are cleared more rapidly from the lung than DPPC. In these studies, radiolabeled lipids were instilled intratracheally, and their recoveries were analyzed with time (16). A mechanism for the difference in turnover rates of DPPC and DPPG has not been established. We hypothesized that alveolar macrophages may be partially responsible for this difference in turnover because it has been shown that alveolar macrophages have the ability to internalize and degrade surfactant-like liposomes in vitro (35). The goal of these studies was to investigate the role of the alveolar macrophage in the metabolism of PG.

METHODS

Materials. DPPC, DPPG, egg PC, cholesterol, and 2-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphoglycerol (NBD-PG) were purchased from Avanti Polar Lipids (Birmingham, AL). The fluorescent lipid 2-(4,4'-difluoro-5-(4-phenyl-1,3-buta dienyl)-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (BODIPY-PC) was purchased from Molecular Probes (Eugene, OR). L-a-dipalmitoyl-[2-palmitoyl-9, 10-3H(N)]-phosphatidylcholine (89 Ci/ mmol) and L-a-dipalmitoyl-[di palmitoyl-1,1-14C]-phosphatidylcholine (110 mCi/mmol) were obtained from DuPont New England Nuclear (Boston, MA). RPMI 1640 medium and phosphate-buffered saline (PBS) were purchased from GIBCO BRL (Life Technologies). Chloroform (CHCl3) and methanol (MeOH) were from Mallinckrodt. Bovine serum albumin (BSA), EDTA, EGTA, ammonium hydroxide (NH4OH), and phospholipase D type I from cabbages were purchased from Sigma (St. Louis, MO). Bicinchoninic acid (BCA) protein assay reagent was purchased from Pierce (Rockford, IL).

Animals. Male Sprague-Dawley rats (200–500 g) were purchased from Charles River Laboratories (Raleigh, NC).

Synthesis of [3H]DPPG from [3H]DPPC. [3H]DPPG was synthesized from [3H]DPPC by the head group transfer reaction with phospholipase D type I from cabbages as previously described (17). Briefly, [3H]DPPC was incubated over night in CHCl3 as the organic phase and 40 mM CaCl2·0.1 M acetic acid buffer, pH 5.6, as the aqueous phase with an excess of glycerol and 400 U of phospholipase D. The reaction was incubated overnight with stirring so that the two phases mixed. After incubation, the organic phase was removed, and 100 μg of unlabeled DPPC and 100 μg of DPPG were added as a carrier. DPPG was separated from DPPC by two-dimensional thin-layer chromatography (TLC) (28). The first solvent phase consisted of CHCl3·CH3OH·H2O·NH4·H2O (70:30:3.2 vol/vol/vol), and the second solvent phase consisted of CHCl3·CH3OH·H2O (65:35:5 vol/vol). DPPG containing silica was scraped from the TLC plate with a razor blade, and the lipids were extracted with the method of Bligh and Dyer (4). The TLC was repeated to ensure purity of the DPPG.

Purification of alveolar proteinosis SP-A. SP-A was isolated from the lavage fluid of patients with alveolar proteinosis by butanol extraction of the sedimented lipids as previously described (34), with the modification of treatment with polymyxin to lower endotoxin levels (25).

Preparation of liposomes. Small unilamellar liposomes were prepared with 52% DPPC, 26% egg PC, 15% DPPG, and 7% cholesterol by weight (34). Liposomes were extruded from a French pressure cell as previously described (14) at a concentration of 1.1 mg lipid/ml. For uptake and degradation studies, 4.4 mg of liposomes were labeled with trace amounts of [3H]DPPG (8 μCi) or a combination of [3H]DPPG (8 μCi) and [14C]DPPC (2 μCi). For confocal studies, liposomes were labeled by replacing DPPG with NBD-PG and trace labeling with BODIPY-PC (2.93 mol/100 ml of DPPC).

