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Thrombin stimulates albumin transcytosis in lung microvascular endothelial cells via activation of acid sphingomyelinase

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Kuebler WM, Wittenberg C, Lee WL, Reppien E, Goldenberg NM, Lindner K, Gao Y, Winoto-Morbach S, Drab M, Mühlfeld C, Dombrowsky H, Ochs M, Schütze S, Uhlig S. Thrombin stimulates albumin transcytosis in lung microvascular endothelial cells via activation of acid sphingomyelinase. Am J Physiol Lung Cell Mol Physiol 310: L720–L732, 2016. First published February 5, 2016; doi:10.1152/ajplung.00157.2015.—Transcellular albumin transport involves caveolae that are abundant in lung microvascular endothelial cells. Stimulation of albumin transcytosis by proinflammatory mediators may contribute to alveolar protein leak in lung injury, yet the regulation of albumin transport and its underlying molecular mechanisms are so far incompletely understood. Here we tested the hypothesis that thrombin may stimulate transcellular albumin transport across lung microvascular endothelial cells in an acid-sphingomyelinase dependent manner. Thrombin increased the transport of fluorescently labeled albumin across confluent human lung microvascular endothelial cell (HMVEC-L) monolayers to an extent that markedly exceeds the rate of passive diffusion. Thrombin activated acid sphingomyelinase (ASM) and increased ceramide production in HMVEC-L, but not in bovine pulmonary artery cells, which showed little albumin transport in response to thrombin. Thrombin increased total caveolin-1 (cav-1) content in both whole cell lysates and lipid rafts from HMVEC-L, and this effect was blocked by inhibition of ASM or de novo protein biosynthesis. Thrombin-induced uptake of albumin into lung microvascular endothelial cells was confirmed in isolated-perfused lungs by real-time fluorescence imaging and electron microscopy of gold-labeled albumin. Inhibition of ASM attenuated thrombin-induced albumin transport both in confluent HMVEC-L and in intact lungs, whereas HMVEC-L treatment with exogenous ASM increased albumin transport and enriched lipid rafts in cav-1. Our findings indicate that thrombin stimulates transcellular albumin transport in an acid sphingomyelinase-dependent manner by inducing de novo synthesis of cav-1 and its recruitment to membrane lipid rafts.

transcytosis; acute lung injury; caveolin-1; acid sphingomyelinase; ceramide

TRANSCYTOSIS THROUGH ENDOTHELIAL cells is increasingly recognized as a salient step in vascular homeostasis that facilitates the extravasation and targeted delivery of endogenous molecules typically >5 kDa (depending on the type of endothelium) such as insulin (5) or immunoglobulins (64) as well as exogenous drugs and nanoparticles (21). In addition, transcytosis has been proposed to play an important role in the regulation of transvascular fluid fluxes by altering interstitial oncotic pressures (50). Endothelial transcytosis occurs characteristically via caveolae and thus requires caveolin-1 (cav-1). One of the first substances that was shown to be actively and specifically transported across pulmonary artery endothelial cell monolayers was albumin (69, 70), which is rapidly transcytosed after binding to the gp60 protein in caveolae (33). Under physiological conditions the transcytotic pathway of albumin transport is regarded as dominating over the paracellular (50) and may also contribute relevantly to increased protein permeability in response to inflammatory stimuli, as suggested by the fact that mice deficient in cav-1 show reduced pulmonary microvascular permeability in the LPS model (51). In addition, studies in the lung and on the blood brain barrier indicate that inflammatory mediators may indeed promote transcytosis (24, 73), yet the significance of endothelial transport under inflammatory conditions and underlying regulatory mechanisms remain to be elucidated.

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Thrombin has been used extensively to study the mechanisms of vascular permeability in pulmonary endothelial cells in vitro and in vivo (8, 48, 76). Thrombin-induced hyperpermeability in endothelial cell culture is mediated via activation of protease-activated receptors (PAR), in particular PAR-1 (8, 47), which causes the recruitment and activation of several heterotrimeric G proteins at the cytoplasmic portion of the receptor (75) and subsequent activation of the small GTPase RhoA. The ensuing formation of stress fibers and destabilization of intercellular junctions finally result in endothelial gap formation (8, 47, 83). Independent of this well-recognized pathway for paracellular protein leak, endothelial transport via caveolae may present an important alternative yet thus far unrecognized pathway underlying thrombin-induced hyperpermeability. Notably, several components of the PAR signaling complex localize to caveolae (38), and thrombin has been shown to stimulate translocation of cav-1 to the plasma membrane in human umbilical vein endothelial cells (66). Given the well-established role of caveolae in transcytosis, we therefore hypothesized that thrombin may stimulate transcytosis in endothelial cells and that this effect may contribute to pulmonary vascular protein leak in response to inflammatory stimuli.

Recently, sphingolipids have emerged as key regulators in the formation and function of caveolae and lipid rafts. Caveolae are enriched in sphingolipids (14), and sphingolipid segregation has been implicated in the biogenesis of caveolae (18). In previous work, we have shown that pulmonary vascular permeability is regulated by acid sphingomyelinase (ASM)-dependent formation of the sphingolipid ceramide from sphingomyelin (22). Notably, exogenous sphingomyelinase also triggers the formation of caveolae-like microdomains in lipid membranes (26). In line with this view, we recently demonstrated that in lung microvascular endothelial cells, ASM activation and subsequent formation of ceramide cause recruitment of key signaling molecules to caveolar lipid rafts including cav-1, endothelial NO synthase (eNOS), and the polymodal receptor potential canonical 6 (TRPC6) (65, 84). ASM activation and ceramide formation are also critically involved in the uptake of Pseudomonas aerugionosa bacteria into lung epithelial cells (23), suggesting that sphingolipid signaling may regulate caveolar composition and formation and facilitate caveolar transcytosis, which may in turn contribute to protein extravasation in response to activation of the ASM-ceramide pathway.

