Pathways for clearance of surfactant protein A from the lung

Deepika Jain, Chandra Dodia, Aron B. Fisher, and Sandra R. Bates

Institute for Environmental Medicine, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania

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Jain, Deepika, Chandra Dodia, Aron B. Fisher, and Sandra R. Bates. Pathways for clearance of surfactant protein A from the lung. Am J Physiol Lung Cell Mol Physiol 289: L1011–L1018, 2005.—Uptake and degradation of 125I-surfactant protein A (SP-A) over a 1-h period was studied in alveolar cells in culture and in isolated perfused lungs to elucidate the mechanism for clearance of the protein from the alveolar space. Specific inhibitors of clathrin- and actin-dependent endocytosis were utilized. In type II cells, uptake of SP-A, compared with controls, was decreased by 60% on incubation with clathrin inhibitors (amantadine and phenylarsine oxide) or with the actin inhibitor cytochalasin D. All agents reduced SP-A metabolism by alveolar macrophages. Untreated rat isolated perfused lungs internalized 36% of instilled SP-A, and 56% of the incorporated SP-A was degraded. Inhibitors of clathrin and actin significantly reduced SP-A uptake by ~54%, whereas cytochalasin D inhibited SP-A degradation. Coincubation of agents did not produce an additive effect on uptake of SP-A by cultured pneumocytes or isolated perfused lungs, indicating that all agents affected the same pathway. Thus SP-A clears the lung via a clathrin-mediated pathway that requires the polymerization of actin.

clathrin-mediated uptake; actin; type II cells; macrophages; degradation; perfused lung

PULMONARY SURFACTANT FORMS a thin film at the air-liquid interface of the alveoli, contributing to alveolar stability and immune functions. The chemical composition of lung surfactant is highly conserved among species and consists of 80–90% phospholipids, 3–10% neutral lipids (primarily cholesterol), and 5–10% surfactant-associated proteins (10). The extracellular metabolic pathway is only partially understood and includes morphological transformations of surfactant forms within the liquid phase (31, 40), as well as clearance of spent surfactant. Possible mechanisms for clearance of surfactant components from the alveoli include extracellular degradation within the air spaces, uptake by various cell types, swallowing after movement up the airways, and removal through the air-blood barrier (49). Previous studies have demonstrated that uptake by type II cells and alveolar macrophages is responsible for the major fraction of surfactant phospholipid and protein removed from the alveolus (5, 12, 18, 20). Surfactant protein A (SP-A), the most abundant protein in pulmonary surfactant, is an ~700-kDa oligomeric hydrophilic glycoprotein belonging to the collectin family. Monomeric SP-A varies from 26 to 36 kDa depending on the degree of glycosylation. Although SP-A is synthesized, secreted, and recycled primarily by type II pneumocytes (30, 47), it also has been immunohistochemically identified in other cell types of the lung, including macrophages (45, 49) and nonciliated bronchial (Clara) cells (45) and cells of the tracheal and bronchial glands (34). SP-A is thought to be secreted constitutively by type II cells into the alveolar lumen, where it associates with surfactant lipid (24).

Several in vitro studies have identified important functions of SP-A in the lung, such as formation of tubular myelin (36), enhancement of the rate of surface adsorption of the lipids (41), prevention of inactivation of the biophysical properties of surfactant by plasma (15), and modulation of various immunologic functions of the surfactant (50). With regard to regulation of extracellular surfactant metabolism, it is well established that SP-A plays an important role in surfactant phospholipid secretion, reuptake, and recycling (18, 20, 27). SP-A has been shown to enhance uptake of phospholipid liposomes by type II cells (3, 43), macrophages (2, 52), and intact lung (25), to modulate the activity of the lysosomal-type phospholipase A2, which degrades dipalmitoylphosphatidylcholine (16), and to inhibit phospholipid secretion (7, 51). Secretagogues that enhance phospholipid secretion and uptake also enhance Ca2+-dependent SP-A binding by the type II cell and recruitment of SP-A receptors to the type II cell membrane (13). However, the exact mechanism for uptake of SP-A is not well defined.

