Identification and characterization of rodent ABCA1 in isolated type II pneumocytes

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1Children’s Hospital of Philadelphia, 2University of Pennsylvania Medical Center, and 3Institute for Environmental Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104; 4Institute of Pharmacological and Biological Sciences, University of Parma, Parma, Italy; and 5Pfizer, Incorporated, Groton, Connecticut 06340

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Bortnick, Anna E., Elda Favari, Jian-Qin Tao, Omar L. Francone, Muredach Reilly, Yuzhen Zhang, George H. Rothblat, and Sandra R. Bates. Identification and characterization of rodent ABCA1 in isolated type II pneumocytes. Am J Physiol Lung Cell Mol Physiol 285: L869–L878, 2003; 10.1152/ajplung.00077.2003.—ATP-binding cassette transporter A1 (ABCA1) promotes transfer of cholesterol and phospholipid from cells to lipid-free serum apolipoproteins. ABCA1 mRNA and protein expression in primary cultures of rodent type II cells was sensitive to upregulation with 5 μM 9-cis-retinoic acid (9cRA) and 6.2 μM 22-hydroxycholesterol (22-OH). The increase in ABCA1 protein levels was time dependent and was maximal after 16 h of exposure to 9cRA + 22-OH. Inducible ABCA1 was also found in transformed cell lines of lung origin: WI38/Va13, A549, and NIH-H441 cells. Stimulation of ABCA1 in rat type II cells by 9cRA + 22-OH resulted in a four- or fivefold enhancement of efflux of radioactive phospholipid or cholesterol, respectively, from the pneumocytes to apolipoprotein AI (apo AI), whereas cAMP (0.3 mM) had no effect. ABCA1-mediated lipid efflux to apo AI was independent of the surfactant secretion pathway, inasmuch as upregulation of ABCA1 resulted in a reduction of secretagogue-stimulated surfactant phospholipid release. These studies demonstrate the presence of functional ABCA1 in type II cells from the lung.

phospholipid; cholesterol; lung; reverse cholesterol transport; surfactant secretion

CHOLESTEROL COMPRIS 10% of pulmonary surfactant lipid by weight (30). It acts to increase the fluidity of phospholipid fatty acyl chains, thereby modulating the viscosity of surfactant and surface tension within alveoli (44, 54). Epithelial pneumocytes (alveolar type II cells) secrete surfactant lipids and proteins from lamellar bodies, which are characteristic intracellular storage sites (30, 48). De novo synthesis of cholesterol by type II cells contributes little to the pool of cholesterol found in surfactant (23). Instead, it is primarily derived from serum lipoproteins: high-density lipoprotein (HDL), low-density lipoprotein, and very-low-density lipoprotein (24), with the latter delivering the majority of surfactant-incorporated cholesterol (21). Type II cells possess receptors for all three lipoproteins (21, 53) and are capable of cholesterol uptake. However, the mechanism for the removal of cholesterol from alveolar pneumocytes remains to be elucidated.

Cholesterol is thought to be mobilized from peripheral tissues and carried to organs of metabolism by a process described as reverse cholesterol transport (RCT) (for review see Ref. 19). HDL is considered to be the particle that carries cholesterol through plasma to the liver or endocrine tissues. Several HDL-binding proteins have been identified on type II cells, including scavenger receptor class B type I, HB2, and glycosylphosphatidylinositol-anchored membrane metalloproteinase. All three appear to be involved in the HDL-mediated uptake of vitamin E by pneumocytes (32, 58). Although the function of scavenger receptor class B type I has been characterized in adrenal cells and hepatocytes (27, 57), the participation of this or other HDL receptors in RCT from lung tissue has not been evaluated.

ATP-binding cassette transporter A1 (ABCA1) has been identified as a major protein involved in receptor-mediated efflux (2, 26, 43). ABCA1 is a transmembrane protein of ~210 kDa with four domains: two with six hydrophobic membrane-spanning regions for substrate transport and two containing highly conserved ATP-binding cassettes (17). It appears to facilitate the initial step in the formation of HDL, namely, the release of cellular cholesterol and phospholipid to apolipoprotein AI (apo AI) (10, 17), the major protein component of HDL, although other exchangeable apolipoproteins (AII, AIV, CI, CII, CIII, and E) also serve as lipid acceptors (9). It is hypothesized that further acquisition of lipid and enzymatic modification of nascent particles result in mature, spherical HDL with a core of esterified cholesterol and triglyceride (43).

