Macrophage and type II cell catabolism of SP-A and saturated phosphatidylcholine in mouse lungs

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Gurel, Okyanus, Machiko Ikegami, Zissis C. Chronos, and Alan H. Jobe. Macrophage and type II cell catabolism of SP-A and saturated phosphatidylycholines in mouse lungs. Am J Physiol Lung Cell Mol Physiol 280: L1266–L1272, 2001.—Type II cells and macrophages are the major cells involved in the alveolar clearance and catabolism of surfactant. We measured type II cell and macrophage contributions to the catabolism of saturated phosphatidylcholine and surfactant protein A (SP-A) in mice. We used intratracheally administered SP-A labeled with residualizing 

125I-dilactitol-tyramine, radiolabeled dipalmitoylphosphatidylycholine (14C)DPPC, and its degradation-resistant analog

[14C]PPC-ether. At 15 min and 7, 19, 29, and 48 h after intratracheal injection, the mice were killed; alveolar lavage was then performed to recover macrophages and surfactant. Type II cells and macrophages not recovered by the lavage were subsequently isolated by enzymatic digestion of the lung. Radioactivity was measured in total lung, lavage fluid macrophages, alveolar washes, type II cells, and lung digest macrophages. Approximately equal amounts of 125I-dilactitol-tyramine-SP-A and [14C]DPPC-ether associated with the macrophages (lavage fluid plus lung digest) and type II cells when corrected for the efficiency of type II cell isolation. Eighty percent of the macrophage-associated radiolabel was recovered from lung digest macrophages. We conclude that macrophages and type II cells contribute equally to saturated phosphatidylycholine and SP-A catabolism in mice.

PULMONARY SURFACTANT is a complex mixture of lipids and proteins that is rapidly turned over and recycled (28). Dipalmitoylphosphatidylycholine (DPPC), a saturated phosphatidylycholine (Sat PC), is the major surfactant component by mass and the primary surface tension-lowering lipid (19). Surfactant protein A (SP-A), an innate host defense collectin, is the most abundant surfactant protein in the lung that also makes surfactant films more resistant to inhibition by other proteins (5). Synthesis, secretion, and reuptake maintain alveolar Sat PC and SP-A homeostasis. A previous study (9) in rabbits implicated alveolar macrophages and type II cells as the major cell types involved in the catabolism and/or recycling of Sat PC and SP-A. To date, no information is available on macrophage and type II cell catabolic activity in mouse lungs. In rabbits, the relative contributions of type II cells and macrophages to overall Sat PC catabolism were measured with a degradation-resistant diether analog of DPPC (DPPC-ether). This analog was previously shown (20) to have alveolar and total lung clearance kinetics and reutilization efficiencies similar to those of DPPC. The macrophages recovered by alveolar lavage took up 10–20% of the radiolabeled DPPC-ether, with the rest being catabolized or recycled by the type II cells (21). In rabbits, measurements of macrophage-associated radiolabel recovery were limited to the macrophages in the alveolar lavage fluid. Therefore, macrophage contributions to Sat PC catabolism may have been underestimated. In this study, we harvested macrophages after enzymatic lung digestion, in addition to those from the alveolar lavage fluid, to estimate with greater accuracy the contribution of macrophages to Sat PC catabolism.

Type II cells and macrophages are also the major cells involved in the alveolar clearance and catabolism of SP-A (2, 29, 31). However, the relative contributions of type II cells and macrophages to overall SP-A catabolism have not been measured. In rabbits, alveolar clearance of SP-A was measured with iodinated SP-A. 125I-SP-A has a short biological half-life of ~6.5 h, and the majority of the radiolabel was recovered from the lung tissue and not from lavage fluid macrophages (27). In this study, we labeled SP-A with 125I-dilactitol-tyramine (DLT), a residualizing label that prolongs the retention of the radiolabel at the site of the catabolism. The use of 125I-DLT-SP-A for the study of surfactant protein catabolism is a strategy similar to the use of DPPC-ether for Sat PC catabolism. We instilled DPPC-ether and residualizing labeled SP-A intratracheally and isolated type II cells and macrophages from the same mice. We asked what relative roles type II cells and macrophages play in the clearance and catabolism of DPPC and SP-A in mouse lungs.