Mammalian cells are known to internalize particles through a number of processes collectively termed “endocytosis.” Endocytic pathways include clathrin-dependent receptor-mediated uptake and clathrin-independent endocytosis, along with other mechanisms such as macropinocytosis, phagocytosis, and caveolae-dependent endocytosis (1, 32). Clathrin is a heteromeric protein that forms a basketlike framework of coated pits on the cytoplasmic face of the membrane. Internalization of clathrin-coated vesicle and its contents occurs in response to receptor cross-linking. Considering that the major endocytic pathways of the type II cell would involve clathrin and/or actin, we previously used specific inhibitors of clathrin and actin to identify the mechanism whereby phospholipid liposomes and phospholipids in natural surfactant were incorporated into the lung and type II pneumocytes (18, 27). We found evidence for participation of clathrin-dependent and clathrin-independent pathways with actin polymerization necessary for uptake of lipids via the clathrin-mediated pathway. Previous studies have provided evidence that SP-A uptake is mediated by clathrin (39, 42, 55), but a possible role for nonclathrin endocytosis has not been evaluated. In the present study, we examined this possibility for uptake and whether actin plays a role in the clathrin-dependent uptake of SP-A. In addition, we have compared the effect of inhibitors on clearance of SP-A by cultured cells in vitro and in the intact lung.

Address for reprint requests and other correspondence: S. R. Bates, Institute for Environmental Medicine, Univ. of Pennsylvania School of Medicine, 1 John Morgan Bldg., 3620 Hamilton Walk, Philadelphia, PA 19104-6068 (e-mail: batekenn@mail.med.upenn.edu).

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Materials and Methods

Sprague-Dawley pathogen-free male rats weighing ~200–250 g were obtained from Charles River Breeding Laboratories (Kingston, NY). Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Phenylarsine oxide (PAO), cytochalasin D (CytoD), amantadine HCl, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO); fatty acid-free BSA from Roche Diagnostics (Indianapolis, IN); and 125I from Amersham Biosciences (Piscataway, NJ).

Isolation of surfactant and purification of SP-A. SP-A was purified from bronchoalveolar lavage (BAL) fluid of alveolar proteinosis patients after therapeutic lavage at the Hospital of the University of Pennsylvania. Whole surfactant was isolated by density gradient centrifugation of cell-free lavage fluid. The surfactant fraction was dialyzed and lyophilized as described previously (3). SP-A was extracted from whole surfactant using 1-butanol and β-o-glucopyranoside (23). Rat lung surfactant was isolated after intrahepatic lavage of rat lungs followed by NaCl-NaBr gradient centrifugation of the cell-free lavage fluid (20).

Iodination. Iodination of SP-A was performed using Iodogen (Pierce, Rockford, IL) according to the manufacturer's directions. Iodinated protein was dialyzed against 5 mM Tris buffer for 24 h with frequent buffer changes to remove free 125I. Specific activity for all preparations was ~200–400 cpd/mg protein. The trichloroacetic acid (TCA) precipitability was 76–98%. The iodinated proteins were stored at 4°C and used within 2–3 wk. Storage of SP-A did not appreciably affect TCA precipitability.

Isolation and culture of type II cells. Alveolar type II pneumocytes were isolated from 200- to 250-g Sprague-Dawley rats as previously described (3). Rat lungs were perfused and subjected to endotracheal lavage before instillation of elastase (Sigma). The lobes were minced with a tissue chopper, and the lysate was sequentially filtered through 160-, 37-, and 10-µm mesh. Macrophages were removed from the resulting cell suspension by plating on 100-mm dishes precoated with rat immunoglobulin G (Sigma). The remaining sonicate was counted in a gamma counter (Beckman). Values were corrected for TCA precipitability of the iodinated SP-A preparations. We did not examine SP-A degradation, inasmuch as available evidence indicates that type II cells in culture do not catabolize SP-A (5, 27).

SP-A uptake by alveolar macrophages. Alveolar macrophages, obtained from lung BAL of pathogen-free male Sprague-Dawley rats (500 g), were plated in 35-mm plastic dishes (Costar, Cambridge, MA) at 37°C in serum-free MEM. After 1 h, the cells were washed three or more times to remove red blood cells and other nonadherent cells; 99% of the cells that adhered to the dish were alveolar macrophages. Experiments on the uptake of 125I-SP-A followed the protocol used for type II cells. Macrophages were solubilized using 0.2 N NaOH, and an aliquot was used for determination of total protein by the method of Lowry et al. (29); another aliquot was counted for radioactivity. To estimate degradation of SP-A by macrophages, medium was collected from the dishes at the end of the experiment and analyzed for the presence of TCA-soluble SP-A as previously described (5).