ABCA1 is likely to play a role in the cholesterol metabolism of many cell types, inasmuch as it is found

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in several human, primate, and murine tissues, including cells of the small intestine, liver, brain, and kidney, as well as the lung, where it was reported to be localized to pulmonary macrophages (33, 34). ABCA1 expression is upregulated in macrophages from atherosclerotic lesions, presumably in response to cholesterol enrichment (33, 34). ABCA1 gene expression is highly responsive to 1) liver X receptor (LXR) agonists such as 22-hydroxycholesterol and 27-hydroxycholesterol in combination with rexinoid receptor (RXR) ligands such as 9-cis-retinoic acid (9cRA), a form of vitamin A (47), 2) cholesterol enrichment (20), and 3) cAMP stimulation, which is especially important for murine macrophages (9, 35).

Mutations of ABCA1 result in the HDL deficiency disorders Tangier disease (homozygous state) and familial hypoalphalipoproteinemia (heterozygous state) (8, 11, 35). In patients with Tangier disease, impaired ABCA1 function leads to the accumulation of cholesterol and sterol esters in tissues. ABCA1 dysfunction has been associated with premature coronary artery disease in some individuals (2). Evidence for the importance of ABCA1 in cholesterol homeostasis also comes from gene-targeted deletion of murine ABCA1 (13, 38). These animals exhibit the Tangier disease phenotype of low total cholesterol, low or absent HDL and apo AI, and accumulation of cholesteryl esters trapped in peripheral tissues. In two studies of ABCA1 gene-targeted mice, the Abca1-/- pups exhibit severe respiratory distress, lung congestion, and bronchopulmonary dysplasia. In addition, the Abca1-/- mice showed accumulation of cholesterol in alveolar macrophages and type II pneumocytes in the lungs at 7, 12, and 18 mo of age (38). Marked hypertrophy and hyperplasia of type II pneumocytes were observed. Type II cells were engorged with lipid-rich, membrane-bound vacuoles, giving them a foamy appearance, while normal, surfactant-containing lamellar bodies were absent. On the other hand, lung pathology was not observed on gene targeting of ABCA1 in a different strain of mice (13).

Given the localization of ABCA1 in the lung, a highly vascular organ, and a source of exchangeable apolipoproteins from the lipolysis of chylomicrons, very-low-density lipoprotein (39) and HDL, we hypothesized that ABCA1 was present on pneumocytes and might play an important role in type II cell cholesterol homeostasis. Our study establishes the localization and activity of ABCA1 in alveolar pneumocytes.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), gentamycin, rat immunoglobulin G (IgG), Fetal red, aprotinin, leupeptin, pepstatin, phenylmethanesulfonfyl fluoride (PMSF), 3’5’-cyclic monophosphate (CPT-cAMP), dimethylsulfoxide (DMSO), 22-R-hydroxycholesterol (22-OH), and 9cRA were purchased from Sigma; organic solvents and scintillation fluid from Fisher Scientific (Pittsburgh, PA); [1,2-3H]cholesterol (specific activity 45 Ci/μmol) from NEN Life Science Products; [methyl-3H]choline chloride (specific activity 90 mCi/mmol) from Amersham (Arlington Heights, IL); plastic tissue culture dishes (35 mm) from Costar (Cambridge, MA); Eagle’s minimum essential medium (MEM) and phosphate-buffered saline (PBS) from CellGro (Hersndon, VA); and fatty acid-free bovine serum albumin (BSA) from Intergent (Purchase, NY).

Animals

Specific pathogen-free Sprague-Dawley male rats (Charles River, Wilmington, MA; 200–250 g body wt) and C57/BL6 male mice (Charles River; 5–6 wk of age, 20–25 g body wt) were used.

Cell Preparation and Maintenance

Isolation and culture of primary type II cells. Type II pneumocytes were isolated from pathogen-free male Sprague-Dawley rats (300 g) using intratracheal elastase (3 U/ml) as described previously (5, 16). The cells were separated by filtration and seeded on rat IgG-coated bacteriological plastic plates for 1 h for removal of macrophages. Partially purified type II cells were seeded at 2.5 x 10⁶ cells/35-mm dish and incubated overnight at 37°C in 5% CO₂ in MEM supplemented with 10% FBS and gentamicin. After removal of nonadherent cells, the purity of the type II cells was >90% by modified Papanicolaou stain, and the viability was >98% by vital dye exclusion. The principal contaminating cells were macrophages.