Here, we show that thrombin enriches caveolae in cav-1 and stimulates albumin transcytosis both in human microvascular endothelial cells in vitro and in intact rat lungs. We further demonstrate that both the recruitment of cav-1 to lipid rafts as well as albumin transcytosis triggered by thrombin are regulated by ASM. These data provide evidence that albumin transcytosis is stimulated by inflammatory mediators, identify ASM signaling as the underlying regulatory pathway, and thus describe a novel mechanism that may contribute relevantly to increased protein permeability in lung inflammation.

**METHODS**

**Materials.** All drugs and chemicals used were obtained from Sigma Aldrich (Taufkirchen, Germany) unless noted otherwise; ceramide-specific antibody (78) was from GlycoTech (Kuekels, Germany).

**Cell culture and albumin translocation assay.** Bovine pulmonary artery endothelial cells (BPAEC) were obtained from the American Type Culture Collection (catalog no. CCL-209, Manassas, VA), primary human microvascular endothelial cells of the lungs (HMVEC-L) were from Cambrex Bio Science (catalog no. CC-2527, Verviers, Belgium). The cell lines were used between the 3rd and 10th passages as described before (41). For measurement of endothelial permeability, cells were seeded on Costar Transwell membrane inserts (12-mm diameter, 0.4-mm pore size, polycarbonate membrane, obtained from Corning, Corning, NY) at a density of 40,000 cells/cm² and cultured for 72 h with daily change of medium until reaching confluence. Four hours before addition of thrombin (human α-thrombin; MP Biomedicals; Santa Ana, CA; catalog no. 194918 with lot numbers 6554F and 2134K; 10-1,000 nmol/l with 100 nmol/l corresponding to ~12.8 U/ml or exogenous ASM and subsequent assessment of trans- and paracellular endothelial permeability, growth medium was changed to low-serum medium containing normal medium supplemented with 2% heat-inactivated fetal calf serum.

Measurements of transcellular endothelial protein permeability were performed as recently described (44). In brief, 1 mg/ml fluorescein isothiocyanate (FITC)-labeled bovine albumin was added to the upper chamber of the Transwell insert after 3-h treatment with thrombin or a similar time period in untreated controls. At the times specified, FITC-albumin content of the lower compartment was quantified by fluorescence spectrometry at excitation and emission wavelengths of 485 and 530 nm, respectively. FITC-albumin translocation rate across an empty (cell-free) membrane was defined as 100%, translocation across an untreated control monolayer as 0%. Zero and 100% translocation rates were determined on each experimental day, and measurements were always performed in triplicate. As translocation rates show natural variations between different batches and passage numbers of primary cells, appropriate controls were included for each experiment, and individual experiments were always performed on identical batches and passage numbers. In a subset of experiments, we additionally discriminated between para- and transcellular transport by simultaneous use of FITC-albumin, which is transported via both routes, and tetramethylrhodamine isothiocyanate-dextran (TRITC-dextran, average molecular weight 65–85 kDa), which is only transported via the paracellular pathway because it lacks a receptor cookie. Both FITC-albumin and TRITC-dextran were added to the upper chamber at 1 mg/ml, and at specified time points, 50-μl aliquots from the lower compartment were examined and spectrophotometrically analyzed for FITC and TRITC fluorescence at excitation wavelengths of 485 and 535 nm, and emission wavelengths of 535 and 595 nm, respectively. Albumin and dextran concentrations were calculated based on linear standard curves with FITC-albumin and TRITC-dextran, and a permeability index (PI) was calculated for each tracer as PI [%] = X–c/(m–c)·100, where X and c are the data for the treated and untreated cells, respectively, and m the data for the membrane alone. The net transcytosis of FITC-albumin was then calculated as the difference between the PIs for FITC-albumin and TRITC-dextran.

**Transcytosis assessment by TIRF microscopy.** In an alternative second in vitro approach, transcytosis across endothelial monolayers was assessed by a recently established total internal reflection fluorescence (TIRF) microscopy assay (3). In brief, human lung microvascular endothelial cells were seeded on 12-well coverslips and were used within 3 days after reaching confluence. Cells were serum starved for 4–6 h in low-serum media containing 2% heat-inactivated fetal bovine serum. Prior to TIRF imaging, cells were incubated with either 10 μg/ml Alexa-488-labeled albumin or 25 μg/ml 70 kDa rhodamine-labeled dextran, respectively, in Roswell Park Memorial Institute (RPMI) 1640 media for 10 min at 2°C. Excess dextran or albumin, respectively, was removed by rinsing the coverslip two times in cold PBS+. Warm RPMI 1640 medium was then added to allow for transcytosis, and 10 TIRF videos for 10 healthy cells randomly chosen by nuclear staining (DAPI) were acquired on an Olympus cell TIRF Motorized Multicolor TIRF module mounted on an Olympus IX81 microscope (Olympus, Hamburg, Germany). Samples were
Imaged with Velocity acquisition software at λ = 491 nm for Alexa-488 albumin, and λ = 561 nm for rhodamine-labeled dextran, respectively, at a frame rate of 10 s⁻¹ from 5 to 13 min after warm medium was added. Transcytotic events were quantified off-line in a blinded fashion using a vesicular detection and tracking algorithm custom-written and automated in MATLAB (5), which tracks individual moving vesicles and identifies fusion events with the plasma membrane, thereby allowing for accurate differentiation between actual transcytotic events from both diffusion across endothelial junctions and transient interactions of vesicles with the basolateral plasma membrane (17).