SP-A uptake in isolated perfused lung. Rats were anesthetized with pentobarbital sodium (50 mg/kg body wt ip). A tracheal catheter was used for instillation of 7.5 µg of 125I-SP-A in 100 µl of PBS into the lungs. This procedure ensured uniform distribution of the labeled SP-A throughout the lung. Of the total SP-A recovered after instillation, 24, 14, 16, 38, and 9% were recovered from the lower-left, middle-left, upper-left, lower-right, and upper-right portions of the lung, respectively; these recoveries are consistent with the sizes of the lobes. Lungs were cleared of blood by perfusion with 5 mM Krebs-Ringer bicarbonate buffer containing 3% BSA and moved to an isolated organ perfusion system (19). Perfusion buffer consisted of Krebs-Ringer bicarbonate with 3% fatty acid-free BSA and 10 mM glucose. Perfusion was constantly gassed with 5% CO2 in air. Lungs were ventilated at 60 cycles/ml, 2 ml tidal volume, and 2 cmH2O end-expiratory pressure. Lungs were perfused for 1 h with buffer alone (control) or with buffer containing 2 µM PAO, 5 mM amantadine, or 10 µM CytoD, and 1-ml aliquots of the perfusate were collected every 10 min. At the end of the 1-h experimental perfusion, lungs were lavaged five times with 10-ml aliquots of ice-cold Ca2+ and Mg2+-free PBS. Lung tissue was homogenized on PBS on ice with a Polytron homogenizer and then with a motorized mortar and pestle. Perfusion, lavage, and lung homogenate were counted for total radioactivity as well as TCA precipitability (5). Results are expressed as a percentage of the total 125I-SP-A counts recovered, which represents ~83 ± 0.7% (n = 22) of the total 125I-SP-A instilled. TCA-soluble counts in the instilled SP-A (2–24% of total) were subtracted from TCA-soluble counts in the lung fractions.

Statistical analysis. Values are means ± SE unless otherwise stated. Results were analyzed statistically by t-test or paired t-test using SigmaStat for Windows (Jandel, San Rafael, CA), where statistical significance is taken as P < 0.05. Multiple-group comparisons were done by one-way ANOVA.

Results

SP-A association with type II cells. Microscopy and biochemical analysis provided evidence that SP-A is found in association with coated pits and is internalized into type II cells (39, 42, 55). We previously showed that PAO, an agent that prevents formation of clathrin-coated vesicles, inhibited the uptake of SP-A by type II cells in culture, suggesting a role for clathrin-coated pits in internalization of SP-A (8). To confirm and extend our data, the present experiments used an additional clathrin inhibitor that works downstream of PAO, i.e., amantadine, a cationic amphiphilic agent that prevents budding of clathrin-coated vesicles. In addition, the effect of actin inhibition on incorporation of SP-A into type II cells was examined by treating the cells with CytoD, an agent that destabilizes the actin cytoskeleton.

Control cells not exposed to agents showed that SP-A binding was similar at 30 and 60 min (Fig. 1A). Neither of the inhibitors had a significant effect on binding of SP-A to type II cells (Fig. 1A). Uptake of SP-A by the pneumocytes increased linearly with time during a 60-min incubation (Fig. 1B). In contrast to the effect on binding, blocking clathrin- or actin-mediated processes resulted in inhibition of SP-A internalization.
tion by type II cells at the initial time point (30 min), with a significant reduction in cellular incorporation of SP-A after 1 h of intervention (Fig. 1B). After 1 h of incubation with 125I-labeled SP-A, PAO decreased SP-A uptake in type II cells to 50% of the control value (Fig. 2), confirming previous results (8). Amantadine and CytoD were as effective as PAO in inhibiting SP-A uptake by type II cells in culture (Fig. 2). Treatment of type II cells with a combination of actin and clathrin inhibitors did not further reduce SP-A incorporation by the cells, indicating a single clathrin-dependent pathway that requires actin polymerization.

