Scavenger receptor class B, type I-mediated uptake of A1AT by pulmonary endothelial cells

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1Division of Pulmonary, Allergy, Critical Care and Occupational Medicine, Department of Medicine, Indiana University, Indianapolis, Indiana; 2Division of Nephrology, Department of Medicine, Indiana University, Indianapolis, Indiana; 3Department of Pharmacology, Rush University, Chicago, Illinois; and 4The Richard L. Roudebush Veterans Affairs Medical Center, Indianapolis, Indiana

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Lockett AD, Petrusca DN, Justice MJ, Poirier C, Serban KA, Rush NI, Kamocka M, Predescu D, Predescu S, Petrache I. Scavenger receptor class B, type I-mediated uptake of A1AT by pulmonary endothelial cells. Am J Physiol Lung Cell Mol Physiol 309: L425–L434, 2015. First published June 19, 2015; doi:10.1152/ajplung.00376.2014.—In addition to exerting a potent anti-elastase function, α-1 antitrypsin (A1AT) maintains the structural integrity of the lung by inhibiting endothelial inflammation and apoptosis. A main serpin secreted in circulation by hepatocytes, A1AT requires uptake by the endothelium to achieve vasculoprotective effects. This active uptake mechanism, which is inhibited by cigarette smoking (CS), involves primarily clathrin- but also caveola-mediated endocytosis and may require active binding to a receptor. Because circulating A1AT binds to high-density lipoprotein (HDL), we hypothesized that scavenging receptors are candidates for endothelial uptake of the serpin. Although the low-density lipoprotein (LDL) receptor-related protein 1 (LRP1) internalizes only elastase-bound A1AT, the scavenger receptor B type I (SR-BI), which binds and internalizes HDL and is modulated by CS, may be involved in A1AT uptake. Transmission electron microscopy imaging of colloidal gold-labeled A1AT confirmed A1AT endocytosis in both clathrin-coated vesicles and caveolae in endothelial cells. SR-BI immunoprecipitation identified binding to A1AT at the plasma membrane. Pretreatment of human lung microvascular endothelial cells with SR-B ligands (HDL or LDL), knockdown of SCARB1 expression, or neutralizing SR-BI antibodies significantly reduced A1AT uptake by 30–50%. Scardb1 null mice exhibited decreased A1AT lung content following systemic A1AT administration and reduced lung anti-inflammatory effects of A1AT supplementation during short-term CS exposure. In turn, A1AT supplementation increased lung SR-BI expression and modulated circulating lipoprotein levels in wild-type animals. These studies indicate that SR-BI is an important mediator of A1AT endocytosis in pulmonary endothelium and suggest a cross talk between A1AT and lipoprotein regulation of vascular functions.

α-1 ANTITRYPSIN (A1AT) is a serpin synthesized primarily in hepatocytes and then secreted in circulation. Although endothelial cells synthesize other serpins, they do not produce A1AT, but take it up from circulation, as shown for both lung and systemic (dermal and human umbilical vein) endothelial cells and in vivo (32, 33). Once internalized, A1AT binds to and inhibits executioner caspases (22) and protects lung endothelial cells against apoptosis, including that induced by cigarette smoke (CS) exposure (2, 41). Intracellular A1AT also inhibits the TNF-α-converting enzyme to reduce TNF-α secretion and sustained proinflammatory responses of lung endothelial cells (21). Both clathrin-coated pits (41) and caveolae (2) were shown to be important for A1AT uptake by the endothelium, but whether a receptor is required for endocytosis remains unknown. Because the intracellular uptake of A1AT is an essential step for vascular protection, identification of such a receptor is of biological and ultimately clinical importance.

A1AT deficiency (AATD) and smoking are the two main risk factors for chronic obstructive pulmonary disease (COPD), a prevalent clinical problem and now the third leading cause of mortality in the U.S. CS not only decreases A1AT antiprotease activity (44) but also significantly inhibits A1AT uptake by endothelial cells in vitro and in vivo (22). The mechanism by which CS inhibits A1AT uptake is not known and its elucidation could lead to interventions to improve A1AT intracellular protective effects.