For type II cells isolated from C57/BL6 mouse lungs, we used the Dobbs procedure (5, 16) outlined above or a modification of the procedure described by Warshamana et al. (55) using dispase. Briefly, after isolated lungs were cleared of blood by perfusion through the pulmonary artery, 2 ml of dispase (50 U/ml) were instilled into the lungs, and the syringe was left in place to prevent leakage of dispase. The lungs were placed in a sterile tube containing dispase (1 ml) and incubated for 45 min at room temperature in order for enzymatic digestion to proceed. Lung tissue was separated from large bronchi by mechanical means with forceps and transferred to a petri dish containing 10 ml of DMEM with 0.01% DNase I for 10 min at 37°C. The cells were filtered sequentially through 100-, 40-, and 25-μm mesh and centrifuged at 130 g for 10 min at 4°C. The cell pellet was resuspended in 10 ml of MEM and plated on mouse IgG (0.75 mg/ml)-coated petri dishes at 37°C for 1 h for removal of macrophages. Cells that were not adhered to the dish were collected by panning the dish and centrifuged at 130 g for 10 min. The cell pellet was resuspended in MEM with 10% FBS and seeded on 100-mm cell culture dishes at 37°C for 1 h for removal of fibroblasts. The final cell isolates were seeded on type I collagen-coated 35-mm dishes in Ham’s F-12 culture medium supplemented with 15 mM HEPES, 0.8 mM CaCl₂, 0.25% BSA, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, and 2% mouse serum. After 24 h, most of the cells were attached. The medium was changed to serum-free Ham’s F-12 supplemented as described above. The isolated type II cells were 95% pure as judged by cytokeratin staining and the presence of Nile red-positive vacuoles.

Cell lines. W138/VA13 cells (simian virus 40-transformed human lung fibroblasts), A549 cells (derived from a lung adenocarcinoma of type II cell origin), and NIH-H441 cells (derived from lung papillary adenocarcinoma of Clara cell origin) were obtained from the American Type Culture Collection (Rockville, MD) and grown in MEM supplemented with 10% FBS and gentamicin.
Apolipoproteins

Lipid-free human apo AI was obtained as described previously (25, 37). Apo AI was solubilized in 6 M guanidine-HCl overnight at 4°C. This solution was extensively dialyzed against a buffer of 10 mM Tris-HCl, 0.15 M NaCl, and 1 mM EDTA (disodium), filtered (0.45 μm; Millipore), and stored at 4°C.

Measurement of Cholesterol and Phospholipid Release Into Media

Efflux to apo AI. After removal of the freshly isolated rat type II cells from the IgG plates, two sets of cells were plated overnight in MEM supplemented with 10% FBS containing [3H]cholesterol (2 μCi/dish) or [3H]choline chloride (0.7 μCi/dish). After 18 h, the cells were washed and incubated with 0.2% BSA with or without 22-OH (6.2 μM) in ethanol and 9cRA (5 μM, in DMSO) for 6–8 h. Because the two agents were always present in combination, they are referred to as 9cRA-22-OH. Control cells were incubated in equivalent levels of BSA and diluent. The final concentrations of DMSO and ethanol were <0.01% and <0.025%, respectively. Next, the cells were washed with PBS and incubated for an additional 6 or 18 h in MEM in the presence or absence of human apo AI (10 μg/ml, 1 ml/plate). For termination of the experiment, the media were removed and centrifuged (2,000 rpm, 10 min, 4°C) to pellet detached cells. For cholesterol efflux, radioactivity was determined in 100-μl aliquots of media by liquid scintillation counting. Cell monolayers were washed with PBS, dried, and extracted with isopropanol alcohol, and the [3H]cholesterol was counted. For phospholipid efflux, methanol was added to the cell monolayer, and the cells were scraped from the dish. The cells and the media were extracted (7) with 500 μg of egg phosphatidylycholine (PC) added as carrier. The [3H]phospholipid in the chloroform layer was counted. The amount of radiolabeled lipid (cholesterol or phospholipid) released into the medium was calculated as the percentage of lipid counts in the medium divided by the total counts of lipid in the cells plus the medium. Data from experiments containing apo AI as an acceptor for the lipid released from the cells into the medium was calculated as described in

Secretion stimulated with ATP. Cell monolayers were radioactively labeled and treated with 9cRA and 22-OH as described in Efflux to apo I. For some experiments, the cells then were washed with PBS and incubated for an additional 2.5 or 18.5 h with or without 1 mM ATP. One set of cells was harvested after 0.5 h and served as control for background phospholipid secretion associated with medium change. The 0.5-h background secretion was subtracted from the data. For the time-course experiments, the [3H]phospholipid-labeled cells were incubated for 0.5–16 h without or with 9cRA-22-OH, washed, incubated for 1 h without or with ATP, and harvested. Radioactivity in the monolayers and media was determined as described in Efflux to apo I. The amount of radiolabeled lipid (cholesterol or phospholipid) released into the medium was calculated as described in Efflux to apo I. Data from experiments containing ATP without a protein lipid acceptor (no apo AI) are expressed as percent secretion. Total [3H]phospholipid per dish did not differ between groups whether or not the cells were incubated with LXR/RXR agonists, indicating that these agents did not affect PC synthesis or degradation.