**Western blotting.** Size fractionation of 40 µl probe volumes was performed by SDS polyacrylamide gel electrophoresis (12.5%). Proteins were transferred on nitrocellulose membranes with the semidyed Hoefler system at 0.8 mA/cm² for 75 min. The blots were washed with TBST (Tris-buffered saline and TWEEN 20) buffer and incubated overnight at 4°C with a primary antibody. After washing, secondary antibodies labeled with infrared fluorochromes were incubated for 1 h. Protein bands were detected at λ = 700 or 800 nm with the Odyssey infrared imaging system and quantified by use of the Odyssey imaging software (LI-COR Biosciences, Bad Homburg, Germany).

**Acid sphingomyelinase activity.** ASM activity was determined both in the supernatant of endothelial cell layers and in the cell pellets by infrared imaging system and quantified by use of the Odyssey imaging system. Antibodies labeled with infrared fluorochromes were incubated for 1 h. The volume of supernatants was reduced from 3 ml to 0.1% Triton X-100, pH 5.0) and homogenized. Samples were centrifuged in an ultracentrifuge (150,000 g; 30 min, 10°C), and protein content was determined by protein assay (25). Fractions, pellets were resuspended in 150 µl 85% sucrose, 800 µl 30% sucrose, and 200 µl 5% sucrose solution in 10 mmol/l Tris·HCl buffer and samples were centrifuged overnight (200,000 g, 4°C, 20 h). Samples of 150 µl were taken from the supernatant (cytosolic fraction), and the pellet was resuspended in 100 µl Triton buffer (1% Triton X-100, 10 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 5 mmol/l EDTA) and incubated at 4°C for 60 min. A sucrone density gradient was established by subsequent addition of 100 µl 85% sucrose, 800 µl 30% sucrose, and 200 µl 5% sucrose solution in 10 mmol/l Tris-HCl buffer and samples were centrifuged overnight (200,000 g, 4°C, 20 h). Volatility of 150 µl was taken from top to bottom, yielding eight raft membrane fractions. For pellet fractions, pellets were resuspended in 150 µl PBS.

**Immunohistochemical analysis of cav-1 membrane distribution.** HMVEC-L cells were grown to confluence on gelatinized glass coverslips and stimulated with thrombin or corresponding solvent (0.9% NaCl) controls on the next day. Stimulated cells and unstimulated controls were fixed with acetone/methanol 1:1 at −20°C. After washing with PBS, cells were incubated with rabbit polyclonal anti-cav-1 antibody 250 µg/ml diluted 1:200 (C16360; BD Biosciences, Erembodegem, Belgium) for 45 min at 37°C in a humid chamber. Washing and incubation was repeated with an Alexa Fluor 488 fluorescence-labeled secondary goat anti-rabbit antibody (2 mg/ml diluted 1:400; Molecular Probes, Eugene, OR). Cells were embedded in 1,4-diazabicyclo[2.2.2]octane and visualized at the appropriate wavelength by fluorescence microscopy.

**Fluorescence imaging in isolated perfused rat lungs.** All experiments in isolated lungs were performed in accordance with the “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal Resources, National Academy Press, Washington, DC 1996) after approval by the animal care and use committee of the local government authorities. Isolated blood-perfused lungs from male Sprague-Dawley rats (348 ± 5 g body wt; Charles River Wiga, Sulzfeld, Germany) were prepared as previously described (10, 37). Lungs were continuously pump-perfused at 14 ml/min with autologous heparinized blood at 37°C and constantly inflated with a gas mixture of 21% O₂, 74% N₂, and 5% CO₂ at a positive airway pressure of 5 cmH₂O. Left atrial pressure was adjusted to 5 cmH₂O yielding pulmonary artery pressures of 12 ± 1 cmH₂O.

Isolated blood-perfused rat lungs were positioned under an upright microscope (Axiovert Xavero 100 HD; Zeiss, Jena, Germany) on a custom-built stage and superfused with normal saline at 37°C and covered with Saran wrap to prevent drying or cooling of the lung surface. A microcatheter (Ref. 800/110/100; SIMS Portex, Kent, UK) was advanced via the left atrium and wedged in a pulmonary vein draining a capillary area on the lung surface. Via the microcatheter, membrane-permeant fura 2-AM (5 µM; Molecular Probes), which deesterifies intracellularly into imperative fura 2, was infused continuously for 20 min and selectively loaded to microvascular endothelial cells by maintaining absorptive conditions (35). Subsequently, lung capillaries were perfused with FITC-labeled albumin (1 mg/ml) for 60 min in the presence or absence of thrombin (500 mmol/l) and the ASM pathway inhibitor imipramine (10 µmol/l), respectively.

**Fluorescence imaging of interendothelial gap formation.** HMVEC-L were grown to confluency on Transwells, then treated with either saline (control) or thrombin (500 mmol/l) for 1 h, in the absence or presence of 10 µmol/l imipramine. Monolayers were fixed and stained for F-actin with rhodamine phalloidin, and imaged at λ = 552 nm by a Leica DM4000 microscope equipped with a Quorum WaveFX-X1 Borealis spinning disc confocal system (Quorum Technologies, Guelph, ON, Canada).