Scavenger receptors class B, including type I (SR-BI), initially identified to internalize LDL in macrophages (12), bind multiple ligands on diverse cell types and are involved in cellular processes ranging from innate immune response regulation to protection against diseases of the cardiovascular system, central nervous system, and the lung (18, 29, 42, 53). This protection has been attributed to removal of cholesterol from the circulation via transfer of HDL-bound cholesterol esters, conferring on SR-BI a cytoprotective role (1, 13, 45). In addition to native LDL and HDL, SR-BI also binds oxidized and acetylated LDL (1, 37) and mediates vitamin E transport to increase the protection against oxidative stress, such as that induced by CS (50). However, CS exposure may eventually cause oxidation and dysfunction of SR-BI (43), including a downregulation of its expression in the lung (49). Because CS also decreases the uptake of A1AT in the lung, it is conceivable that this effect occurs via downregulation of SR-BI. Also supporting a shared receptor between A1AT and lipoproteins is the recovery of A1AT and oxidized A1AT in complexes of circulating HDL and LDL, respectively (24, 30). In addition to SR-BI, SR-BII is another splice variant of the Scarb1 gene. Whereas both receptors clear cholesterol in association with caveolae (4, 26), SR-BII receptor is the main isoform localized in clathrin-coated pits (9). Using studies in cultured primary lung endothelial cells and in mice, we show that SCARB1-encoded receptors are necessary for the uptake of a significant amount of A1AT by lung endothelial cells via direct interactions with A1AT protein.
MATERIALS AND METHODS

Reagents. A1AT, LDL, and HDL, all purified from human plasma, were obtained from Sigma (St. Louis, MO), as were the majority of chemical reagents, unless otherwise specified. Select experiments were performed or confirmed using pharmaceutical grade human purified A1AT, provided through a grant from Baxter Healthcare (Deerfield, IL). SR-BI neutralizing and immunoblotting antibodies were purchased from Novus Biologicals (Littleton, CO), anti-A1AT was obtained from Bethyl Laboratories (Montgomery, TX), and anti-vinculin was obtained from Calbiochem (La Jolla, CA).

Cell culture experiments. Primary human lung microvascular endothelial cells (HLMVEC) from male and female donors were purchased from Lonza (Walkersville, MO) and maintained in essential basal medium (EBM2) supplemented with 5% fetal bovine serum and growth factors. Primary human pulmonary artery endothelial cells (HPAEC) purchased from Cascade Biologics (Grand Island, NY) were maintained in Medium 200 containing low-serum growth supplement. Primary rat lung microvascular endothelial cells (RLMVEC) were a kind gift from Troy Stevens (University of South Alabama, Mobile, AL) and were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All cell lines were cultured and treated at 37°C in 5% CO2. Before treatment, confluent monolayers of endothelial cells were serum-deprived for 2 h to exclude endocytosis of exogenous A1AT present in fetal bovine serum. Cells were pretreated with purified human LDL (25–200 µg/ml) or HDL (8–50 µg/ml) for 15 min or with SR-BI neutralizing antibody for 30 min before the addition of purified human A1AT (50 µg/ml) for 1 h. For select experiments, A1AT was fluorescently labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR) before treatment. At the end of the experiment, cells were rinsed once with cold PBS and harvested for Western blotting or flow cytometry. For treatment. At the end of the experiment, cells were rinsed once with cold PBS and harvested for Western blotting or flow cytometry. For

Western blotting, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma) containing protease and phosphatase inhibitors. Western blotting, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma) containing protease and phosphatase inhibitors.

Fig. 1. α-1 Antitrypsin (A1AT) association with caveolae and clathrin-coated pits at the plasma membrane of primary rat lung microvascular endothelial cells. Representative transmission electron microscope (TEM) images of primary rat lung microvascular endothelial cells treated with A1AT (A520 = 0.4, 15 min) show A1AT associated with clathrin-coated pits (CCP; A and B) and caveolae (Cav; C) (black arrow) and intracellular (white arrow). Direct magnification, ×120,000–150,000. D: quantification of TEM images showing the number of gold-A1AT particles per vesicle per cell. ns, Not significant.