Isolation of alveolar macrophages. Alveolar macrophages were isolated from the lung lavage fluid of male Sprague-Dawley rats weighing 200–500 g (32). Briefly, rats anesthetized with pentobarbital sodium were killed by exsanguination; the lungs were removed and lavaged with PBS containing 0.2 mM EGTA. Macrophages were removed from the lavage fluid by centrifugation at 228 g for 10 min. Incubation conditions. For lipid uptake assays with cells in suspension, isolated cells were resuspended in PBS with 2 mM Ca2+ and 0.1% BSA at a concentration of 2 × 106 cells/ml. Liposomes were added to a concentration of 20 μg lipid/ml, and in some cases, SP-A was added. Cells were incubated at 37 or 4°C with gentle agitation. After incubation, the cells were washed three times by centrifugation at 228 g for 10 min in a Beckman GS-6R centrifuge. After the first two centrifugations, the cells were resuspended in PBS with 2 mM EDTA and transferred to a clean microfuge tube to minimize nonspecific adsorption of radioactive activity. After the third centrifugation, the cells were resuspended in 400 μl of 150 mM NaCl, 50 mM sodium phosphate buffer, 2 mM EDTA, and 0.5% Nonidet P-40 and vortexed vigorously. A 300-μl aliquot was taken for scintillation counting, and protein concentration was analyzed with the BCA protein assay reagent. For degradation studies, the cells were resuspended in 800 μl of ethanol and dried under nitrogen. The lipids were extracted with the method of Bligh and Dyer (4), followed by two-dimensional TLC.

For lipid uptake assays with adherent cells, isolated macrophages were resuspended in RPMI 1640 medium at a concentration of 5.0 × 106 cells/ml. Cells were plated on two-chamber Lab-Tek slides at a concentration of 1 × 106 cells/chamber and allowed to adhere for 90 min. The cells were washed twice with PBS with 2 mM Ca2+ and 0.1% BSA and covered with 2 ml of the same. Liposomes were added to a concentration of 20 μg lipid/ml, and in some cases, SP-A was added. Cells were then incubated at 37 or 4°C. After incubation, the medium was removed, and the cells were washed twice with PBS with 2 mM Ca2+ and 0.1% BSA, followed by two washes with PBS with 2 mM Ca2+. For assays with radiolabeled lipids, cells were collected from the slides with 400 μl of 150 mM NaCl, 50 mM NaPO4, 2 mM EDTA, and 0.5% Nonidet P-40 by scraping the slides with a disposable transfer pipette. For analysis of [3H] and [14C], a 300-μl aliquot was added to a scintillation vial containing 4 ml of ICN Cytoscan scintillation cocktail, and the sample was analyzed in a Beckman LS 500 TD scintillation counter. Protein was analyzed with the BCA protein assay reagent.

For degradation assays, cells were plated on Lab-Tek one-chamber slides at a concentration of 2 × 106 cells/chamber. After incubation with liposomes, the cells were collected by scraping into 800 μl of ethanol. After being dried under nitrogen, the lipids were then extracted with the method of Bligh and Dyer (4), followed by two-dimensional TLC.

Two-dimensional TLC. Lipids from cells incubated with liposomes were extracted with the method of Bligh and Dyer.
(4). Nonradioactive lipid extracted from a homogenate of rat lung was added as a carrier, and the lipids were separated by two-dimensional TLC as previously described (28). The first solvent phase consisted of CHCl₃-CH₃OH-H₂O-NH₄OH (70: 30:3:2 vol/vol), and the second solvent phase consisted of CHCl₃-CH₃OH-H₂O (65:35:5 vol/vol). Iodine vapor was used to visualize the lipids. Individual lipid species were scraped with a razor blade into scintillation vials, and radioactivity was determined by scintillation counting. Lipids were identified by comparison to known lipid standards separated under the same conditions.