**Isolation of membrane microdomains.** Membrane microdomains were isolated with slight modifications as previously described (4, 49). Briefly, cells were grown to confluence in T-175 tissue flasks, stimulated, and harvested by using Accutase for detachment; 2.5 × 10⁶ cells were lysed in lysis buffer (42 mmol/l KCl, 10 mmol/l HEPES, pH 7.4, 5 mmol/l MgCl₂ + proteinase block “Complete”) by sonification. After centrifugation (250 g, 4°C, 5 min), supernatants were centrifuged in a ultracentrifuge (150,000 g, 4°C, 30 min) to separate membranes. An aliquot of 150 µl was taken from the supernatant (cytosolic fraction), and the pellet was resuspended in 100 µl Triton buffer (1% Triton X-100, 10 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 5 mmol/l EDTA) and incubated at 4°C for 60 min. A sucrone density gradient was established by subsequent addition of 150 µl 85% sucrose, 800 µl 30% sucrose, and 200 µl 5% sucrose solution in 10 mmol/l Tris-HCl buffer and samples were centrifuged overnight (200,000 g, 4°C, 20 h). Volume of 150 µl were taken from top to bottom, yielding eight raft membrane fractions. For pellet fractions, pellets were resuspended in 150 µl PBS.

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After washout of intravascular FITC-albumin by local infusion of HEPES buffer, fura 2 and FITC fluorescence in lung venular capillaries was excited at $\lambda = 340$, 360, and 380 nm or 470 nm, respectively, by monochromatic illumination (Polychrome IV; TILL.L. Photonics, Martinsried, Germany). Fluorescence was collected through an achromat objective (UAPON X40 W2/340; Olympus, Hamburg, Germany) and appropriate dichroic and emission filters (FT 425 and BP 505–530 or DCLP 500 and LP 515; Zeiss, Jena, Germany) by a CCD camera (Sensicam; PCO, Kehlheim, Germany), and subjected to digital image analysis (TILLvisION 4.01; TILL.L. Photonics). In every lung, 10–15 individual venular capillaries were viewed at a focal plane corresponding to their maximum diameter (14–30 μm). Images of both fura 2 and FITC fluorescence were recorded in random sequence. Changes in the endothelial Ca$^{2+}$ concentration ([Ca$^{2+}$]i) were calculated from the 340/380 fura 2 ratio and expressed relative to baseline. For endothelial albumin uptake, the mean ratio of FITC-over-fura 2 fluorescence, measured at the isosbestic wavelength $\lambda = 360$ nm, was calculated in capillary endothelial cells for each experiment, and cross-capillary line profiles perpendicular to the main vessel axis were analyzed individually for fura 2 and FITC fluorescence concentration. Preparation of albumin-gold complexes. The production of BSA-gold complexes was performed as described previously (25, 34). In brief, solution A [4.740 ml H2O, 60 ml 1% (wt/vol) HAuCl4 in H2O] was heated to 60°C and quickly mixed with solution B [900 ml H2O, 240 ml 1% trisodium citrate, and 12 ml 1% tannic acid]. In a first concentration step, the gold colloid was gently boiled down to 25% of its original volume, and 25 ml of a 0.2 M sodium phosphate buffer were added to 500 ml of colloidal gold with rapid stirring to adjust pH to 6.1. The minimal amount of BSA that would stabilize the gold against electrolyte-induced aggregation was determined by serial dilutions of a 5% (wt/vol) BSA solution as 0.0634 mg/ml of the concentrated gold solution. The crude complex was then prepared by rapidly mixing 525 ml of concentrated gold solution with 0.675 ml of 5% BSA in 10 mM sodium phosphate buffer. For further concentration, batches of the BSA-gold suspension were centrifuged for 90 min at 34,000 g. The supernatant was discarded, and the final volume of the suspension was reduced by recentrifugation for 100 min to 8–10 ml. After dialysis against Ringer solution (8.0 g NaCl, 0.2 g CaCl2 × 6 H2O, 0.1 g KCl, and 0.1 g NaHCO3 per 1,000 ml H2O, pH 7.4), the concentrate was frozen in liquid nitrogen and stored at −80°C. Before use, the gold solution was warmed to body temperature and passed through 0.2-μm filters (Sertifil Paed, B. Braun, Melsungen, Germany). Five milliliters of concentrated albumin-gold complexes were added to 35 ml of lung perfusate and infused for 30 min into isolated perfused rat lungs in the presence or absence of thrombin stimulation.

Electron microscopy of fixed lungs. Following perfusion with albumin-gold complexes, isolated lungs were fixed by vascular infusion of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.2 M HEPES buffer. After storage of the lungs in the same fixative at 4°C for at least 24 h, tissue blocks were chosen by uniform random sampling for embedding in epoxy resin as described previously (54). Briefly, samples were washed, postfixed in 1% osmium tetroxide for 2 h, rinsed in H2O, and dehydrated in an ascending ethanol series and finally embedded in epoxy resin. Ultrathin sections were cut from these tissue blocks per animal and stained with uranyl acetate and lead citrate. The sections were investigated with use of a LEO 902 transmission electron microscope (Zeiss, Oberkochen, Germany). Test fields for quantification were obtained by uniform random sampling. Whenever a test field contained structures belonging to interalveolar septa, the number of gold particles inside the capillary lumen or inside capillary endothelial cells was determined (46, 53).

Statistics. Unless stated otherwise, all data are presented as means ± SE. Data were analyzed by Mann-Whitney U-test or one-way ANOVA followed by one-sided t-tests. Multiple comparisons were accounted for by the false discovery rate in R2.50 (61). The distribution of gold particles was analyzed by $x^2$ test.