mRNA Isolation and Quantitative Real-Time Polymerase Chain Reaction Analysis

Cells were lysed, and total RNAs were extracted by the single-step method using TriZol (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. Reverse transcription was performed using DNase-treated RNA (1 μg), random hexamers, and superscript II reverse transcriptase (Invitrogen) at 40°C for 50 min. The amount of cDNA generated was measured using real-time polymerase chain reaction (PCR) with the TaqMan Syber green quantitative PCR assay with a sequence detector system (7700 ABI PRISM, Perkin-Elmer Life Sciences) according to the manufacturer’s instructions. Reaction conditions were 95°C for 10 min followed by 40 cycles of amplification (95°C for 15 s and 60°C for 1 min). The level of ABCA1 gene expression was calibrated as gene expression rates and normalized to expression rates for GAPDH (for mouse) or β-actin (for human) retrieved from the same sample. The mouse ABCA1 oligonucleotide primers used in this assay were gggectcaacctttagcaaa (forward) and gagcgaatacttctccca (reverse). The HPLC-purified detection probe was cccacaccttggaagcgttac. The mouse GAPDH primers were gecctcctctgtagaacaacaa (forward) and ggcacaactctcactctcttgc (reverse) and the probe was cagcgccacactaagccca. The human ABCA1 gene primers were gcaccagctggttgagctgtagtatg (forward) and cgagcactctctcactctctgtgc (reverse) and the HPLC-purified probe was acagcacaagcactctctgtgtg. The nuclear actin primers were ctcttcctctctctgtgact (forward) and tcgctactctctcactctgtgtag (reverse), and the probe was cactctctctctctctgtgta.

Antibodies

The methodology for the production of monoclonal antibody (Mab) 3C9 has been previously described (40). Mab 3C9 recognizes ABACA3, a 180-kDa lamellar body membrane protein (40, 60). The antibody identifies the outer membrane of lamellar bodies in rat and mouse lung tissues as well as in cultures of type II cells. Mab 3C9 has been shown to be specific for type II cells and does not label alveolar macrophages or other lung cell types (60). Two anti-ABCA1 antibodies were used in these studies: a rabbit anti-mouse ABCA1 raised against the COOH-terminal end of the murine protein (9) and a rabbit anti-rodent/human ABCA1 purchased from Novus Biologicals (Littleton, CO) (41).

Protein Isolation and Western Blot Analysis

Cellular extracts were lysed in buffer (0.5% NP-40, 10 mM Tris, 1 mM MgCl₂, 1% Triton X-100, pH 7.5), supplemented with protease inhibitors [aprotinin (10 μg/ml), leupeptin (1 μg/ml), pepstatin (1 μg/ml), and PMSF (0.2 mM)], and then centrifuged at 12,000 rpm for 4 min for removal of debris. Supernatants were loaded onto the gel, electrophoresed under reducing conditions on 3–8% NuPAGE Tris acetate gels with Tris-acetate running buffer (Novex, San Diego, CA), and transferred to nitrocellulose membranes as described previously (5, 9). ABCA1 was detected with a rabbit site-directed primary antibody (1:1,000 dilution; Novus Biologicals). Membranes were then incubated with a goat anti-rabbit IgG conjugated to horseradish peroxidase with visualization by enhanced chemiluminescence (ECL Plus, Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

Statistical Significance

Values are means ± SE; n is the number of separate experiments performed. Statistical significance was deter-
RESULTS

Expression of ABCA1 mRNA was detected in isolated primary cultures of rodent type II alveolar pneumocytes. Real-time PCR analysis revealed low levels of ABCA1 mRNA in mouse type II cells 24 h after isolation (Fig. 1). After 8 h incubation with the combination of RXR and LXR ligands 9cRA (5 μM) and 22-OH (6.2 μM), respectively, ABCA1 mRNA levels were increased 6.5-fold (Fig. 1). WI38/VA13 cells, transformed human lung fibroblasts, served as positive controls. In addition, A549 cells, derived from a human lung adenocarcinoma of type II cell origin, contained mRNA for ABCA1 that was significantly induced by 8 h exposure to 9cRA-22-OH (Fig. 1). Finally, on exposure to LXR/RXR agonists under the same conditions, ABCA1 mRNA in NIH-H441 cells, a human transformed Clara cell line originating from the bronchiolar epithelium, was significantly elevated by 4.0-fold (n = 3, real-time PCR performed as for WI38/VA13 and A549 cells).

Western blot techniques were also used to detect expression of ABCA1 protein in type II pneumocytes. After 8 h of treatment with 9cRA-22-OH, a specific band at 220 kDa, consistent with ABCA1 protein, was induced in rat and mouse type II cell cultures to levels comparable to those of the positive control, WI38/VA13 human lung fibroblast (Fig. 2). Inducible ABCA1 protein was also found in A549 cells (data not shown). We characterized the time course of expression of ABCA1 protein in type II cells obtained from rats after exposure to 9cRA-22-OH. On stimulation with the RXR/LXR agonists, ABCA1 protein rose from 3 to 6 h, peaked at 16 h of incubation, and began to fall thereafter (Fig. 3).