**Phospholipase assay.** Phospholipase activity in alveolar macrophage homogenate was measured with the method of Fisher et al. (9) with modifications. Macrophage homogenate was prepared by resuspending lavaged cells in 40 mM sodium acetate (pH 4.0, 4°C) containing either 10 mM CaCl₂ or 5 mM EDTA at a concentration of 2.5 × 10⁵ cells/ml. The suspension was sonicated with a Virsonic 60 ultrasonic cell disrupter set at 40% power in six intervals lasting 5 s and homogenized with five passes of a motor-driven Potter-Elvehjem homogenizer. Briefly, 1 ml of alveolar macrophage homogenate was incubated with 1 μM liposomes labeled with [³H]DPPC and [¹⁴C]DPPG at 37°C for 60 min; in some instances, SP-A was added at 10 or 100 μg/ml. The reaction was started by the addition of substrate and stopped by the addition of 1 ml of CHCl₃-MeOH (2:1). The samples were vortexed and centrifuged at 228 g. The organic phase was dried under N₂, resuspended in 40 μl of CHCl₃-MeOH (2:1), and spotted on a TLC plate. The samples were separated by one-dimensional TLC with hexane-diethyl ether-acetic acid (6:4:1) for the first half of the plate, followed by the same solvents at 90:10:1 for the second half (10). Lipids were identified by comparison to known standards and scraped from the plate for scintillation counting with a Beckman LS 500 TD scintillation counter for single-labeled samples. Phospholipase activity was calculated from the specific activity of the liposome preparation. Protein concentrations in the homogenate (200 μg/ml) were determined by BCA assay before incubation with liposomes.

**Confocal microscopy.** Alveolar macrophages were plated on Lab-Tek two-chamber slides (Nunc) at a density of 1 × 10⁶ in 2 ml of RPMI 1640 medium (GIBCO BRL) for 90 min in an atmosphere of 5% CO₂. The cells were then washed twice with PBS with 2 mM Ca²⁺ and 0.1% BSA. The cells were then incubated with 20 μg/ml of unilamellar liposomes labeled with NBD-PG and BODIPY-PC with and without 5 μg SP-A/ml. After 180 min of incubation, the cells were washed twice with PBS with 2 mM Ca²⁺ and 0.1% BSA and twice with PBS with 2 mM Ca²⁺. The cells were then fixed for 10 min with freshly prepared 4% formaldehyde in PBS. The cells were washed with PBS and mounted with 2.5% (wt/vol) 1,4-diazabicyclo[2.2.2]octane in 90% glycerol in PBS. The distribution of NBD-PG and BODIPY-PC was examined with a Zeiss 410 confocal microscope with a ×63 objective.

**Data analyses.** Uptake of lipid in picomoles per microgram of cell protein was calculated from the specific activity of DPPC and DPPG in the liposomes and data from the BCA protein assays. Data were compared by Student’s t-test for unpaired samples or analysis of variance and Tukey’s test when appropriate; n is the number of independent experiments. Error bars not visible are occluded by the data point marker.

**RESULTS**

**SP-A enhances uptake of DPPC and DPPG by alveolar macrophages.** Alveolar macrophages in suspension bound and internalized both DPPC and DPPG at 37°C. The uptake of both lipids was enhanced by SP-A (Fig. 1). The presence of SP-A increased the association of lipid with macrophages in suspension by ~670% for DPPC and 500% for DPPG. The effects of SP-A on the metabolism of DPPC and DPPG were also analyzed with adherent alveolar macrophages. SP-A stimulated uptake of both DPPC and DPPG by adherent alveolar macrophages incubated at 37°C for 3 h in a dose-dependent manner (Fig. 2) at concentrations of SP-A > 5 μg/ml. At 15 μg/ml of SP-A, 16.0 ± 1.1 pmol DPPC/μg cell protein and 4.5 ± 0.3 pmol DPPG/μg cell protein associated with macrophages. This represented an increase in association of DPPC and DPPG of ~400% over the no-SP-A control. The stimulation of association observed at 37°C was 350% greater than that observed at 4°C.