A1AT coloidal gold labeling and electron microscopy. Colloidal gold was prepared as previously described (35). The gold particles (5–7 nm) were stabilized with A1AT and then diluted with PBS to A520 = 0.4 for cell culture treatments. Endothelial cells grown to 90–100% confluence on 35-mm dishes were exposed to 5- to 7-nm gold-conjugated A1AT for 15 min at 37°C, followed by washing with 1 M Na-cacodylate (3 times), fixation with 1.5% glutaraldehyde, and postfixation with 1% osmium tetroxide, followed by negative staining with 2% uranyl acetate, dehydration (70%, 80%, 95% ethanol 5 min each and 100% ethanol 3 × 5 min), and embedding with 100% EPON 812 (Electron Microscopy Sciences, Washington, PA) as previously described (31). Electron microscopy was performed by the Indiana University School of Medicine Electron Microscopy Center (Indianapolis, IN).

Proximity ligation assay. RLMVEC were cultured on 0.2% gelatin-coated glass coverslips to ~90% confluence before treatment with A1AT (50 µg/ml) on ice. Cells were then fixed with 4% paraformaldehyde (15 min, 24°C), followed by permeabilization using 0.1% Triton X-100 (10 min, 24°C). The Duolink in situ proximity ligation assay (PLA) kit (Sigma) was used according to the manufacturer’s protocol to detect direct A1AT and SR-BI interaction. Briefly, after blocking with Duolink blocking solution, mouse anti-A1AT antibody (0.01 µg/µl) and rabbit anti-SR-BI antibody (0.01 µg/µl) diluted Duolink dilution buffer were added simultaneously to cells overnight at 4°C. The anti-rabbit and anti-mouse PLA probes were then added, followed by probe ligation and amplification. The slides were mounted in Duolink in situ mounting medium with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), and the orange PLA signal was detected using a confocal/2-photon Leica TCS SP8 system (Leica Microsystems, Buffalo Grove, IL) available at the Indiana Center for
Biosological Microscopy (Indianapolis, IN) with a Leica HC PL APO ×40/1.3 NA oil-immersion objective. Images were collected in a sequential illumination mode using 405- and 552-nm excitation laser lines. Fluorescent emission was collected in two HyD spectral detectors with filter range set up to 420–500 nm and 600–650 nm for DAPI and PLA, respectively. Series of sections through the depth of cells (Z stacks) were collected using optimal step size settings (0.35 μm; images comprised 1,024 × 1,024 pixels (92.26 × 92.26 μm). A minimum of three representative areas of each experimental condition were randomly selected for scanning. Z stacks were processed with Imaris 7.7 image analysis and visualization software (Bitplane USA, South Windsor, CT) to extract statistical parameters for PLA-positive spots. The segmented images of nuclei and PLA spots were generated, and the average sum intensity of PLA spots per cell was quantified. At least three fields were averaged for each treatment.

**SR-B knockdown.** The Accell human **SCARB1** small interfering RNA (siRNA) pool (Thermo Scientific Dharmacon, Pittsburgh, PA) was delivered according to the manufacturer’s instructions. Briefly, HLMVEC were grown to 60–70% confluence before the growth medium was replaced with Accell serum-free siRNA delivery medium and siRNA (0.5–1 μM; 72 h). To prevent endothelial cell death, after 24 h, the Accell delivery medium was supplemented with serum-containing growth medium to a final serum concentration of 2.5% for the remaining 48-h incubation. At the end of 72 h, cells were serum-deprived for 2 h in EBM2 medium before treatment with unlabeled or labeled A1AT. SR-BI knockdown was verified by Western blot and by Real-Time PCR using the RT2 qPCR Primer Assay from Qiagen (Valencia, CA).