MATERIALS AND METHODS

Labeling of SP-A with 125I-DLT. SP-A was isolated from the bronchoalveolar lavage fluid of patients with alveolar proteinosis with octylglucopyranoside used according to the methods of Hawgood et al. (8). DLT was iodinated by the...
methods of Strobel et al. (25), with minor modifications. In an IODO-GEN-coated polypropylene tube, 10 nmol of DLT and 25 μl of 0.5 M potassium phosphate buffer (pH 7.0) were reacted with 1 mCi of Na\textsuperscript{125}I for 25 min. The mixture was then transferred to another tube containing 4 U of galactose oxidase in 2 μl of 0.1 M potassium phosphate buffer (pH 7.0) and incubated for 45 min at 37°C. SP-A was then added to the mixture at a concentration of 1 mg/ml and incubated with 20 mM NaCNBH\textsubscript{3} for 1.5 h at 37°C. The protein-bound label was separated from the free label, first with a Sephadex G-25 column, then by ultrafiltration with a Centricon YM 10 centrifugal filter (Millipore, Bedford, MA). The final filtrate was 97% precipitable with 7% trichloroacetic acid (TCA) and was used in experiments within 24 h. The percentage of TCA-precipitable counts did not change with overnight storage. The specific activity of the \textsuperscript{125}I-DLT-SP-A was 0.011 precipitable counts.

**Lung Perfusion Models.** Eight-week-old female specific pathogen-free C57BL/6 mice (Charles River Laboratories, Wilmington, MA) with an average body weight of 16.0 ± 0.3 g were anesthetized with methoxyflurane and orally intubated with 30 and 40 mmHg was necessary to clear the lungs of blood. A 20-gauge intravenous catheter was inserted into the trachea and secured tightly with sutures.

**Macrophage and type II cell isolation.** Lungs were lavaged with three 1-ml aliquots of phosphate-buffered saline at 4°C, and the pooled alveolar lavage samples from each animal were layered over 0.8 M sucrose in 0.9% NaCl and centrifuged at 500 g for 15 min. The supernatant (alveolar surfactant) and cell pellets (lavaged macrophages) were further processed to determine radioactivity. After the lavage, lungs were carefully removed from the chest cavity, and type II cell isolation was performed as previously described by Corti et al. (6), with minor modifications. The lungs were filled with 2 ml of Disperse (Collaborative Research, Bedford, MA) via the tracheal catheter followed by 1 ml of low-melt agarose, and the lungs were immediately covered with crushed ice for 2 min. The lungs were then placed in 2 ml of Disperse in a polypropylene tube and incubated for 45 min at room temperature. In a 60-mm petri dish, 7 ml of Dulbecco’s modified Eagle’s medium with 0.01% deoxyribonuclease I (both from Sigma, St. Louis, MO) and 20-μm nylon mesh (Tetko, Lancaster, NY). The filtered suspension was centrifuged at 130 g for 8 min at 4°C and resuspended in 10 ml of culture medium. A small aliquot was saved for a differential cell count. This crude cell suspension was plated on a 100-mm tissue culture dish that had previously been coated overnight with 16 μg of purified monoclonal rat anti-mouse anti-CD16/CD32 and anti-CD45 (PharMingen, San Diego, CA). After 30 min at 37°C, the nonadherent cells were removed from the plate and used for determination of radiolabel recovery in type II cells. A small aliquot was saved for a differential cell count.

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**Characterization of cell isolates.** Cell counts were determined with a hemacytometer counting chamber (Fisher Scientific, Pittsburgh, PA), and viability was monitored by ex-
clusion of the vital dye trypan blue. Purity of the type II cells, the macrophages recovered by lavage, and the lung digest was determined by 200-cell differential counts on cytocentrifuge slides that were stained by the modified Papanicolaou technique.