SP-A stimulated uptake of both DPPC and DPPG by adherent macrophages in a time-dependent manner (Fig. 3). At 5 μg SP-A/ml and 37°C, the time course of association of both DPPC and DPPG was approximately linear. In the absence of SP-A, the association of lipid with cells was linear as well. After 3 h of incubation at 37°C with 5 μg SP-A/ml, the association of DPPC and DPPG had increased by ~570 and 550%, respectively. In the absence of SP-A, the increase was ~460% for DPPC and 450% for DPPG.

To investigate the differential metabolism of DPPC and DPPG by alveolar macrophages both in suspension and adhered to a plastic surface, the ratio of the two
lipids at 180 min of incubation in the presence and absence of SP-A was measured (Fig. 4). By comparing the ratio of DPPC to DPPG internalized by the cell with the ratio of DPPC to DPPG in the liposomes (3.41), it is possible to determine whether one lipid species is being internalized preferentially over the other. Interestingly, in the absence of SP-A, the ratio of the two lipids associated with the macrophages in suspension after 180 min of incubation was significantly less than the ratio of the lipids in the liposomes added to the cells at the beginning of the experiment, signifying greater DPPG uptake with respect to DPPC. In contrast, in the presence of 5 μg SP-A/ml, the ratio of DPPC to DPPG associated with the macrophages (∼3.8) was significantly greater than the ratio of DPPC to DPPG in the liposomes, signifying greater DPPC uptake with respect to DPPG. For macrophages adhered to a surface, the ratio of cell-associated radioactivity was greater than in the starting liposomes in both the presence and absence of SP-A, signifying greater uptake of DPPC compared with DPPG under these conditions (Fig. 4). These data suggest that macrophages internalize DPPC and DPPG differently and that SP-A and adherence to a surface affect this process.

**Degradation of DPPC and DPPG by alveolar macrophages.** Two-dimensional TLC was used to analyze the degradation of DPPC and DPPG by alveolar macrophages and to determine whether SP-A affected the amount of lipid degraded (Table 1). In the presence of 5 μg SP-A/ml, 85 ± 8% of the 14C counts migrated with DPPG in the liposomes, signifying greater DPPC uptake with respect to DPPG. For macrophages adhered to a surface, the ratio of cell-associated radioactivity was greater than in the starting liposomes in both the presence and absence of SP-A, signifying greater uptake of DPPC compared with DPPG under these conditions (Fig. 4). These data suggest that macrophages internalize DPPC and DPPG differently and that SP-A and adherence to a surface affect this process.
Degradation of lipid by adherent alveolar macrophages

Table 1. Degradation of lipid by adherent alveolar macrophages in suspension

<table>
<thead>
<tr>
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<th>Fraction of Total Radioactivity, %</th>
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<tr>
<td></td>
<td>DPPC</td>
</tr>
<tr>
<td></td>
<td>+SP-A</td>
</tr>
<tr>
<td>PC</td>
<td>85.1 ± 7.7</td>
</tr>
<tr>
<td>PG</td>
<td>0</td>
</tr>
<tr>
<td>Solvent front</td>
<td>10.3 ± 4.8</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>Other</td>
<td>4.3 ± 3.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3 independent experiments. Alveolar macrophages were suspended in PBS with 2 mM CaCl₂ and 0.1% BSA at a concentration of 2 × 10⁶ cells/ml. The cells were then incubated in the presence (+) and absence (−) of 5 μg surfactant protein A (SP-A)/ml and 20 μg phospholipid/ml labeled with trace amounts of [14C]dipalmitoylphosphatidylcholine (DPPC) and [3H]dipalmitoylphosphatidylglycerol (DPPG) at 37°C for 180 minutes. The cells were washed by centrifugation, and lipids were extracted and separated by 2-dimensional TLC. Individual lipid species were isolated and analyzed for radioactivity. PC, phosphatidylcholine; PG, phosphatidylglycerol.