**In vivo A1AT delivery and CS exposure.** Mouse studies were approved by the Animal Care and Use Committee of the Indiana University School of Medicine. Male and female SR-BI-null homozygous (B6;129S2-**Scarb1**tm1Kri/J) mice and wild-type (WT) mice were acquired from Jackson’s Laboratory (Bar Harbor, ME). Mice were administered intravenous human A1AT (20 mg/kg in PBS daily; from either Baxter or Sigma) or PBS vehicle control. Immediately after A1AT injection, mice were exposed to CS (5 h/day) or ambient air via whole body exposure, for 3 days, using a Teague TE-10 exposure apparatus (Teague Enterprises, Woodland, CA). Research cigarettes 3R4F were obtained from the (former) University of Kentucky Tobacco Research Institute (Lexington, KY). Lung processing was performed as previously described (7).

**HDL and LDL/VLDL measurement.** Plasma was isolated from mouse blood via centrifugation (2,400 g, 10 min, 4°C). Plasma levels of HDL and LDL/VLDL were measured using the Biovision HDL, LDL/VLDL measurement kit (Biovision, Milpitas, CA) according to the manufacturer’s instructions.

**A1AT ELISA.** Plasma was isolated as described above, and levels of human A1AT were measured using the Bethyl human A1AT ELISA kit (Bethyl Laboratories, Montgomery, TX) in diluted plasma samples (dilution factor 10^4) according to the manufacturer’s instructions.

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Fig. 2. Scavenger receptor B type I/II (SR-BI/II) ligands competitively inhibit A1AT uptake by primary human lung microvascular endothelial cells. 

A: representative Western blot (n = 3) of intracellular A1AT in cells pretreated with HDL (at indicated concentrations; 15 min) before A1AT treatment (50 μg/ml; 1 h). Vinculin was used as a loading control. B: the intensity of A1AT immunoblotting was quantified by densitometry, normalized to that of vinculin, and then expressed as fold change compared with the normalized densitometry of cells not treated with HDL. C: quantification of fluorescently labeled A1AT uptake, measured by flow cytometry following similar conditions as in A, and expressed as fold change compared with cells not treated with HDL. Values are means ± SE (n = 3–4). *P < 0.05 vs. cells treated with A1AT alone. D: representative Western blot (n = 3) of intracellular A1AT in cells pretreated with LDL (at indicated concentrations; 15 min) before A1AT treatment (50 μg/ml; 1 h) and of vinculin as a loading control. E: the intensity of A1AT immunoblotting was quantified by densitometry, normalized to that of vinculin, and then expressed as fold change compared with the normalized densitometry of cells not treated with LDL. Values are means ± SE. *P < 0.05 vs. cells treated with A1AT alone. F: quantification of fluorescently labeled A1AT uptake, measured by flow cytometry following similar conditions as in D, and expressed as fold change compared with cells not treated with LDL. Values are means ± SE (n = 3). *P < 0.05 vs. cells treated with A1AT alone.

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Statistical analysis. Statistical analysis was performed using Prism software (Irvine, CA). The differences between groups were compared using unpaired Student’s t-test or ANOVA. Statistically significant difference was set at \( P < 0.05 \).

RESULTS

A1AT association with both clathrin-coated pits and caveolae of pulmonary endothelial cells. Multiple cell types, such as macrophages, pancreatic beta cells, and epithelial and endothelial cells, internalize A1AT. Aldonyte et al. and our laboratory have shown that A1AT uptake by the pulmonary large vessels (2) and microvasculature (41) occurs primarily via caveola- and clathrin-mediated endocytosis, respectively. Whereas these studies relied on functional assays, a precise morphological assessment of the internalization of A1AT in endothelial cells has not been performed. We used transmission electron microscopy (TEM) of RLMVEC treated 15 min with colloidal gold-labeled A1AT and detected the tracer in both clathrin-coated pits (Fig. 1, A and B) and caveolae (Fig. 1C). Quantification of the number of A1AT-gold particles per each vesicle type revealed no statistically significant difference between the utilization of two endocytosis pathways (Fig. 1D), suggesting that lung microvascular endothelial cells utilize both endocytic structures to internalize A1AT.