Data analysis. All values are given as means ± SD. Differences between groups were tested by Student’s two-tailed t-test. Significance between more than two groups was determined by the Student-Newman-Keuls multiple comparison procedure. Significance was accepted at P < 0.05. For radio-label recovery, curvilinear regression curves and analysis of variance were computed.

RESULTS

Type II cell and macrophage isolates. The purity of the type II cell isolates ranged from 90 to 94%, with an average of 92.3 ± 1.5%. The cell purity of the lavaged macrophages ranged from 96 to 99%, with an average of 97.5 ± 1.7%, and the purity of the macrophages recovered from the lung digest ranged from 33 to 51%, with an average of 42.0 ± 7.3%. Other cell types captured on the antibody plates by the Fc receptor-specific antibodies were lymphocytes (average 39.1 ± 7.3%) and neutrophils (average 17.8 ± 1.9%). The cell yields for all isolates, corrected for the cell purity, are shown in Table 1. On average, 20-fold more macrophages were harvested from the lung digest than from the alveolar lavage fluid.

Recovery of [3H]DPPC, [14C]DPPC-ether, and 125I-DLT-SP-A in alveolar lavage fluid and the total lung. Percent recovery of [3H]DPPC and its degradation-resistant analog [14C]DPPC-ether from lavaged macrophages, alveolar lavage fluid (lavaged macrophages plus alveolar surfactant), and the total lung (alveolar lavage plus lung tissue) is given in Fig. 2, A and B. Because some of the instilled radiolabel is inevitably lost in the syringe, the needle, or the trachea, recovery curves for each radiolabeled probe are expressed as a percent of the radioactivity at the 15-min time point. At 15 min, 73% of the instilled 125I-DLT-SP-A and 68% of the instilled [14C]DPPC-ether radioactivity was recovered in the lungs. When normalized for isotope recovery at 15 min, by 48 h, only 3.8% of the [3H]DPPC remained in the lung compartment, yielding a biological half-life of 7 h for DPPC. In comparison, 45% of the [14C]DPPC-ether was recovered from the lung at 48 h, resulting in an increase in the biological half-life to 47 h. Total lung recovery of the 125I-DLT-SP-A was similar to that of the [14C]DPPC-ether (Fig. 2C). The residualizing DLT increased the biological half-life of the radiolabel in the total lung compartment to 29.1 h relative to the SP-A iodinated by the Bolton-Hunter method that our laboratory (13) previously reported to have a biological half-life of 6 h in mouse lungs.

Recovery of [14C]DPPC-ether and 125I-DLT-SP-A from type II cells and macrophages. To allow direct comparisons of radioactivity recovery from type II cells and lavaged and lung digest macrophages, the total recovered radioactivity from each cell population was normalized to cell number. [14C]DPPC-ether and 125I-DLT-SP-A recoveries per 10^9 cells from each cell pop-

Table 1. Cell counts in mouse lungs

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Type II Cells</th>
<th>Lavage</th>
<th>Lung digest</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>5.2 ± 1.0</td>
<td>0.12 ± 0.05</td>
<td>5.6 ± 1.0†</td>
</tr>
<tr>
<td>7</td>
<td>6.1 ± 0.3</td>
<td>0.25 ± 0.07*</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td>19</td>
<td>6.0 ± 0.2</td>
<td>0.16 ± 0.03</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>29</td>
<td>5.9 ± 0.9</td>
<td>0.17 ± 0.06</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>48</td>
<td>5.4 ± 0.9</td>
<td>0.13 ± 0.04</td>
<td>2.7 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ± SD of no. of cells × 10^6; n = 4 lungs/group. *P < 0.05 vs. 0.25- and 48-h groups. †P < 0.01 vs. all other groups.
ulation are shown in Fig. 3. Recovery for [3H]DPPC is not shown because the radiolabel was rapidly eliminated from both cell populations, and cell specific accumulation was not measurable after the first few time points. The lavaged macrophages contained the highest per cell radioactivity of [14C]DPPC-ether and 125I-DLT-SP-A among the three cell populations. During the study period, the radiolabel concentration in the lavaged macrophage isolates remained unchanged (125I-DLT-SP-A) or slightly decreased ([14C]DPPC-ether), whereas the lung digest macrophage isolates accumulated both radiolabels over time.