Table 2. Degradation of lipid by adherent alveolar macrophages

<table>
<thead>
<tr>
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<th>Fraction of Total Radioactivity, %</th>
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<tr>
<td></td>
<td>DPPC</td>
</tr>
<tr>
<td></td>
<td>+SP-A</td>
</tr>
<tr>
<td>PC</td>
<td>94.4 ± 1.9</td>
</tr>
<tr>
<td>PG</td>
<td>0</td>
</tr>
<tr>
<td>Solvent front</td>
<td>3.1 ± 1.4</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>2.5 ± 1.3</td>
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Values are means ± SE; n = 3 independent experiments. Alveolar macrophages were adhered to 1-chamber slides at a concentration of 2 × 10⁶/chamber for 90 minutes. The cells were then incubated in the presence and absence of 5 μg SP-A/ml and 20 μg phospholipid/ml labeled with trace amounts of [14C]DPPC or [3H]DPPG at 37°C for 180 min. The cells were washed, and lipids were extracted and separated by 2-dimensional TLC. Individual lipid species were isolated and analyzed for radioactivity.

3H counts migrated with PC and PG, respectively. In the presence of 5 μg SP-A/ml, 95 ± 1.2% of the 14C counts migrated with PC, whereas 90 ± 7% of the 3H counts migrated with PG. With no SP-A present, 90 ± 2% of the 14C counts migrated with PC, whereas 85 ± 5% of the 3H counts migrated with PG. In all cases, the majority of counts not migrating with authentic PC or PG migrated with the solvent front.

**SP-A inhibits phospholipase activity in alveolar macrophage homogenate.** Phospholipase activity was measured in the alveolar macrophage homogenate at pH 4.0 in the presence of 10 mM CaCl₂ or 5 mM EDTA. Inhibition of phospholipase activity was examined by including 10 or 100 μg SP-A/ml (Table 3). Phospholipase activity in the alveolar macrophage homogenate was less than that previously reported for the lung homogenate (8) or macrophage homogenate (19); this is possibly due to differences in incubation conditions. For all three lipids tested, phospholipase activity in the presence of 5 mM EDTA was two- to fivefold higher than the corresponding sample in 10 mM Ca²⁺. There was a higher phospholipase activity toward sn-2-labeled DPPC. Activ-
labeling. The BODIPY-PC also localized to the periphery of the cells and was visible in pseudopodia in some instances. Neither label localized to the nucleus.

DISCUSSION

The goal of this study was to further characterize the role of alveolar macrophages in the maintenance of surfactant homeostasis and to specifically investigate the role of the alveolar macrophage in the metabolism of PG. Our hypothesis was that macrophages contribute to the previously reported finding that PG is cleared from the alveolar space more rapidly than DPPC (16). We found that the metabolism of DPPG is affected by SP-A and adherence of cells to a plastic surface. The ratio of DPPC to DPPG internalized by macrophages in suspension demonstrates that DPPG is internalized to a greater extent than DPPC in the absence of SP-A. The percentage of cell-associated DPPC or DPPG degraded is greater in the absence of SP-A than in the presence of SP-A. In addition, exposure to SP-A and/or adherence to a plastic surface abolishes this differential uptake, suggesting that PG metabolism by alveolar macrophages is a process that can be regulated.

Experiments with alveolar macrophage homogenate to measure phospholipase activity also supported a role for differential metabolism and regulation. The macrophage homogenate displayed higher phospholipase activity toward sn-2-labeled DPPG compared with DPPC labeled at the sn-2 position. Phospholipase activity was higher in the absence of calcium than in the presence of calcium. In the presence or absence of calcium, phospholipase activity was inhibited by 100 μg SP-A/ml, supporting a role for SP-A in regulating the metabolism of lipids in the alveolar macrophage. SP-A enhances the association and internalization of liposomes by alveolar macrophages. In these experiments, it was shown that SP-A stimulated uptake and internalization of both DPPC and DPPG from unilamellar liposomes by alveolar macrophages. The level of SP-A stimulation of uptake was much greater for cells in suspension than for adherent cells. SP-A enhanced lipid uptake by cells in suspension ~13-fold but only stimulated uptake by adherent cells ~2-fold. Although