**Effect of lipid on SP-A uptake.** SP-A in lung surfactant in vivo is associated with phospholipid, especially phosphatidylcholine. To elucidate whether association of SP-A with lipid has an effect on the mechanism of uptake, whole rat surfactant isolated from lung lavage or Survanta, a commercially available SP-A-free surfactant preparation, was mixed with iodinated SP-A in the ratio of 9:1 (9 μg phospholipid/μg 125I-SP-A). Isolated type II cells preincubated with or without inhibitors for 30 min were further incubated with this SP-A-phospholipid mixture for 1 h. With rat surfactant, results were corrected for the presence of endogenous SP-A, estimated to be 1.5 μg of SP-A in 9 μg of surfactant phospholipid (44). The presence of rat surfactant phospholipid resulted in 1.9-fold augmentation of SP-A uptake (Fig. 3). As with lipid-free SP-A, uptake of the SP-A-phospholipid complex was blocked by clathrin and actin antagonists. Addition of a combination of agents had no further effect (Fig. 3). Similar results were seen with addition of Survanta phospholipid (data not shown), demonstrating that the inhibitory effect of clathrin antagonists and CytoD was the same, irrespective of differences in the source of phospholipid.

**SP-A metabolism by alveolar macrophages in culture.** Alveolar macrophages have been shown to endocytose and de-
grade SP-A (4, 5, 53). Although macrophages have receptors for SP-A (14) and would be expected to utilize a clathrin-coated pit pathway for internalization, macrophages are also active phagocytes, a process requiring actin polymerization. Clathrin or actin antagonists were utilized to determine possible effects on the uptake of SP-A by macrophages. In addition, alterations in macrophage degradation of SP-A were followed. Alveolar macrophages were isolated from lung lavage, and cells were preincubated with the inhibitors before addition of 125I-SP-A to the medium. As seen for type II cells, clathrin inhibitors and CytoD inhibited total uptake of SP-A (intact SP-A + degradation products) by macrophages in culture (Fig. 4A). Exposure to clathrin and actin inhibitors was not additive, which is indicative of a single clathrin-dependent uptake pathway requiring actin activity. Macrophages without agents degraded SP-A rapidly, with ~85% of the total SP-A recovered from the culture as TCA-soluble products (Fig. 4B). All agents demonstrated a minor (30%) effect on the extent of SP-A degradation once SP-A was taken up by the cells (Fig. 4B), indicative of a possible effect on an intracellular degradative process.

Metabolism of SP-A in isolated perfused lung. Because in vitro cell culture may result in alteration of metabolic pathways, extrapolation to results in vivo can be problematic. To examine the in vivo situation more closely, we utilized the isolated perfused lung model. Iodinated SP-A was instilled into lungs before they were removed from the chest, then the lungs were perfused for 1 h in the presence or absence of inhibitors (see MATERIALS AND METHODS). Uptake of SP-A from the alveolar space into the lung cells was defined as the sum of the TCA-precipitable SP-A products recovered from lung tissue and the TCA-soluble SP-A recovered from the lung homogenate, lavage, and perfusate. There was no TCA-precipitable SP-A in the perfusate. Total recovery of instilled 125I-SP-A was ≥85% after 1 h of perfusion.

Catabolism of SP-A by the lung, as indicated by the appearance of TCA-soluble 125I-labeled amino acids in the perfusate, was fairly rapid (Fig. 5A). SP-A degradation products appeared in the perfusate within the initial time period (10 min) and accumulated linearly over the subsequent 60-min perfusion period. At 1 h after instillation, significant uptake of SP-A, as measured by the sum of the TCA-precipitable 125I disintegrations per minute in the lung, together with the TCA-soluble counts in the lavage, perfusate, and lung, had occurred. Uptake of SP-A into control lungs was 2.63 ± 0.18 μg of SP-A or 36% of SP-A instilled. Amantadine- and PAO-treated lungs internalized 1.02 ± 0.03 and 1.07 ± 0.13 μg of SP-A, respectively, ~40% of control values. Uptake of SP-A by CytoD-treated lungs was 1.07 ± 0.14 μg (P < 0.05 vs. control). Addition of PAO + CytoD had no significant further effect on SP-A uptake overall of either the inhibitors alone (Fig. 5B).

To determine whether 125I-SP-A reached type II cells and alveolar macrophages via the intratracheal instillation procedure, type II cells (isolated as described in MATERIALS AND METHODS) and alveolar macrophages (isolated from the lung lavage fluid) were obtained from rat lungs 1 h after instillation of 125I-SP-A. A direct comparison of the quantity of SP-A associated with the two cell types is not meaningful because of differences in the protocols. The isolation of type II cells from the rat lungs took several hours, and the cells were treated with enzymes at 37°C during the procedure, whereas the isolation of macrophages was performed quickly and at cold temperatures. However, 125I-SP-A was associated with both cell types (43 ± 6 and 751 ± 158 ng SP-A/mg cell protein for type II cells and macrophages, respectively, n = 3), demonstrating that 125I-SP-A reached type II cells and macrophages via our instillation procedure.