RESULTS

Albumin translocation rate in HMVEC-L and BPAEC. Treatment of HMVEC-L and BPAEC monolayers with thrombin increased the translocation of FITC-albumin from the upper to the lower chamber across the Transwell membrane insert in a concentration-dependent manner (Fig. 1, B and C). In HMVEC-L, albumin translocation rate increased above 100% at thrombin concentrations ≥30 nmol/l and reached ~50% at 500 nmol/l. In principle, albumin translocation reflects a composite readout of the combined effects of passive paracellular leak and active transcytosis of albumin. Translocation rates below 100%, which is defined as the passive diffusion rate across a cell-free membrane and, hence, the maximum possible rate of paracellular leak, may be caused to varying degrees by both paracellular and transcellular transport. Yet rates >100% can only be explained by active transcellular albumin transport and thus signify endothelial transcytosis (Fig. 1A). In contrast, albumin translocation rate in BPAEC did not exceed 50% even at the highest concentrations of thrombin applied (Fig. 1C). Thrombin-induced transcytosis was confirmed by a double-indicator dilution technique, using TRITC-dextran as indicator of paracellular transport, whereas FITC-albumin reflects both para- and transcellular pathways. Following thrombin stimulation, translocation of FITC-albumin exceeded that of TRITC-dextran, thus confirming thrombin-induced transcytosis, which was conserved at a steady rate for at least 3 h as indicated by the near-linear increase of net transcytosis (defined as difference between FITC-PI and TRITC-PI) as a function of time (Fig. 1D). A similar marked preponderance of albumin versus dextran transcytosis across HMVEC-L was evident in an independent second assay based on TIRF microscopy and automated vesicular tracking (Fig. 1E), which also confirmed the stimulation of albumin transcytosis across HMVEC-L in response to thrombin (Fig. 1F). Online video supplements show representative image sequences of Alexa-488-labeled albumin transport obtained by TIRF microscopy at the basolateral endothelial surface in the absence (Supplemental Video S1; Supplemental Material for this article is available online at the Journal website) or presence (Supplemental Video S2) of thrombin stimulation.

Regulation of albumin transcytosis via the acid sphingomyelinase pathway. In HMVEC-L, stimulation with thrombin (500 nmol/l) increased the activity of ASM and ceramide synthase within 5 and 30 min, respectively (Fig. 2, A and C), resulting in an increased content of C16- and C18-ceramide (Fig. 2D). In contrast in BPAEC, thrombin increased neither ASM activity nor ceramide content (Fig. 2, B and E). Extracellular ASM activity in the supernatants of either cell type was not altered (data not shown). To elucidate whether ASM and ceramides contribute to the thrombin-induced transcytosis of albumin, we examined the effects of structurally dissimilar inhibitors of the sphingomyelinase pathway, imipramine and D609, in this setting. In HMVEC-L, both inhibitors markedly attenuated the thrombin-induced increase in albumin translocation (Fig. 3), suggesting that endothelial transcytosis was mediated by ASM. Regulation via ceramide synthase was ruled out, because the ceramide synthase inhibitor fumonisin did not alter thrombin-induced albumin translocation (data not shown). In BPAEC, imipramine or D609 had no effect on thrombin-induced albumin translocation, consistent with the notion that
both albumin transcytosis and activation of the ASM pathway were undetectable in this cell type (data not shown).

To exclude that ASM inhibition reduced albumin translocation by attenuating paracellular permeability, we examined whether the ASM-dependent pathway is additive to the Rho/Rho kinase pathway that regulates thrombin-induced endothelial cell permeability via phosphorylation of endothelial myosin light chain kinase. The specific Rho-kinase inhibitor Y27632 alone reduced the thrombin-induced increase in albumin translocation to a similar degree as the ASM inhibitor imipramine or a highly specific anti-ceramide antibody (cer-Ab) (16). Yet monolayer pretreatment with a combination of imipramine or a highly specific anti-ceramide antibody (cer-Ab) (16). Yet monolayer pretreatment with a combination of Y27632 and imipramine or cer-Ab abrogated thrombin-induced albumin translocation almost completely (Fig. 3A), suggesting that the ASM-regulated pathway was additive to the Rho/Rho-kinase mediated paracellular pathway. In line with this notion, imipramine did not prevent the characteristic formation of interendothelial gaps, a hallmark of paracellular permeability, in response to thrombin (Fig. 3B).

**Endothelial albumin uptake in intact lungs.** To address endothelial albumin transport in intact pulmonary microvessels, we visualized the uptake of FITC-labeled albumin in venular capillaries of the isolated blood-perfused rat lung. Effective thrombin delivery to the microvascular area of observation was verified by concomitant fura 2 ratio imaging, which revealed an increase in endothelial [Ca^{2+}], by 96.9 ± 14.3% from baseline (P < 0.05; n = 5). Whereas little uptake of FITC-labeled albumin was detectable after 60 min of FITC-albumin perfusion in unstimulated lung microvessels, FITC fluorescence accumulated markedly in the vascular wall following thrombin stimulation (Fig. 4A) resulting in an increased FITC-over-fura 2 fluorescence ratio (Fig. 4B). Representative cross-capillary line profiles of fluorescence intensities demonstrate that FITC accumulation in thrombin-stimulated microvessels colocalized with fura 2 loaded endothelial cells (Fig. 4C).

In unstimulated microvessels, endothelial FITC fluorescence intensity did not exceed background values as determined in the adjacent alveolar areas.

The rise in endothelial FITC fluorescence did not result from altered optical tissue properties as indicated by unchanged fluorescence intensities of fura 2 exemplified in Fig. 4C. Enhanced albumin uptake was also not attributable to an
increased microvascular surface area, since diameters of the visualized lung venular capillaries did not change during thrombin infusion. Inhibition of the ASM pathway by imipramine blocked the increase in microvascular FITC-over-fura 2 fluorescence ratio (Fig. 4B), further supporting the notion that thrombin-stimulated endothelial transcytosis of albumin is regulated by ASM.