Because the number of macrophages isolated after the lung digest was 20-fold more than that obtained by alveolar lavage, the relative contributions of the macrophages from the lavage fluid and the lung digest were calculated as percentages of the total macrophage-associated radiolabel recovery. At 48 h, >80% of the [14C]DPPC-ether and 125I-DLT-SP-A was recovered from the macrophages from the lung digest (Fig. 4).

Based on a previous morphometric estimate that lungs of similar size (average wet lung weight of 0.23 vs. 0.22 g in this study; P = 0.54) from C57BL/6-FVB/N mice contain ~21 x 10^6 type II cells (10), we recovered ~25% of the type II cells from the lung digests. We estimated the amount of radiolabel recovered in the type II cell population and corrected for the efficiency of the type II cell recovery (Fig. 5). The estimates of accumulation of both [14C]DPPC-ether and 125I-DLT-SP-A were similar for the total macrophage population and for the type II cells after 10 h. This result indicates that the two cell populations contributed equally to the catabolism of DPPC and SP-A.

TCA precipitation of 125I label. The ability of TCA to precipitate 125I radioactivity was used as an indirect measure of protein catabolism because the 125I-DLT-SP-A was TCA precipitable and the 125I-DLT was TCA soluble. The percentage of TCA-precipitable radioactivity in the alveolar fluid ranged from 89 to 97%, with an average of 93.0 ± 4.0% for the first 19 h of the study, indicating that 125I-DLT-SP-A was not degraded within the alveolar space. At later times, radioactive label recovery in the alveolar surfactant was too low to reliably measure TCA precipitation. In contrast, the precipitable counts in the lung tissue decreased exponentially from 93% at 15 min to 21% at 48 h (R = 0.99), indicating that the SP-A attached to 125I-DLT was
degraded within the lung tissue. Nevertheless, the retention of the $^{125}\text{I}}$-DLT allowed us to estimate the localization of catabolic activity. The half-life of the TCA-precipitable label in the lung tissue was 6.2 h, which was not different from the previously reported half-life for $^{125}\text{I}}$-SP-A labeled with the Bolton-Hunter reagent and $^{35}\text{S}}$-SP-A labeled in vivo with $[^{35}\text{S}}]$methionine (13, 27).

**DISCUSSION**

Despite extensive knowledge of the overall alveolar and lung clearance kinetics of surfactant components, little is known about the cell-specific distribution of catabolic activity in vivo. Information obtained from rabbits and rats indicates that type II cells and macrophages are the major sites of surfactant lipid catabolism. However, their relative contributions to surfactant protein catabolism remain unknown (11). To date, cell populations other than type II cells and macrophages have not been shown to have a significant role in alveolar surfactant uptake and catabolism. Recently, Savov et al. (23) found that biotinylated SP-A given to rats by intratracheal injection accumulated in lamellar bodies in type II cells, but no similar accumulation was observed in Clara cells. Therefore, we focused on the type II cells and macrophages and their contributions to Sat PC and SP-A catabolism. We used DPPC-ether and residualizing labeled SP-A to increase intracellular retention of the radiolabels after uptake by the cells. After intratracheal administration of radiolabeled DPPC-ether and SP-A, we isolated macrophages and type II cells. When corrected for the efficiency of recovery of the cells, type II cells and macrophages accumulated similar amounts of SP-A and DPPC.