Total degradation of SP-A was taken as the sum of TCA-soluble 125I-SP-A disintegrations per minute recovered from the homogenate, lavage, and perfusate. Approximate recoveries of degraded SP-A, expressed as a percentage of the total TCA-soluble 125I disintegrations per minute, were 20% from the lung homogenate, 33% from the BAL, and 47% from the perfusate. The location for recovery of the degraded SP-A products did not change on treatment with agents. Of the total radioactivity recovered from control lungs after 1 h of perfusion, ~44% was associated with TCA-precipitable protein (intact SP-A), and the remaining radioactivity (56%) was in the...
TCA-soluble fraction. Because all the agents inhibited the uptake of SP-A into the lung, the extent of degradation of SP-A (as μg SP-A/lung) also was reduced by the drugs (Fig. 5B). However, when SP-A degradation is expressed as a percentage of uptake (Fig. 5C), alteration of the actin cytoskeleton with CytoD resulted in a reduction of SP-A degradation in the absence or presence of PAO, whereas perfusion of the lungs in the presence of the clathrin inhibitors PAO and amantadine did not affect degradation (Fig. 5C), implying a role for actin filaments in the subsequent transport of the internalized SP-A to the site of degradation.

DISCUSSION

Previously, using radioactive liposomes, we evaluated the endocytic pathways involved in the uptake of surfactant phospholipid and determined that the incorporation of surfactant lipid into the lung and type II cells was sensitive to inhibitors of clathrin-coated pit formation and to the polymerization of actin fibers (38). Our results indicated two distinct pathways for endocytosis of liposomes, clathrin dependent and clathrin independent, with some involvement of actin filaments in the clathrin-mediated uptake. To confirm that SP-A uptake is strictly clathrin dependent and that this is the case for pneumocytes in culture as well as for the lung in vivo, we have performed a parallel experiment designed to study the mechanisms utilized for endocytosis of SP-A. Our data indicate that SP-A uptake in vitro in primary cultures of type II pneumocytes and alveolar macrophages and, importantly, in vivo in the isolated perfused lung occurred only through a single clathrin-mediated pathway that required a functioning actin polymerization process.

We began by examining the role of clathrin and actin integrity in internalization of SP-A by alveolar type II cells in vitro. SP-A has been morphologically localized in clathrin-coated pits in type II cells by microscopic studies (39, 42). We reported previously the involvement of clathrin-coated pits in SP-A internalization by type II cells in culture (8). In the present study, we have extended our observations about clathrin-mediated SP-A uptake by using two functionally different clathrin inhibitors, amantadine and PAO. Amantadine is a cationic amphiphilic drug that stabilizes clathrin-coated vesicles and prevents their budding, whereas PAO blocks clathrin-mediated endocytosis by cross-linking clathrin and preventing the formation of clathrin-coated vesicles. In addition, we blocked actin-mediated SP-A uptake by using a broad-spectrum disrupter of actin polymerization, CytoD, which caps F-actin filaments and prevents actin polymerization. We found that both classes of clathrin inhibitors blocked SP-A uptake in cultured type II cells by ∼50%. These results confirm previous studies which showed that intracellular potassium depletion inhibits SP-A uptake, compatible with a clathrin-mediated process (42). As for the clathrin-independent, actin-dependent pathway, we saw that the actin inhibitor CytoD also inhibited uptake by 50% in type II cells. However, when the cells were treated with a combination of clathrin and actin inhibitors, there was no cumulative effect, showing that each of these inhibitors acted via the same pathway. Thus there was no evidence for SP-A endocytosis independent of clathrin. The observation that an intact actin cytoskeleton plays an important role in the clathrin-mediated uptake of SP-A by pneumocytes supports several recent reports that actin assembly has a critical role in clathrin-coated vesicle formation and receptor-mediated endocytosis (28, 54). The results add emphasis to several reports that found an important contribution of actin assembly to coated pit-dependent uptake processes (9, 21, 28).