Experiments were performed to determine whether the ABCA1 protein was functional and capable of promoting release of cholesterol to apo AI. Isolated rat type II cells were labeled with [3H]cholesterol for 24 h. ABCA1 expression was upregulated in some cells by incubation with 9cRA-22-OH for 6 h followed by a
A

B

Fig. 3. Time course for induction of ABCA1 protein by 9cRA-22-OH in rat type II cells. Type II cells were incubated without or with 9cRA (10 μM)-22-OH (6.25 μM) with 0.2% BSA for 0–24 h; 24 μg of total protein were loaded per lane. A: Western blot of a representative experiment. B: optical density of scanned gels in A. Results were obtained by subtracting optical density of the gels without 9cRA-22-OH from that of the gels with agents at the same time point. Data are representative of 3 experiments, each with similar results.

Further 6 h of incubation with or without apo AI (10 μg/ml). The response of type II cells to an additional regulator of ABCA1, CPT-cAMP (0.3 mM), was also examined, inasmuch as cAMP has been found to be a potent enhancer of ABCA1 activity in several cell types, especially murine macrophages (9). Cells and media were assayed for radioactive lipid (see MATERIALS AND METHODS). Release of [3H]cholesterol from type II cells in the presence of 9cRA-22-OH or CAMP (~2% efflux) did not differ from untreated cells (no treatment, Fig. 4). Incubation with apo AI alone also did not affect cholesterol release. Only the pneumocytes exposed to the combination of 9cRA-22-OH and apo AI demonstrated a significant stimulation of cholesterol efflux (Fig. 4). The lack of effect of CAMP indicated that the low numbers of macrophages in the primary cultures of type II cells (<10%) probably did not contribute to the release of radiolabeled cholesterol into the medium (9). A cell line derived from type II cells, A549 cells, also demonstrated ABCA1-mediated cholesterol efflux. On pretreatment of A549 cells with 9cRA-22-OH, cholesterol release to apo AI was stimulated by greater than fivefold over control values: 0.8 ± 0.2 vs. 3.9 ± 0.3% cholesterol efflux (mean ± SE, triplicate determinations from a representative experiment).

Upregulation of ABCA1 activity in the presence of a cholesterol acceptor also resulted in the depletion of cholesterol mass from the alveolar pneumocytes by 36%. The cholesterol content of type II cells incubated in medium with apo AI plus 9cRA-22-OH for 6 h reduced cholesterol mass from 23.9 ± 2.88 (with apo AI alone) to 15.1 ± 0.5 (SE) μg cholesterol/mg cell protein (with apo AI + 9cRA-22-OH, triplicate determinations from a representative experiment). No cholesterol ester was detected in these cells.

ABCA1 activity has been reported to stimulate apo AI-dependent phospholipid as well as cholesterol efflux (12). To examine phospholipid efflux, pneumocytes were prelabeled overnight with [3H]choline (to label cellular phospholipids) or [3H]cholesterol (to label cellular cholesterol), washed, and incubated with 9cRA-22-OH for 8 h. After 18 h of apo AI exposure, ligands alone had little effect on lipid release into the medium (Fig. 5). The 2% efflux of phospholipid or cholesterol after apo AI addition to the cultures was enhanced four- or fivefold, respectively, from cells exposed to 9cRA-22-OH, evidence that ABCA1 catalyzes the removal of both lipids from type II cells.

Fig. 4. cAMP does not affect apolipoprotein (apo) AI-dependent cholesterol efflux. Type II cells were prelabeled with [3H]cholesterol (2 μCi/dish) for 24 h in medium containing 10% FBS and incubated without additions (no treatment), with 8-(4-chlorophenylthio)-adenosine 3’,5’-cyclic monophosphate (0.3 mM), or with 9cRA (10 μM)-22-OH (12.5 μM) with 0.2% BSA for 8 h. Medium was removed and replaced with apo AI (10 μg/ml) for an additional 6 h. Values are means ± SE; n = 3. *Significantly different from apo AI or 9cRA-22-OH alone (P < 0.05).