Electron microscopic analyses of gold-labeled albumin in unstimulated lungs revealed colloidal gold particles either inside the lumen of septal capillaries or within the abundant intracellular vesicles at the luminal side of the endothelium (Fig. 5A). Sixty minutes after thrombin stimulation, uptake of gold particles into endothelial vesicles was markedly increased as exemplified by representative electron microscopic images (Fig. 5B) and quantified by a contingency table analysis (Fig. 5D). Furthermore, in thrombin-simulated yet not in unstimulated lungs, occasional transcytosis of gold particles to the basal membrane of endothelial cells was detectable (Fig. 5C), substantiating the stimulation of endothelial albumin transcytosis by thrombin in the intact lung.

Recruitment of cav-1 into lipid rafts. Since albumin transcytosis occurs via caveolae, we investigated the effects of thrombin on the presence of the signature molecule cav-1 in detergent-resistant membrane fractions (lipid rafts) of cultured HMVEC-L. In the absence of a valid caveolar housekeeping protein to serve as loading control, gels were consistently loaded with equal volumes (20 μl) of the sucrose gradient fractions as previously described (65). Thrombin treatment...
increased the amount of cav-1 in the characteristic lipid raft fractions (fractions 1–4 in Fig. 6, A and B) in a time-dependent manner. Cav-1 recruitment to the endothelial plasma membrane in response to thrombin stimulation was further confirmed by immunohistochemistry (Fig. 6C).

Pretreatment of HMVEC-L cells with D609 or imipramine reduced the thrombin-induced accumulation of cav-1 in lipid rafts (Fig. 7A), suggesting that thrombin stimulates cav-1 recruitment to caveolae via an ASM-dependent mechanism. To examine whether this process depends on recruitment of preformed or newly synthesized cav-1, we blocked protein translation with cycloheximide. Cycloheximide inhibited the thrombin-induced accumulation of cav-1 in lipid rafts indicating that cav-1 recruitment resulted predominantly from de novo protein synthesis (Fig. 7A). This notion was further confirmed by the analysis of total cellular cav-1 levels in whole cell lysates demonstrating an increase in endothelial cav-1 contents after 180 min of thrombin stimulation, which was again sensitive to both cycloheximide and the transcriptional inhibitor actinomycin D (Fig. 7B).

To provide further evidence that ASM can recruit cav-1 into lipid rafts and regulate albumin transcytosis in endothelial cells, we exposed HMVEC-L to exogenous ASM (1 U/ml) for 120 min. ASM treatment caused cav-1 recruitment to lipid rafts (Fig. 8, A and B) and increased albumin translocation rate to >100% (Fig. 8C), thus confirming that ASM activation is able and sufficient to stimulate albumin transcytosis in response to thrombin, presumably by recruiting newly synthesized cav-1 into lipid rafts to form caveolae.

**DISCUSSION**

Here, we demonstrate that the proinflammatory mediator thrombin can directly stimulate endothelial transcytosis of albumin in vitro in both Transwell and TIRF assays as well as in intact lungs as demonstrated by in situ fluorescence microscopy and electron microscopy. Thrombin-induced albumin transcytosis was shown to be mediated by activation of ASM and subsequent formation of ceramide. Activation of the ASM pathway recruits de novo synthesized cav-1 to the plasma membrane, thus providing a functional link between thrombin-stimulated sphingolipid signaling and the activation of a caveolin-dependent trafficking pathway. Since endothelial transcytosis of albumin and other osmotically active solutes alters transmural oncotic pressure gradients, this newly identified regulatory pathway may play a role in protein and fluid extravasation in inflammatory diseases.

**Thrombin-induced endothelial barrier dysfunction.** Thrombin is a protease and a proinflammatory mediator that is considered to play an important pathophysiological role under inflammatory conditions such as sepsis and acute lung injury (6, 19, 31). Therefore, thrombin has been widely used in vitro to study the mechanisms underlying increased endothelial cell permeability in inflammatory diseases (8, 32, 47). Thrombin-induced contraction of endothelial cells in culture depends on calcium and is regulated by activation of Rho kinase resulting in endothelial gap formation and increased paracellular permeability (50, 56, 77). To study cellular mechanisms underlying the thrombin-induced endothelial permeability increase, two fundamentally different methods have been applied in confluent endothelial cell monolayers: either the passage of tracer molecules such as labeled albumin, dextran (55), or transferrin (9) or the transendothelial electrical resistance (TEER) across the monolayer (68, 74) are determined. The latter method provides a high temporal resolution but disregards transcellular transport unless fused vesicles would form a transcellular continuum. The kinetics of tracer molecules, on the other hand,
depends on both para- and transcellular transport, provided that the tracer is actively transported by transcytosis as is albumin. Thus tracer passage per se cannot differentiate between both pathways and has a lower temporal resolution compared with TEER.

Previous studies using TEER in bovine pulmonary artery or human umbilical vein endothelial cells have shown that blockade of the Rho kinase pathway with Y27632 almost completely prevents thrombin-induced gap formation and paracellular permeability (30, 83). In contrast, Y27632 attenuated only approximately one-third of the total thrombin-induced translocation of albumin across HMVEC-L in the present study. These divergent results suggest the existence of an additional mechanism for albumin translocation that is not detectable by TEER and independent from Rho kinase signaling. As we will discuss, the findings presented herein strongly suggest that this additional mechanism is ASM-dependent endothelial transcytosis.