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Label Position</th>
<th>Phospholipase Activity, pmol/μg protein in 60 min</th>
<th>10 mM Ca²⁺</th>
<th>5 mM EDTA</th>
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<tr>
<td></td>
<td></td>
<td>+SP-A</td>
<td>-SP-A</td>
<td>Inhibition, %</td>
</tr>
<tr>
<td>[³H]DPPC</td>
<td>sn-1</td>
<td>0.37 ± 0.03‡</td>
<td>0.65 ± 0.05§</td>
<td>43</td>
</tr>
<tr>
<td>[³H]DPPC</td>
<td>sn-2</td>
<td>0.29 ± 0.02</td>
<td>0.43 ± 0.05†</td>
<td>31</td>
</tr>
<tr>
<td>[³H]DPPG</td>
<td>sn-2</td>
<td>0.45 ± 0.04§</td>
<td>0.81 ± 0.06§</td>
<td>44</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 3 independent experiments. Phospholipase activity in alveolar macrophage homogenate (2.5 × 10⁶ cells/ml) was measured by incubation of homogenate with 1 μM liposomes containing [³H]DPPC or a combination of [³H]DPPG and [¹⁴C]DPPC at 37°C for 60 min; SP-A was added at 100 μg/ml to some samples. The reaction was started by the addition of substrate and stopped by the addition of 1 ml of CHCl₃-MeOH (2:1). Lipids were extracted from the samples and separated by TLC. Radioactivity of each lipid species was quantified by scintillation counting. Significantly different from +SP-A: *P < 0.05; †P < 0.1. Significantly different from sn-2-labeled DPPC: ‡P < 0.1; §P < 0.05.
SP-A does not bind to DPPG (23), it has been shown to aggregate PC-containing liposomes (21). Liposome aggregation could lead to increased uptake of lipid aggregates by macrophages. However, two groups (26, 30) have shown that specific point mutations in SP-A can abolish the stimulation of lipid uptake by type II cells while maintaining the ability of SP-A to aggregate lipid vesicles. If the mechanism for SP-A-stimulated lipid uptake by alveolar macrophages is similar to that of type II cells, these data would suggest that aggregation of lipids is not sufficient to explain the stimulation of lipid uptake by alveolar macrophages.

Adherence of the cells significantly enhanced lipid uptake via a mechanism independent of SP-A. Adherent macrophages internalized ~10 times as much lipid as cells in suspension with no SP-A present. In contrast, Bates et al. (3) showed that stimulation of cells by overnight adherence had no effect on lipid uptake. However, that study compared lipid uptake of cells that were adhered for 1 or 24 h, whereas our study compared the uptake of lipids by freshly isolated cells in suspension and cells adhered for 1.5 h. Thus the difference in lipid uptake characteristics between freshly isolated cells and cells in culture for 1.5 h are greater than those observed in cells adhered for 1 or 24 h.

Association of both DPPC and DPPG with alveolar macrophages was greater at 37°C than at 4°C in both the presence and absence of SP-A, suggesting that active uptake of the liposomes is occurring. This is supported by confocal microscopy results showing internalized fluorescent label. One caveat that must be considered when interpreting the microscopy studies is that it was necessary to replace all of the DPPG with the fluorescent analog NBD-PG due to the rapid quenching of the NBD signal. It is possible that the metabolism and cellular distribution of the NBD-PG is not representative of DPPG.

Effects of adherence and SP-A on degradation by macrophages. The percentage of internalized lipid that was degraded by alveolar macrophages in suspension decreased approximately fivefold in the presence of 5 μg SP-A/ml; this same concentration of SP-A did not inhibit degradation in adherent cells. To compare the absolute mass of lipid degraded, we calculated the moles of lipid degraded from the TLC analysis. As shown in Table 4, stimulation of lipid uptake by SP-A may affect degradation of phospholipids by alveolar macrophages due to higher amounts of lipid entering the degradative pathway. Even though in the presence of SP-A degradation decreases as a percentage of total lipid, the molar amount of lipid degraded increases compared with the mass of lipid degraded in the absence of SP-A. In macrophages in suspension, SP-A increases uptake to a much greater extent than it stimulates degradation. Therefore, degradation may be the rate-limiting step in the metabolism of internalized lipid. It is possible that this process cannot be stimulated further by SP-A when the cells are already stimulated by adherence. SP-A does not increase the mass of degraded lipids in adherent cells, possibly because degradation is already maximally enhanced by adherence.