Fig. 5. Upregulation of ABCA1 results in release of lipids to apo AI from type II cells. Type II cells were radiolabeled with [3H]choline (0.7 μCi/dish) or [3H]cholesterol (2 μCi/dish) for 24 h in medium containing 10% FBS, and radiolabeled cells were incubated with 9cRA (5 μM)-22-OH (6.2 μM) for 8 h in medium containing 0.2% BSA. Medium was removed and replaced with medium containing the same compounds in the presence or absence of apo AI (10 μg/ml) for an additional 18 h. Efflux that occurred in the absence of any additions (medium alone) was subtracted from the data. In medium alone, phospholipid efflux was 6.4 ± 0.3%. Values are means ± SE; n = 3. *Significantly different from apo AI alone (P < 0.05).
Type II cells are capable of basal and secretagogue-stimulated release of surfactant lipid, composed predominantly of phospholipid, from lamellar bodies. In the assay of apo AI-mediated ABCA1 activity of type II cells, efflux of lipid into the medium in the absence of added agents, probably due to surfactant secretion, occurred under all experimental conditions and was subtracted from the data (Fig. 5). However, to ensure that the lipidation of apo AI on upregulation of ABCA1 was not due to effects on surfactant secretion, the specific effects of 9cRA and 22-OH on secretagogue-induced phospholipid and cholesterol release were examined. The effects of secretagogues on surfactant secretion from type II pneumocytes in culture are routinely measured after 1 or 2 h of incubation, inasmuch as the secretory process responds rapidly and changes are readily demonstrable over that time period (48). ATP is a potent stimulator of type II cell secretion. As an agonist for P2 purinoceptors, ATP acts via several signaling cascades, resulting in the activation of protein kinase C, calcium mobilization, and movement of the lamellar body to the plasma membrane, followed by formation of a fusion pore and release of surfactant from the lamellar body to the extracellular milieu (48).

Rat type II cells prelabeled with [3H]choline or [3H]cholesterol were incubated in medium alone or with 9cRA-22-OH for 6 h and then incubated without or with the secretagogue ATP (1 mM) for 2 h. Cholesterol release was not significantly affected by any treatment after this brief incubation time (Fig. 6A). For phospholipid secretion, basal phospholipid release after 2 h of incubation was not affected by 9cRA-22-OH. Interestingly, the 4.3-fold stimulation of phospholipid secretion, due to the action of ATP, was significantly reduced by a prior exposure of the alveolar cells to 9cRA-22-OH (Fig. 6A). Addition of apo AI (10 μg/ml) to the medium did not affect control or stimulated phospholipid secretion whether or not ABCA1 was upregulated.

Additional experiments were conducted for 18 h to determine whether the inhibitory effect of 9cRA-22-OH on ATP-induced secretion continued over the time frame utilized in the assays to measure ABCA1 biological activity. After 18 h of incubation, phospholipid and cholesterol release was enhanced by exposure to ATP, with phospholipid demonstrating the greater response (Fig. 6B). Treatment of the type II cells with 9cRA-22-OH had no effect on basal lipid release but significantly reduced ATP-enhanced phospholipid release. The reduction in cholesterol release with exposure to 9cRA-22-OH did not reach statistical significance. Thus the upregulation of ABCA1, due to incubation with 9cRA-22-OH, enhanced apo AI-dependent phospholipid and cholesterol efflux and correlated with the inhibition of secretagogue-induced phospholipid secretion.

Finally, we measured the time course of the effects of incubation with 9cRA-22-OH and the resultant induction of ABCA1 protein (Fig. 3) on the inhibition of surfactant secretion from type II cells over a 16-h period (Fig. 7). [3H]phospholipid-labeled type II cells were incubated for the indicated time, washed, exposed to medium without or with 1 mM ATP for 1 h, and harvested. PC secretion at each time point is expressed as a percentage of maximum secretion, determined by incubation of the cells with ATP at the same time point. Control secretion (~20% of maximum) was not affected by upregulation of ABCA1 by RXR/LXR agonists. However, the presence of 9cRA-22-OH inhibited ATP-stimulated PC secretion in a time-dependent fashion, and, after 16 h of exposure to the agents, ATP was no longer able to stimulate PC secretion above control values (Fig. 7). The extent of the ability of the type II cell secretory pathway to respond to ATP decreased in parallel with the increase in the level of ABCA1 protein, providing supportive data for an interaction between the two events.

DISCUSSION

Alveolar type II cells play an important role in lung function, inasmuch as they produce and secrete surfactant, the lipid-rich material that lines the alveolar surface and serves to maintain surface tension. Although the phospholipid metabolism of type II cells is well described, the mechanisms controlling the turnover of cholesterol have been less extensively examined. ABCA1 has been shown to be important in the removal of cholesterol from many organs, cells, and cell
ABCAl IN TYPE II PNEUMOCYTES