SR-BI/II ligands competitively inhibit A1AT uptake. Primary HLMVEC were pretreated with HDL or LDL, and A1AT internalization was determined by Western blotting (Fig. 2, A, B, D, and E) and flow cytometry (Fig. 2, C and F). Both lipoproteins inhibited A1AT uptake. Whereas HDL exerted a relatively more potent inhibition, up to \( \sim 40\% \) for 50 \( \mu \)g/ml HDL (Fig. 2C), LDL had a more clear dose-dependent inhibitory effect on A1AT uptake, up to \( \sim 30\% \) for 200 \( \mu \)g/ml LDL (Fig. 2D–F). Similar results were obtained in primary rat lung microvascular cells (data not shown).

SCARB1 knockout decreases A1AT uptake. Lung endothelial cells were treated with siRNA against SCARB1 (up to 1 \( \mu \)M) vs. nontargeted siRNA control, followed by efficiency analysis by real-time PCR (Fig. 3A) and Western blotting (Fig. 3B). The specificity for SCARB1 knockdown was demonstrated by real-time RT-PCR against the closely related scavenger receptor class B family member Scarb2 (CD36), the expression of which was not significantly affected following Scarb1 siRNA treatment (Fig. 3A). SCARB1 knockdown resulted in robust dose-dependent decreases in SR-BI expression (Fig. 3B) concomitant with parallel decreases of intracellular A1AT uptake (Fig. 3, B and C).

SR-BI antibodies inhibit A1AT uptake and pull down A1AT. To limit the availability of SR-BI to interact with ligands, we used a blocking antibody followed by measurement of A1AT intracellular uptake by Western blotting (Fig. 4A) and flow cytometry (Fig. 4B). Titers of 1:250, but not 1:500, of neutralizing SR-BI antibody significantly inhibited A1AT uptake by pulmonary endothelium (Fig. 4, A and B) to levels comparable to those achieved by treatment with HDL or LDL, leading to \( \sim 40\% \) inhibition (Fig. 4B). To determine if A1AT interacts with SR-BI, we used immunoprecipitation of SR-BI followed by immunoblotting for A1AT. Endothelial cells were placed on ice before A1AT treatment to prolong the binding of A1AT at the cell surface. After immunoprecipitation with anti-SR-BI antibody or IgG control antibody, immunoblotting with anti-A1AT antibody showed that A1AT pulls down in association with SR-BI (Fig. 4C). To confirm this interaction, we used a complementary method of detection of protein-protein interactions by proximity ligation assay (PLA). PLA generates a fluorescent signal, detected as orange spots, only when the probes used to detect two proteins bind in close proximity. As negative controls for the assay and the specificity of the

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**Fig. 3.** SCARB1 knockdown inhibits A1AT uptake by primary human lung endothelial cells. A: expression of SR-BI encoding gene SCARB1 and a closely related gene, SCARB2, in human pulmonary artery endothelial cells, measured by real-time PCR following treatment with SCARB1-targeting small interfering RNA (siRNA) or a nontargeting (NT) siRNA control (each at 1 \( \mu \)M; 72 h) and expressed relative to control cells. Values are means ± SE. *\( P < 0.05 \) vs. respective NT siRNA-treated cells. B: representative Western blot of SR-BI and intracellular A1AT in human lung microvascular endothelial cells treated with A1AT (50 \( \mu \)g/ml; 1 h) following the indicated siRNA treatment (72 h). Vinculin was used as a loading control. C: the intensity of A1AT immunoblotting was quantified by densitometry, normalized to that of vinculin, and then expressed as fold change compared with the normalized densitometry of cells treated with NT siRNA. Values are means ± SE. *\( P < 0.05 \) vs. A1AT-NT siRNA-treated cells.
Fig. 4. Anti-SR-B1 antibodies inhibit A1AT uptake and pull down A1AT in primary human lung microvascular endothelial cells. 

A: the intensity of A1AT immunoblotting was quantified by densitometry, normalized to that of vinculin, and then expressed as fold change compared with the normalized densitometry of cells treated with control IgG at the indicated titers. Cells were treated with neutralizing antibodies against SR-BI (a-SR-BI) or isotope control (30 min) before addition of A1AT (50 μg/ml; 1 h). Values are means ± SE (n = 3). *P < 0.05 vs. IgG-treated cells. #P < 0.05 vs. a-SR-B1 (1:500)-treated cells. 