Bates et al. (3) showed that the levels of uptake and degradation in macrophages adhered to plastic for 1 h were similar to the levels we observed with cells in suspension and much less than what we measured in adherent macrophages. Two possible experimental features may account for the differing results. First, Bates et al. used medium containing 10% fetal calf serum for the lipid uptake experiments, whereas our medium was serum free; any lipid present in the calf serum would dilute the labeled liposomes. Second, in Bates’ study, DPPC was labeled with [methyl-3H]choline, not [3H]palmitate, resulting in a different set of traceable degradation products and dissimilar results.

Inconsistencies between our current work and previously reported results (35) may be due to variations in conditions between the two sets of experiments, such as time of incubation, lipid concentration, and the preparations of SP-A. The difference between lipid uptake in the presence of 15 μg SP-A/ml and in the absence of SP-A was not significantly different in previous work by Wright and Youmans (35), although SP-A at concentrations >15 μg SP-A/ml did enhance lipid uptake (see Fig. 1 in Ref. 35). In addition, there was no difference in DPPC degradation (see Table 1 in Ref. 35). In our present experiments, however, there was a significant difference in lipid uptake and degradation in the presence of 5 μg SP-A/ml compared with that in samples without SP-A (Fig. 2, Table 4). It is possible that the SP-A currently being used is more potent at stimulating lipid uptake than the SP-A that was used previously. It is also possible that differences in results are due to differences in the location of the radiolabel on the DPPC molecule. Our present analysis with intact macrophages was performed with sn-1-labeled DPPC, and previously reported data by Wright and Youmans (35) utilized sn-2-labeled DPPC.

Phospholipase activity in alveolar macrophage homogenates is higher for DPPG than for DPPC and is

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**Table 4. Lipid uptake or association and degradation at 3 h for macrophages in suspension**

<table>
<thead>
<tr>
<th>SP-A, μg/ml</th>
<th>Uptake, pmol/μg protein</th>
<th>Degradation %</th>
<th>pmol/μg protein</th>
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<tbody>
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<td>0</td>
<td>0.41 ± 0.21</td>
<td>80</td>
<td>0.33</td>
</tr>
<tr>
<td>5</td>
<td>5.31 ± 2.74</td>
<td>15</td>
<td>0.79</td>
</tr>
<tr>
<td>0</td>
<td>0.13 ± 0.06</td>
<td>70</td>
<td>0.09</td>
</tr>
<tr>
<td>5</td>
<td>1.45 ± 0.77</td>
<td>15</td>
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Values are means ± SE; n > 3 independent experiments. Alveolar macrophages were suspended in PBS with 2 mM Ca²⁺ and 0.1% BSA. The cells were then incubated with 5 μg SP-A/ml and 20 μg phospholipid/ml labeled with trace amounts of [¹⁴C]DPPC or [³H]DPPG at 37°C for 180 min. The cells were washed, and lipid uptake was analyzed by scintillation counting and standardized according to total protein. For degradation studies, lipids were extracted and separated by 2-dimensional TLC. Individual lipid species were isolated and analyzed for radioactivity. The amount of uptake and percentage of radioactivity not migrating with DPPC or DPPG were used to calculate degradation.
inhibitable by SP-A. Regulation of surfactant phospholipid levels may involve enzymatic degradation of internalized lipid species by type II cells and alveolar macrophages. It is believed that type II cells remodel phospholipids containing unsaturated fatty acids to species containing saturated fatty acids as a method of recycling internalized surfactant components (reviewed in Ref. 20). It has also been shown that alveolar macrophages convert surfactant PG to bis(monoacylglycerol)phosphate through a process involving removal of fatty acid on the sn-2 position and transacylation of the lysophosphatidylglycerol (1). Bates et al. (3) showed that SP-A (10 μg/ml) was capable of inhibiting PLA2 activity in rat whole lung homogenate and isolated lamellar bodies at pH 4.0; SP-A also inhibited PLA2 activity in type II cells that internalized lipid in vitro (8). We found that phospholipase activity in alveolar macrophage homogenate was not inhibited by 10 μg SP-A/ml (data not shown), although it was inhibitable by 100 μg SP-A/ml (Table 3). These data would suggest that alveolar macrophage PLA2 may not be regulated in the same manner as lung tissue or lamellar body PLA2. Data from phospholipase activity assays with alveolar macrophage homogenate support differential degradation of phospholipid species (Table 4). PLA2 activity toward DPPG was approximately twofold greater than PLA2 activity toward DPPC depending on the conditions. Type II cells are responsible for the recycling of surfactant lipid species, and if type II cell PLA2 activity is also higher toward DPPG with respect to DPPC, this could also lead to a shorter half-life for DPPG in the lung.