Fig. 7. Time-dependent decrease in the ability of type II cells to respond to ATP on exposure to 9cRA-22-OH. Type II cells were radiolabeled with [3H]choline (0.7 μCi/dish) for 24 h in medium containing 10% FBS, and radiolabeled cells were incubated without or with 9cRA (6 μM)-22-OH (6.2 μM) in medium containing 0.2% BSA for 6–16 h. Cells were then washed and incubated without or with 1 mM ATP for 1 h. Data are expressed as percentage of maximum secretion (secretion with ATP, without 9cRA-22-OH pre-incubation, set equal to 100%). Phosphatidylcholine (PC) secretion with ATP was 6.5 ± 1.1% (n = 8). Values are from 8 experiments performed in duplicate, 2 time points in each experiment. Each data point is the mean and range of 2 experiments or, for 6 h, mean ± SE of 6 experiments. Range bars that are not visible are within the symbols.

lines (for review see Ref. 42). Previous studies demonstrated that mRNA and protein for ABCA1 were present in the lung (36, 56), specifically in alveolar macrophages and the epithelium of small bronchi, with a corresponding expression of ABCA1 protein (56). Using cell culture models, we found that ABCA1 mRNA and protein were expressed in the cells lining the alveolar space that are responsible for the production of surfactant, the type II pneumocytes. Inducible ABCA1 mRNA and/or protein was also detected in two lung cell lines of human origin, A549, a transformed Clara cell line, and NIH-H441, a transformed Clara cell line, a cell type found in small bronchioles.

ABCA1 can be upregulated by various agents. Exposure of type II cells to CPT-cAMP, under experimental conditions that would have resulted in appreciable increases in ABCA1 levels in macrophages (9), showed that this agent did not affect the biological activity of ABCA1 in the pneumocyte cultures. Instead, type II cells seem to be more sensitive to the LXR/RXR heterodimer mode of regulation. The combination of oxysterols and vitamin A regulates the transcription of ABCA1 by activating the nuclear hormone receptors LXR and RXR, respectively (14, 47). Treatment of the type II cells for 6–8 h with 9cRA and 22-OH served to significantly raise mRNA, as determined by real-time PCR, and protein levels, as determined by Western blot techniques. Protein expression was induced in rat type II cells to an extent comparable to that seen on upregulation of ABCA1 in WI38/VA13 transformed human lung fibroblasts. Variable glycosylation (18) and phosphorylation (51) of ABCA1 may account for differences in electrophoretic migration. Elevating ABCA1 protein levels in pneumocytes enhanced the release of phospholipid and cholesterol to lipid-free apo AI, biological activity consistent with an ABCA1-mediated pathway, because an extracellular lipoprotein acceptor was necessary for lipid efflux. After treatment with 9cRA-22-OH, there was no release of lipid over background levels in the absence of lipid-free apo AI. Upregulation of ABCA1 also resulted in a measurable reduction of cholesterol mass when pneumocytes were incubated in the presence of lipid-free apo AI or a source of apolipoproteins such as FCS.

It is clear that LXR/RXR activation enhances the expression of many genes, including other ABC genes such as ABCG family members (for review see Ref. 50). Notably, the mRNA for ABCG5 and ABCG8 has not been found in the lung (6), although ABCG1 message is present (31). Similar to ABCA1, ABCG1 is regulated by sterols in macrophages (31) and in the human intestinal cell line Caco-2 (41). Therefore, it is possible that other LXR/RXR-sensitive genes and their protein products could influence lipid release by type II cells. Nevertheless, ABCA1 is the only ABC protein described that mediates the release of cholesterol and phospholipid specifically to apolipoproteins. ABCA1 is also the only ABC transporter that is known to modulate HDL levels in animals (1, 38) and humans (2). An ABC protein of unknown function, ABCA3, is found in the limiting membrane of lamellar bodies in type II cells (40, 59, 60). A monoclonal antibody to ABCA3, MAb 3C9, labels lamellar body membranes and has been shown to be specific for type II cells in the lung and in primary cell cultures (60). The commercially available anti-ABCA1 antibody (Novus Biologicals) recognizes ABCA1 and several ABCA1-related proteins (by Western blot) and is, thus, not appropriate for studies designed to specifically localize ABCA1. However, in preliminary immunohistochemical studies, we found that anti-ABCA1 antibody-labeled proteins were present in the cytoplasm of type II cells but did not colocalize with MAb 3C9 in lamellar body membranes, providing initial data that the two ATP-binding cassette proteins in type II cells are found in separate intracellular compartments.

The subcellular localization of ABCA1 is important for its function. It is logical to assume that ABCA1 would be located on the basolateral surface of the type II cells to be in contact with apo AI in the plasma or the lymph. Given that we observed release of lipid to apo AI from type II cells in culture, either apo AI was able to reach the ABCA1 protein on the lower cell surface between the cells and the tissue culture dish or ABCA1 has moved to the apical surface of the cultured cells. Inasmuch as type II cells in culture for 24 h on permeable membranes do not form tight junctions, the answer will depend on the development of a monospecific anti-ABCA1 antibody for immunohistochemical studies.