B: comparative efficiency of fluorescently labeled A1AT uptake measured by flow cytometry in cells treated with ligands of SR-BI (HDL and LDL) and blocking antibody against SR-BI and exposed to labeled A1AT (50 μg/ml; 1 h). Values are means ± SE. *P < 0.05 vs. vehicle (Veh)-treated cells.

C: Western blot of A1AT following immunoprecipitation of SR-BI with a specific antibody (a-SR-BI) compared with an isotype control (IgG) in cells untreated (−) or treated (+) with A1AT. Input represents cell lysates before immunoprecipitation. Note a distinct band at the predicted molecular mass of 52 kDa (arrow), above the molecular mass measured by the 50-kDa marker (line) following immunoprecipitation with a-SR-BI, but not with control IgG, in A1AT-treated cells. 

D: proximity ligation assay (PLA) showing interaction of A1AT with SR-BI in endothelial cells treated with A1AT on ice (50 μg/ml; time as indicated) followed by staining with specific antibodies for A1AT and SR-BI. Arrows point to orange spots that indicate protein-protein (A1AT–SR-BI) interaction. 

E: images were quantified to measure the intensity of the spots by A1AT and SR-BI interaction, expressed as average sum intensity per cell. Values are means ± SE. *P < 0.05. White lines were drawn to indicate cell plasma membrane boundaries detected by light microscopy.
interaction, we used conditions lacking antibodies or lacking A1AT, respectively. Using fluorescence microscopy, we detected a time-dependent increase in the amount of orange fluorescence signals, indicating direct intermolecular association of A1AT with SR-BI (Fig. 4D) as indicated by the intensity of the orange spots (Fig. 4E).

Scarb1 requirement for lung A1AT uptake. Purified human A1AT protein (hA1AT) or vehicle control (PBS) was delivered once daily via tail vein injection in mice deficient of Scarb1 (Scarb1−/−) or in WT littermate controls (Scarb1+/+), and hA1AT uptake was analyzed in lung homogenates either at 2 h after the first injection or at 3 days (after 3 daily injections), following exsanguination and rinsing of pulmonary circulation with PBS to remove circulating residual hA1AT. In the lungs of Scarb1 knockout mice, compared with WT mice, there was a marked decrease in hA1AT (Fig. 5, A and B), suggesting decreased hA1AT uptake from the circulation. To ensure an equal amount of hA1AT was injected (intravenously) into mice, we measured, via ELISA, hA1AT concentrations in mouse plasma (at 3 days) and found no significant differences in circulating hA1AT in Scarb1 knockout mice compared with littermate controls (Fig. 5C).

Functional impact of A1AT uptake via SR-BI. To investigate whether the uptake of A1AT by SR-BI is of functional significance, we exposed a separate group of mice to CS (or ambient air control) immediately after intravenous hA1AT administration (or vehicle PBS) for 3 days. As expected, hA1AT inhibited CS-induced inflammation, as measured by TNF-α concentration of the bronchoalveolar lavage fluid (BALF) via ELISA in WT mice (Fig. 6A). However, A1AT did not reduce CS-induced TNF-α in the lungs of mice deficient in Scarb1 (Fig. 6A), which exhibited an ∼10-fold increase in this inflammatory cytokine compared with WT mice exposed to the same short duration of smoking. As a complementary study of the functional impact of A1AT–SR-BI interactions, we measured the lung response to acute injury via Western blotting of the danger-associated molecular pattern (DAMP) molecule high-mobility group protein B1 (HMGB1) in whole lung lysates (Fig. 6B). CS doubled HMGB1 levels in WT mice, but not in Scarb1 knockout mice, indicating that SR-BI may mediate responses to acute CS exposures. Intravenous supplementation of A1AT attenuated CS-induced HMGB1 elevations (Fig. 6B, 3rd vs. 7th lane) in WT mice but had less impact in Scarb1 knockout mice, suggesting that the A1AT effect was in part mediated by SR-BI (Fig. 6B, 5th vs. 6th lane and 6th vs. 8th lane). A1AT administration alone increased HMGB1 above vehicle (Fig. 6B, 1st vs. 5th lane), which may be due to A1AT initiating acute phase response in naïve (CS unexposed) animals. However, implications of HMGB1 modulation on overall lung injury outcomes cannot be concluded from this study, especially in the context of immune system abnormalities in Scarb1-deficient mice (11, 46, 51).