Potential relevance of these studies to the whole animal. It has been shown in vivo that the clearance of DPPG from the lung is faster than that of DPPC in 3-day-old rabbits (16). We found that macrophages in suspension in the absence of SP-A preferentially internalize DPPG over DPPC; the ratio of DPPC to DPPG associated with macrophages is significantly lower than in the starting liposomes. The ratio taken up in the absence of SP-A was 25% lower after 3 h of incubation than it was in the presence of SP-A. Thus uptake by the macrophage could, over time, contribute to the faster disappearance of PG from the alveolar space. Interestingly, adherent macrophages did not exhibit this preferential uptake. In addition, homogenate from alveolar macrophages showed increased PLA2 activity toward DPPG compared with DPPC; differential lipid degradation by PLA2 could further contribute to the faster disappearance of DPPG from the lung. Although these in vitro data suggest a role for SP-A in vivo, it has been shown that mice deficient in SP-A have nominal differences in their surfactant systems (22). It is possible that other coregulatory or compensatory mechanisms exist in the lung that provide sufficient regulation of surfactant metabolism in the absence of SP-A.

It is unclear whether adherent or suspension macrophages most accurately represent the in vivo situation. It has been shown by scanning electron microscopy that resting macrophages in the alveoli are in contact with the epithelium but are not spread out like adherent macrophages in vitro (12). We have not determined whether macrophages adhered to plastic slides are activated with respect to processes other than lipid uptake and degradation. Most comparisons between activated and resting cells have been done between groups of cells allowed to remain attached in culture for increasing amounts of time (3), not in freshly isolated cells and cells allowed to remain in culture. In addition to adherence, cells can be stimulated by exposure to serum proteins (11) or lipopolysaccharide (2, 6, 13, 18). In these cases, the signal leading to stimulation would come as a result of an immune challenge or lung injury. It remains to be seen whether lipid metabolism in alveolar macrophages primed by injury differs from that of cells from a healthy animal. It is possible that adherence to plastic may mimic the characteristics of macrophages from an injured lung, whereas using cells in suspension may be a good model for resting macrophages in the alveolar space.

In summary, macrophages in suspension preferentially internalize DPPG over DPPC when SP-A is not present, and the degradation of the lipid is approximately proportional to the amounts of DPPC and DPPG internalized. Macrophage homogenate exhibited greater degradation of DPPG compared with DPPC. If alveolar macrophages in vivo internalize lipid in a manner similar to suspension cells in vitro and if surfactant lipids that are internalized are depleted of SP-A (15), then alveolar macrophages may be partially responsible for faster clearance of DPPG with respect to DPPC from the alveolar space. The differential clearance of DPPC and DPPG could also result from differences in the sorting and recycling characteristics of type II cells. Future studies will be required to investigate this possibility.

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