Previous in vivo studies have indicated that type II cells are the major contributors to alveolar surfactant clearance and catabolism. In the rabbit, after intratracheal instillation, 65% of the Sat PC was type II cell associated as opposed to 10–20% of that recovered from the alveolar macrophages (21). In contrast, there is ample in vitro evidence (2, 16) showing that alveolar macrophages are capable of taking up DPPC and SP-A. Metabolic studies with transgenic mouse models with altered surfactant homeostasis suggest that alveolar macrophages play a greater role in surfactant clearance than previously suspected. For example, granulocyte-macrophage colony-stimulating factor (GM-CSF) gene-ablated [GM-CSF(−/−)] and GM-CSF receptor β-chain (βc)-deficient mice have foamy, lipid-laden alveolar macrophages along with increased surfactant phospholipid and protein pool sizes (13, 22). Alveolar macrophages isolated from GM-CSF(−/−) mice were shown to have impaired Sat PC and SP-A catabolism in vitro (30). In vivo, GM-CSF(−/−) mice were unable to clear DPPC-ether and iodinated SP-A from the alveolar space after intratracheal injection (13). In GM-CSF receptor βc(−/−) mice, bone marrow transplantation improved surfactant homeostasis by providing donor macrophages with normal GM-CSF receptors (17). Similarly, patients with idiopathic pulmonary alveolar proteinosis, an illness associated with abnormal macrophage function, have markedly increased alveolar phospholipid and SP-A concentrations attributed to reduced clearance rather than increased secretion rates (1). These findings suggest that macrophages play a critical role in the alveolar clearance and catabolism of surfactant in vivo, and perhaps type II cells are not able to compensate for altered macrophage catabolic activity. Healthy lungs, on the other hand, are capable of compensating for the acute increase in surfactant pool size that results from a large dose of intratracheally administered surfactant. Recently Kramer et al. (15) demonstrated that in mice an increase in the surfactant pool size is rapidly compensated for by the increased Sat PC catabolic rate, leading only to transient changes in surfactant homeostasis.

Surfactant catabolism in macrophages has been difficult to study in vivo, especially with radiolabeled SP-A, because alveolar macrophages are capable of rapid uptake and degradation, thus preventing intracellular accumulation of the radiolabel. Young et al. (32) reported that iodinated SP-A associated with alveolar macrophages within 6 min after intratracheal injection, but 75% of the radioactivity was lost by 1 h. To prolong the retention of the SP-A-associated radio-

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**Fig. 5.** Accumulation of $[^{14}\text{C}}]$DPPC-ether (A) and $^{125}\text{I}}$-DLT-SP-A (B) in lavage fluid plus lung digest macrophages and type II cells corrected for the 25% efficiency of type II cell isolation; $n = 4$ animals/time point.
label at the site of catabolism, we labeled SP-A with the residualizing label 125I-DLT. Using the TCA precipitability of 125I as a measure of protein degradation, we demonstrated that residualizing labeled SP-A had a total lung clearance and half-life similar to that of iodinated SP-A and the precursor-labeled native 35S-labeled SP-A (13, 27). Residualizing labels do not interfere with the degradation of the labeled protein, but they are themselves resistant to lysosomal hydrolysis. Because of its relatively large size, ~800 Da, 125I-DLT is unable to diffuse through the lysosomal and/or cell membranes even after the attached protein has been degraded. Therefore, 125I that is attached to the residualizing label is retained at the site of catabolism. Previously, residualizing labels have been used for studies of plasma protein catabolism and for the identification of the catabolic pathways of lipoproteins (26). To our knowledge, this is the first report on the use of residualizing labels in the study of surfactant protein catabolism.