When measuring SR-BI levels in Scarb1-deficient mice and A1AT levels following A1AT supplementation, we noted the expected inhibition of SR-BI expression and A1AT uptake in lungs of CS-exposed WT mice (Fig. 6C). Unexpectedly, hA1AT supplementation increased SR-BI expression in control ambient air-exposed mice and prevented the decrease in SR-BI lung expression in CS-exposed mice (Fig. 6C). This led us to investigate a potential reciprocal modulatory effect of A1AT supplementation on SR-BI function by measuring plasma HDL and LDL cholesterol levels following hA1AT injection. Using ELISA, we noted a trend toward increased HDL (Fig. 6D) and significantly increased LDL (Fig. 6E) levels in plasma at 3 days after hA1AT administration in WT mice. However, these levels did not surpass those of Scarb1 knockout mice (Fig. 6, D and E), a hallmark of this genotype (38) and consistent with SR-BI involvement in the clearance of these lipoproteins.

Fig. 5. Absence of Scarb1 decreases lung levels of intravenously (i.v.) administered purified human (h)A1AT in mice. A and B: representative Western blot (n = 3) of hA1AT and SR-BI in the lungs of wild-type (+/+), and homozygous Scarb1-deficient (−/−) mice 2 h (A) or 3 days (B) after hA1AT injection (20 mg/kg; administered once daily). Mice were humanely killed and exsanguinated, and the lungs were flushed with PBS before collection to remove any remaining intravascular A1AT. Vinculin was used as a loading control. Numerical values indicate the densitometry units of A1AT normalized to vinculin in wild-type vs. Scarb1-deficient mice. C: plasma levels of circulating hA1AT collected before the vasculature was flushed in indicated mice treated with hA1AT as described in B and measured by ELISA. Values are means ± SE. NS, nonsignificant.
DISCUSSION

Our data indicate for the first time that a scavenging receptor encoded by Scarb1 is an important determinant of A1AT endocytosis in the vascular endothelium. Whereas critical protective functions of A1AT against emphysema development are exerted extracellularly by inhibiting neutrophil-released proteinases (15, 28, 48), intracellular vasculoprotective effects contribute to anti-inflammatory and antiapoptotic roles of A1AT in maintaining the structural integrity of the lung (21, 22, 32, 33). Furthermore, we have recently shown that endothelial cells transcytose A1AT following active endocytosis, potentially contributing to its availability in the interstitium in homeostatic conditions (20). These properties may explain protective A1AT effects in systemic diseases characterized by parenchymal inflammation and endothelial cell apoptosis, such as cardiovascular disease (47), human immunodeficiency virus (HIV) infection (40), and diseases of the pancreas such as inflammation (36), diabetes (39, 56), and transplant rejection (54). Therefore, our finding may be of interest for a variety of conditions where optimal A1AT intracellular availability is important to achieve.

Our results enhance the understanding of the mechanisms of A1AT uptake, which in microvascular lung endothelial cells required primarily clathrin mediated with minor contributions of caveola-mediated endocytosis (41). In addition, Aldonyte et al. (2) have shown that in pulmonary artery endothelial cells, A1AT can be internalized via caveolae. We have shown in the present study, using TEM of gold-labeled A1AT, that in primary lung endothelial cells both clathrin and caveolae take up A1AT. This dual localization raised the possibility that A1AT uptake might not be occurring via a single receptor. We therefore focused on the SR-B family of receptors, which can be localized in both clathrin-coated pits and caveolae, and using complementary methods such as competitive inhibition, knockdown, knockout, and neutralization, we have shown SR-B involvement in up to 50% of A1AT intracellular uptake.