The SP-A used in this study was isolated from alveolar proteinosis patients because of the limited amounts of SP-A available from the lavage of rat lungs. Previous studies have shown no qualitative differences in binding rat vs. human SP-A
by rat alveolar cells in vitro (55). Several reports from our laboratory and others have shown that iodination of purified SP-A retains its biological activity as assessed by its ability to inhibit phosphatidylcholine secretion by type II cells (7) and to induce chemotaxis of macrophages in vitro (48), thereby making it a convenient marker to study uptake in the present experiment.

Because phospholipid uptake occurred via a clathrin-dependent pathway and lipids are not known to employ any receptors, it was hypothesized that phospholipid transport utilizes SP-A receptors (38). Our present study supports the suggestion that SP-A could facilitate internalization of the SP-A-lipid complex through the clathrin-mediated pathway. The pathway of SP-A incorporation into type II cells was not affected by the presence of surfactant isolated from rat lung or Survanta, suggesting that the mechanism of SP-A uptake was not influenced by the presence of lipid. Phospholipid uptake also utilized a non-clathrin, actin-dependent pathway. It was possible that SP-A might bind to surfactant phospholipids and be carried into the type II cells via this non-receptor-mediated pathway. That this might be the case is demonstrated by the slightly greater, but significantly different, inhibitory effect of CytoD over amantadine on SP-A uptake in the presence of surfactant.

Available evidence indicates that alveolar macrophages make a major contribution to the degradation of SP-A (5, 22, 49). SP-A uptake by alveolar macrophages is mainly via the clathrin-coated pit pathway (2). Because phagocytosis by alveolar macrophages occurs through clathrin-mediated processes (33), our study using the inhibitors of clathrin (amantadine and PAO) on the metabolism of SP-A by alveolar macrophages in culture cannot differentiate between coated pit- and phagocytosis-dependent pathways. However, as with type II cells, amantadine and PAO significantly inhibited internalization of SP-A by macrophages. Interestingly, CytoD also reduced uptake by ~60% and had no additive effect with clathrin inhibitors, indicating an important role for actin in coated pit movement in macrophages as well. Once SP-A was internalized, all these agents had a slight, but significant, inhibitory effect on degradation of SP-A. Thus clathrin and actin may play a role in the transfer of SP-A to macrophage-degradative compartments.

Most studies of the trafficking of SP-A have primarily utilized isolated type II cells or alveolar macrophages in cell culture systems that serve as in vitro models of the lung. To more closely approximate the physiological environment in the lung, we have utilized an isolated perfused lung model. The advantages of this system are that the functional and structural properties of the organ are preserved and cell-cell interactions are maintained (25, 38). With this system, whole organ metabolism can be examined under controlled conditions (17). To study the metabolism of SP-A by the isolated perfused lung, iodinated SP-A was instilled intratracheally, and rat lungs were perfused in the presence or absence of inhibitors. The amount of instilled SP-A, representing ~10% of the endogenous SP-A (44), distributed evenly to the lung lobes. Thus we assume that the instilled SP-A mixed with the endogenous pool and, thereby, represents the turnover of SP-A in natural surfactant. After uptake and degradation of SP-A by pneumocytes, the degraded products may remain in the lung or appear in the alveolar space (lavage fluid) or the circulation (perfusion). Therefore, we define total uptake as the sum of intact SP-A associated with lung tissue and degraded SP-A products recovered from lung homogenate, alveolar lavage, and perfusate. As in isolated alveolar cells in culture, uptake of SP-A by intact lung was sensitive to clathrin (Amant, PAO) and actin (CytoD) inhibitors. Addition of inhibitors together failed to produce an additive effect, suggesting that SP-A is internalized mainly via a clathrin-dependent pathway and that actin plays a pivotal role in the clathrin-coated pit mechanism of endocytosis in the lung, as was found for the pneumocytes in primary culture. Thus we can distinguish between SP-A incorporation into the lung and incorporation of surfactant phospholipids, since the latter showed an additive effect of clathrin inhibitors and CytoD (38).

Once SP-A was internalized, the intact lung rapidly degraded the surfactant protein. Control lungs catabolized ~56% of the internalized SP-A during 1 h of perfusion. Although treatment of the lungs with clathrin or actin inhibitors blocked uptake of SP-A, the ensuing degradation of the internalized SP-A, expressed as a percentage of uptake, was only affected by actin inhibition. Thus movement of the internalized SP-A to the degradation compartment seems to require a functioning cytoskeleton. However, treatment with PAO or amantadine reduced SP-A degradation by macrophages, indicating that macrophages are not the only cell type involved in SP-A catabolism in the lung, leaving open the question as to the role of type II cells in this process.