Regulated endothelial transcytosis of albumin. The direct quantification of FITC-albumin translocation across endothelial monolayers allowed for the identification of a novel function of thrombin, i.e., the induction of endothelial albumin transcytosis. Several lines of evidence support this conclusion: 1) In the presence of thrombin, albumin translocation across HMVEC-L markedly exceeded passive albumin diffusion across empty Transwell membranes, a finding that can only be explained by active transendothelial albumin transport. 2) Thrombin-stimulated translocation of FITC-albumin was more pronounced compared with that of TRITC-dextran of similar molecular weight. Since previous studies (12) as well as our own analyses by TIRF microscopy demonstrate that transcytosis of dextran is modest to negligible compared with that of albumin, these data provide further evidence that thrombin stimulates transcytosis. 3) Thrombin-stimulated albumin transcytosis was similarly evident in an independent second assay based on TIRF microscopy and automated vesicular tracking. 4) Stimulation of endothelial albumin uptake in the vasculature of intact rat lungs treated with thrombin, but not in untreated control lungs, was also demonstrated by two independent techniques, real-time fluorescence in situ imaging and electron microscopy of gold particles bound to albumin in perfused rat lungs. With respect to the latter technique, it must be kept in mind that electron microscopic analysis of two-dimensional sections cannot unequivocally differentiate between free-standing cytosolic vesicles and caveolar invaginations still in open contact with the extracellular space. Importantly, however, the combination of real-time fluorescence imaging and electron microscopic analyses in intact lungs strongly suggests that this technical issue does not impact on the conclusion that thrombin stimulation increased the uptake of gold-labeled albumin particles into endothelial vesicles in the perfused rat lung.

Transcytosis of macromolecules is a key function of caveolae. In the endothelium of lung microvessels, macromolecules

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**Fig. 5.** Thrombin-stimulated endothelial uptake of gold-labeled albumin. Representative electron microscope images show pulmonary microvessels of either unstimulated lungs (A) or lungs that had been stimulated for 60 min by thrombin (B; 500 nmol/l). Intracellular vesicles were abundant in endothelial cells of both thrombin and unstimulated lungs (arrows). Yet albumin-bound gold particles with a size of 8 nm were more frequently detected intracellularly (white arrowheads) in thrombin-stimulated lungs compared with untreated lungs where gold particles were mostly localized at the luminal surface of the endothelium (black arrowheads). Abluminal release of gold-labeled albumin into the subendothelial interstitium was occasionally seen in thrombin-treated (C), but never in unstimulated lungs. **D:** Quantitative analysis shows the fraction of intracellular vs. total (intracellular + luminal) gold particles in unstimulated and thrombin-treated lungs. *P < 0.01 vs. unstimulated (data from a total of 4,769 analyzed gold particles in n = 4 lungs). cap, Capillary lumen; alv, alveolar lumen; rbc, red blood cell; EC, endothelial cells; coll, interstitial collagen.
are transported via abundant caveolae from the luminal side to the subendothelial space (60). This process is absent in cav-1-deficient mice lacking caveolae (50). Our present observation that thrombin-stimulated transcytosis coincided with the translocation of cav-1 to the cell membrane is in keeping with both a critical role of cav-1 in endothelial transcytosis and the previously reported dynamic trafficking of cav-1 between different subcellular compartments (15). In endothelial cells, laminar shear stress has been shown to recruit cav-1 to the apical plasma membrane (72), where it is localized to newly formed caveolae (62), consolidating the notion that expression and redistribution of cav-1 are critical regulators in the formation of caveolar microdomains. This concept is in line with the finding that siRNA-induced knockdown of cav-1 reduces the number of caveolae per endothelial cell volume (52), while expression of cav-1 is sufficient to induce de novo biogenesis of caveolae in the erythroleukemic cell line K562 (57) or in SF21 insect cells (39). In the present study recruitment of cav-1 to the apical plasma membrane in response to thrombin was demonstrated by immunoblotting of caveolar membrane fractions and by immunohistochemistry, and is in line with previous findings demonstrating that thrombin stimulates the interaction of cav-1 with transmembrane proteins such as TRPC1 (38). Although not shown directly, we speculate that redistribution of cav-1 to the apical membrane leads to the formation of new caveolae. This notion is substantiated by the fact that changes in albumin transcytosis across the endothelial monolayer were paralleled by respective changes in the recruitment of cav-1 to lipid rafts. Importantly, transcytosis of macromolecules through caveolae is a constitutive and ubiquitous characteristic of endothelial cells (50). It is therefore conceivable that a further augmentation of endothelial transcytotic transport

Fig. 6. Thrombin-induced enrichment of caveolin-1 in detergent-resistant endothelial lipid rafts. A: caveolin-1 in detergent-resistant endothelial membrane fractions. Representative immunoblot of the cytosol (c), fractions 1–8 and pellet (P) in untreated HMVEC-L (control; top) and 180 min after addition of 500 nmol/l thrombin to 2.5×10⁶ cells. B: quantification of caveolin-1 content in fractions 1 to 4 of HMVEC-L stimulated with 500 nmol/l thrombin for different time intervals relative to untreated cells at time zero (control). *P < 0.05 vs. control (n = 3–5 independent experiments each). C: representative fluorescence microscopic images (1:400) of HMVEC-L stained with an anti-caveolin-1 antibody and an Alexa fluorescence-secondary antibody. Cell layers of control and thrombin (500 nmol/l) stimulated cells are shown after 120 min. Representative images were replicated in n = 3 experiments.