The SR-B belongs to the large family of scavenger receptors, which are expressed on multiple cell types and are
involved in host immunity, atherosclerosis, and central nervous system disorders (53). The SR-B, encoded by Scarb1 (which produces both SR-BI and its splice variant, SR-BII) and Scarb2 (CD36), are structurally distinct from other classes of scavenger receptors but are closely related to each other, having two short intracellular tails and an extracellular loop (25). All these receptors bind HDL and LDL, but unlike SR-BI and SR-BII, CD36 does not efficiently mediate cholesterol ester uptake (17). Both SR-BI and SR-BII associate with caveolae to clear cholesterol, whereas only SR-BII has been found to carry out this function via association with clathrin (9). SR-BI is primarily expressed at the cell surface, where it binds and anchors HDL extracellularly, whereas SR-BII is rapidly endocytosed and carries HDL intracellularly (10). The functional inhibition approaches that targeted both SR-BI and II (e.g., with HDL or LDL treatment, Scarb1 siRNA, or Scarb1-deficient mice) achieved a relatively similar degree of inhibition of A1AT endocytosis as specifically targeting SR-BI (e.g., SR-BI neutralizing antibody), suggesting that most A1AT uptake mediated by class B scavenger may occur via SR-BI. SR-BI-mediated A1AT endocytosis, which accounts for 30–50% of its total uptake, is expected to occur via association with caveolae, where this receptor primarily resides. This precise mode of transfer of A1AT from its SR-BI-bound form to intracellular compartments remains to be investigated. We have shown that endocytosed A1AT can have multiple fates, such as transcytosis, as well as intracellular processing (20). Future studies will have to elucidate the fate of A1AT that is endocytosed by SR-BI mechanisms and determine what receptor is responsible for A1AT clathrin-mediated uptake.

Because CS has been shown to decrease the lung expression of SR-BI (49) and that of A1AT uptake, the involvement of SR-BI in A1AT uptake may explain why CS exposure decreases the availability of A1AT for vascular-protective functions and those dependent on A1AT endothelial transcytosis to other lung compartments. Tools to increase SR-BI expression or its efficiency of A1AT uptake may improve A1AT bioavailability and enhance its protective effects in the lung. This paradigm may extend to other conditions, as well. Studies using Scarb1 knockout and overexpressing transgenic mice have demonstrated that SR-BI loss negatively affects cardiovascular disease (5), lung immune function (14), and bone metabolism (23), whereas its ectopic expression is protective against atherosclerosis (3). These vascular protective effects are primarily attributed to the HDL cholesterol clearance by SR-BI expressed on endothelial cells (27, 55). Our results support the contribution of endocytosed A1AT to these protective effects. Indeed, circulating native or posttranslationally modified A1AT is found complexed to HDL or LDL, respectively (24, 30). Whereas oxidized or cleaved A1AT binding to LDL is associated with increased monocyte activation, proinflammatory signaling (16), and atherosclerosis (24), binding of A1AT to HDL has been associated with beneficial functions. For example, some of the A1AT protective properties are extended to HDL, such as inhibition of elastase and prevention of apoptosis of human vascular smooth muscle cells (30). In turn, HDL may confer antiatherogenic properties to A1AT given that decreased A1AT function or abundance is identified in atherothrombotic lesions (8, 19). Our evolving knowledge of the scope of HDL mechanisms further suggests symbiotic functions with A1AT. Studies conducted in HDL-deficient apolipoprotein A-I null mice and in humans with decreased HDL cholesterol demonstrated destructive effects in the lung that include increased inflammation, oxidative stress, airway hyperresponsiveness (52), and decreased lung function (6). Interestingly, our data suggest that A1AT treatment can modulate SR-BI expression and function, through as yet unreported mechanisms. It is therefore important to continue investigations of the complementary functions and interactions of A1AT and HDL/SR-BI, because these may lead to understanding of risk factors and management strategies for both lung diseases, including AATD or emphysema, and cardiovascular diseases.

In conclusion, we have identified a novel mechanism that is responsible for up to 50% of A1AT lung endothelial uptake, which is mediated by the scavenging receptor type B, SR-BI, and is modulated by its ligand, HDL. The A1AT-SR-BI interactions may be of functional importance for the anti-inflammatory and vasculoprotective effects of A1AT.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