A substantial portion of SP-A uptake (~40%) was insensitive to the effects of clathrin and actin inhibitors in pneumocytes in culture and in the isolated lung. This internalization of SP-A, despite the presence of inhibitors, could be due to the lag time necessary for the inhibitor to reach the critical cells in the lung or to take effect in intracellular compartments of the cells in vivo or in vitro. Other possible explanations include the following: 1) PAO inhibited formation of clathrin-coated pits but allowed internalization of pits that had formed before PAO exposure. 2) Amantadine disrupted clathrin function by preventing budding of the clathrin-coated vesicles. However, SP-A may have bound to membrane invaginations that were inaccessible to the cell-washing procedures. This residual uptake might represent SP-A bound to a trypsin-insensitive site on the cell surface or just inside the cell or SP-A transported utilizing an alternative internalization pathway (see explanation 5). 3) Loss of cytoskeletal integrity by disruption of actin with CytoD might result in nonspecific internalization of SP-A. 4) The pharmacological agents used in this study may not completely inhibit cell processes. 5) It is tempting to speculate that the newly described pathway utilizing uncoated nonclathrin caveolae-independent endocytic vehicles might play a role in the uptake of SP-A (26). Our previous study on the uptake of surfactant-like liposomes showed that the inhibition of the incorporation of lipids into the lung by these same agents also was incomplete. In that study, uptake via fusion of the phospholipid liposomes with the plasma membrane or via exchange of phospholipids was a possibility, which would not be the case with SP-A. Thus the newly described endocytic vehicle pathway might transfer protein and lipid from the alveolar space into the lung.

This study was not designed to address the relative contributions of macrophages and type II cells to the processing of SP-A in the intact lung. Using a nondegradable analog of SP-A as a radiolabeled marker, we observed that ~10% of the instilled SP-A was internalized by the isolated lung in the absence of inhibitors. Although this study was not designed to address the relative contributions of macrophages and type II cells to the processing of SP-A in the intact lung, it does provide support for the hypothesis that SP-A is transported from the alveolar space into the lung by a clathrin-dependent mechanism.

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dipalmitoylphosphatidylcholine or flow cytometry, others found that although macrophages take up liposomes in vivo, type II cells make a greater contribution to the uptake and metabolism of lipids (35, 37). In this study, we found radioactivity associated with macrophages and type II cells after instillation of 125I-SP-A and perfusion of the lung for 1 h followed by isolation of the cells. The data suggest that both cell types take up instilled SP-A. Furthermore, although there is no evidence that type II cells in culture degrade SP-A (5, 27), it is quite possible, even likely, that they contribute to the degradation of SP-A in the lung, because there is abundant evidence that these cells take up SP-A. Type II cells may lose their ability to degrade SP-A under tissue culture conditions utilizing plastic dishes and calf serum, as is the case for several other differentiated type II cell characteristics (6). Gurel et al. (22) concluded that macrophages and type II cells degraded SP-A, inasmuch as radiolabel was found in type II cells and macropages isolated from lungs exposed to SP-A labeled with a marker designed to indicate sites of catabolism. The additional point that type II cells are known to take up and release intact SP-A via retroendocytosis (46) means that our measurement of SP-A uptake by these cells, as well as by the intact lung, may well be an underestimate of the extent of SP-A processing, because we could not quantitate the retroendocytic pathway.

In conclusion, we have shown in this study that 1) SP-A uptake in intact lungs, as well as in alveolar cells in vitro, utilizes a clathrin-mediated pathway, 2) actin polymerization has an important role in the clathrin-mediated process, 3) in contrast to uptake of phospholipid, SP-A is not internalized by an actin-dependent, clathrin-independent pathway, 4) because the uptake of neither SP-A (this study) nor phospholipid liposomes (38) was completely halted by the inhibitors of clathrin and actin, we speculate that alternate pathways, such as the novel caveolin-independent pathway, may play a minor role. Once SP-A is incorporated by the lung, a majority of the SP-A is degraded. Thus the predominant mechanism for the uptake of SP-A by isolated pneumocytes was confirmed by studies using the intact lung.

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REFERENCES

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