Our results indicate that the macrophage contribution to surfactant catabolism in mice is greater than that previously reported in rabbits. However, there are several technical differences between the studies in rabbits (20, 21) and this study in mice. Most importantly, in the rabbit studies, no effort was made to harvest macrophages that were not recovered by the limited alveolar lavage performed before type II cell isolation. A previous study in mice (14) showed that even an extensive alveolar lavage removes at best 30% of the total alveolar macrophage pool. In preliminary experiments, we determined that a more extensive alveolar lavage procedure dramatically decreased subsequent type II cell yield and purity to <80%. Therefore, we harvested macrophages that were not recovered by the alveolar lavage after a subsequent enzymatic digestion of the lung and isolated, on average, 20-fold more macrophages from the lung digest than from the alveolar lavage fluid. Our total macrophage recovery was within the previously reported range for total pulmonary macrophage number in mice of several different strains (3, 7). Another procedural difference between surfactant accounting studies in rabbits and the current study in mice is the method of type II cell isolation. The type II cell isolation with Percoll gradients in rabbits had higher percentages of contaminating cells, including macrophages that co-sedimented with the type II cells. This may have contributed to an overestimation of the type II cell-associated radiolabel recovery (21). In addition to the technical differences, there are possible species differences between mice and rabbits. For example, the alveolar macrophage number per kilogram of body weight is about threefold higher in mice than in rabbits (24).

By harvesting the macrophages not recovered by alveolar lavage in addition to the macrophages in alveolar lavage fluid, we increased radiolabel recovery from the total alveolar macrophage compartment because, over time, a smaller proportion of the total macrophage-associated radioactivity was recovered in macrophages isolated from the lavage fluid (Fig. 4). Radiolabel accumulation occurred with time in the lung digest macrophages but not in the lavaged macrophages. We assumed that all macrophage-associated radiolabel recovery was from the alveolar macrophage pool because neither surfactant nor alveolar macrophages are likely to gain entry into the interstitium under normal circumstances (4). In Fig. 5, we corrected radiolabel recovery for the efficiency of the type II cell isolation procedure but did not correct for any possible losses in macrophage isolation because our total macrophage numbers were within the range of published data for mice. However, it is possible that this approach may underestimate the role macrophages play in surfactant catabolism.

DPPC and SP-A taken up by type II cells are either sorted for resecretion or degraded by lysosomes. In the adult animal, it is estimated that ~50% of the DPPC is recycled and 50% is catabolized (20). De novo synthesized SP-A seems to be constitutively secreted, for the most part, independently of lamellar bodies, and the SP-A in lamellar bodies may come primarily from reuptake and recycling (12, 18). It is not known if the SP-A that is recycled differs from the SP-A that is catabolized. For example, the characteristics of the glycosylation of SP-A could be different between the two pathways (11). In this study, we used residualizing labeled human SP-A and found that net catabolism based on TCA precipitation was equivalent to the previously reported rates of catabolism for iodinated SP-A and SP-A labeled in vivo with 35S-amino acids. Therefore, we assumed 125I-DLT took the same intracellular pathways as the native mouse SP-A. The accumulation of the residualizing label and [14C]DPPC-ether in type II cells at early time points (Fig. 5) includes surfactant within the recycling and catabolic pathways. Whereas type II cells resecrete surfactant that is not degraded, macrophages can only take up and catabolize surfactant. Presumably, because both type II cells and macrophages compete for the same recycled surfactant, the decrease in the specific activity of the alveolar radiolabeled surfactant with time should not lead to a relative underestimation of catabolic activity for one or the other cell population at later time points. However, after intratracheal injection, the accumulation of radiolabel in type II cells at the initial time point overestimates catabolic activity because much of the label will be recycled. The amount of the radiolabeled surfactant is relatively small by 48 h; consequently, most of the radioactivity in the alveolar lavage fluid was recovered in the lavaged macrophages. By 48 h, the net result was that approximately equal amounts of the residualizing DLT label and DPPC-ether were associated with type II cells and macrophages (Fig. 5).

In conclusion, we demonstrated that labeling SP-A with the residualizing DLT label is useful for studying SP-A catabolic pathways. In mouse lung, alveolar type II cells and macrophages contributed equally to the alveolar clearance and catabolism of Sat PC and SP-A. Our results also indicate that lavaged macrophage-associated radiolabel recovery was not representative
of the overall surfactant uptake behavior of the total macrophage compartment.

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