Reports of lung pathology, involving type II pneumocytes in ATP-binding cassette A1-null mice, prompted
the present study, because this protein appeared to be important for the lipid metabolism of these cells (38). We found that induction of the protein in the presence of apo AI affected the efflux of lipid from the type II cells. Such data suggest that the lack of ABCA1 might result in a disruption of cholesterol turnover and precipitate lipid accumulation. Whether the absence of ABCA1 is the primary cause of the foamy appearance of lung type II cells or whether it acts through a secondary effect remains to be determined. Patients with Tangier disease have not been reported to suffer from significant lung disease, although lung autopsy data are sparse and lung function studies on these patients have not been reported (4). Focal areas of disease, as seen in ABCA1-null mice, may not be symmetric, or compensatory mechanisms may have prevented serious disease. Our data on the effects of ABCA1 activity on surfactant secretion indicate that further investigation is warranted.

Because the secretion of surfactant lipid by type II cells is sensitive to many agents (48), it was possible that the combination of 9cRA-22-OH with apo AI was affecting the release of surfactant. Thus parallel experiments were performed comparing lipid release over an 18-h period in the presence of 9cRA-22-OH with a known secretagogue, ATP, instead of apo AI. With secretagogue treatment, phospholipid release from the pneumocytes was substantially greater than that of cholesterol at 2 and 18 h. In addition, elevation of ABCA1 inhibited ATP-stimulated phospholipid secretion. Consequently, surfactant secretion and ABCA1-mediated efflux are two distinct pathways for the removal of lipid from type II cells. To further substantiate this point, cells derived from type II cells that contain few lamellar bodies and, thus, cannot secrete substantial levels of surfactant lipid, A549 cells, demonstrated ABCA1-mediated efflux of lipid to apo AI.

Unexpectedly, upregulation of ABCA1 by 9cRA-22-OH, in the absence of an apolipoprotein lipid acceptor, resulted in the inhibition of secretagogue-stimulated release of surfactant phospholipid from pneumocytes. Although the mechanism responsible for this result is unknown, two functions of ABCA1 provide potential explanations: its effects on the cytoskeleton and transport of phosphatidylserine (PS). It was of interest that ABCA1 has been found to alter actin organization (52), a cell function that is believed to play a role in the process of surfactant release from type II cells (for review see Ref. 48). Hence, rearrangement of the actin cytoskeleton may alter the movement of lamellar bodies. Furthermore, it is possible that the transfer of PS to the outer leaflet of the plasma membrane, due to the PS flippase activity of ABCA1, affects fusion of the lamellar body to the plasma membrane, inasmuch as the location of PS is known to affect membrane curvature (46). Of course, the prospect of a direct effect of 9cRA and/or 22-OH on portions of the secretory pathway also should be considered. Our results obtained with primary cultures of type II cells raise the intriguing possibility that ABCA1 activity might play a role in the regulation of surfactant secretion in the lung.

The observation that ATP-binding cassette A1, a protein so vital for HDL synthesis, is present and active in lung tissue suggests that the lung may contribute to HDL formation. The lung represents 30% of the vascular bed of the body, with type II and type I cells in close juxtaposition with the circulation (15). Serum lipoproteins are taken up by the lung, as demonstrated by several groups who examined the uptake and/or degradation of 125I-labeled lipoproteins by the isolated, perfused rat lung (24, 45). Lipases (22, 27) and phospholipid transfer protein (29) are present in the lung and are capable of remodeling lipoproteins, possibly releasing lipid-free or lipid-poor apolipoproteins in the process (39) and rendering them available for interaction with ABCA1 on type II or type I pneumocytes. Type II pneumocytes demonstrate plasma membrane receptors for serum lipoproteins (21, 32, 58). Intriguingly, 27-hydroxycholesterol, an LXR ligand shown to upregulate ABCA1 in macrophages (20), is predominantly of pulmonary origin (3). If the lung is, in fact, a source of HDL, then our data also have interesting pharmacological implications in terms of LXR/RXR activation of pneumocytes.

In conclusion, this study demonstrates that the alveolar pneumocyte is another cell type that participates in RCT. ABCA1 was found in type II cells and the A549 type II cell-derived cell line. ABCA1 levels in type II cells were inducible by LXR/RXR nuclear hormone ligands in a time-dependent fashion and were not affected by increases in intracellular cAMP levels. Elevation of ABCA1 resulted in efflux of phospholipid and cholesterol to apo AI, removal of cholesterol mass from the alveolar cells, and reduction of surfactant release. Thus the regulation of cholesterol homeostasis through alterations of ABCA1 expression in alveolar type II cells may make a heretofore unrecognized contribution to the control of surfactant secretion.

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DISCLOSURES
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