Fig. 7. Mechanism of thrombin-induced recruitment of caveolin-1 to lipid rafts. Quantitative analyses show the effect of various inhibitors on the thrombin-induced increase of caveolin-1 in detergent-resistant lipid raft fractions 1–4 (as defined in Fig. 6) of HMVEC-L (A) or in whole cell lysates (B). In each assay, 2.5×10⁶ cells were pretreated with either imipramine (10 µmol/l), D609 (300 µmol/l), or cycloheximide (CHX; 1 µg/ml) for 30 min prior to stimulation by 500 nmol/l thrombin for 180 min and subsequent isolation of the detergent-resistant lipid raft fraction. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. thrombin (n = 3–5 independent experiments each).
ASM largely unclear. Here, we provide several lines of evidence for underlining the activation of endothelial transcytosis remain stimuli has previously been reported, molecular mechanisms inflammatory (28), hormonal (12), or pharmacological (27) by inhibition of either transcription or translation. whole cell lysates, and cav-1 content not only in endothelial lipid rafts but also in findings further support this view expression of cav-1 was shown to coincide with elevated caveolar of cav-1. In line with this view, increased endothelial expres- sion of caveolin-1 is in agreement with our finding that inhibition of the ASM pathway by imipramine did not prevent the characteristic formation of interendothelial gaps in response to thrombin. The parallel induction of both transcytosis and paracellular leak may constitute a specific feature of the inflammatory response and the subsequently emerging vascular leak, as in the absence of inflammatory stimuli para- and transcellular transport pathways seem to counterregulate each other (2, 12, 13). A highly specific ceramide-specific antibody (16) similarly reduced thrombin-induced albumin translocation, and again this effect was additive to the inhibitory action of Y27632. 5) In contrast, inhibition of ceramide synthase activity with fumoni- sin did not block the thrombin-induced albumin transcytosis, thus further supporting the notion that ceramide formation and subsequent albumin translocation were attributable to increased ASM activity. 6) Finally, exogenous ASM stimulated cav-1 recruitment to caveolae and triggered albumin trans- cytosis in HMVEC-L cells.

Taken together, these findings provide strong evidence that thrombin stimulates endothelial albumin transcytosis by ASM-dependent formation of ceramide that in turn promotes cav-1 recruitment to the apical cell membrane. This concept is in line with our recent findings that proinflammatory mediators regulate caveolar composition in an ASM-dependent manner (36, 65, 84). Importantly, activation of ASM and subsequent endo- thelial transcytosis not only may be of relevance in thrombin-stimulated microvessels but may provide a unifying concept regulating transendothelial albumin permeability. E.g., increased transendothelial albumin permeability has been detected in isolated rat lungs exposed to the volatile anesthetic isoflurane (27), which directly stimulates sphingomyelinase activity in cell-free ASM extracts (42).

Regulation of endothelial transcytosis by the ASM pathway. Although stimulation of transendothelial albumin transport by inflammatory (28), hormonal (12), or pharmacological (27) stimuli has previously been reported, molecular mechanisms underlying the activation of endothelial transcytosis remain largely unclear. Here, we provide several lines of evidence for a critical role of ASM in the signaling pathway underlying the thrombin-induced increase in albumin transcytosis: 1) ASM activity increased within 30 min of thrombin stimulation and coincided with elevated ceramide contents in HMVEC-L. 2) Thrombin activated the ASM pathway only in HMVEC-L, but not in BPAEC, and the induction of albumin transcytosis was similarly restricted to HMVEC-L. 3) Two structurally unrelated inhibitors of the ASM pathway, D609 (45, 67) and imipramine (1, 29), blocked cav-1 recruitment to the endothelial membrane and attenuated thrombin-induced albumin transcytosis both in vitro and in intact microvessels of the isolated perfused lung. Notably, in HMVEC-L cells both inhibitors reduced thrombin-induced albumin translocation typically to values ranging between 100 and 150%, with 100% representing free tracer diffusion across the empty Transwell. Values below 100% were only observed when imipramine was combined with a Rho-kinase inhibitor, indicating that the transcyto- totic and the paracellular pathway are additive and act in parallel in thrombin-induced hyperpermeability. This interpre- tation is in agreement with our finding that inhibition of the ASM pathway by imipramine did not prevent the characteristic formation of interendothelial gaps in response to thrombin. The parallel induction of both transcytosis and paracellular leak may constitute a specific feature of the inflammatory response and the subsequently emerging vascular leak, as in the absence of inflammatory stimuli para- and transcellular transport pathways seem to counterregulate each other (2, 12, 13). 4) A highly specific ceramide-specific antibody (16) similarly reduced thrombin-induced albumin translocation, and again this effect was additive to the inhibitory action of Y27632. 5) In contrast, inhibition of ceramide synthase activity with fumoni- sin did not block the thrombin-induced albumin transcytosis, thus further supporting the notion that ceramide formation and subsequent albumin translocation were attributable to increased ASM activity. 6) Finally, exogenous ASM stimulated cav-1 recruitment to caveolae and triggered albumin transcytosis in HMVEC-L cells.

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measure of increased paracellular permeability in the microvasculature (“microvascular leakage”) under both clinical and experimental conditions would no longer be valid, since these techniques fail to differentiate between active transcytosis and passive leakage of albumin. The microvascular effects of thrombin may be a case in point in this respect: in isolated lungs thrombin stimulates albumin extravasation (59) and slightly increases the vascular filtration coefficient (79) but causes no or only minor weight gain in isolated lungs if hydrostatic pressure is maintained constant (76, 80). Although initially seemingly contradictory, these findings may be reconciled if thrombin predominantly stimulated albumin transcytosis in intact lungs, a notion that is supported by our finding that ASM inhibitors prevented the thrombin-induced accumulation of FITC-labeled albumin in lung microvessels almost completely.

In the clinical scenario, albumin solutions are widely used in fluid resuscitation of critically ill patients. The perceived advantages of albumin are effective volume replacement and maintenance of the intravascular colloid osmotic pressure and transport functions that allow for the reversible binding of various substances that are active or toxic in the free form. Our findings give rise to the possibility that albumin may also have undesirable and potentially detrimental effects on the transendothelial oncotic pressure gradient in the critically ill: in these patients, the proinflammatory protease thrombin is commonly activated (20) and may hence propagate albumin transcytosis across the pulmonary endothelium. Increased transport of albumin from blood into the interstitium will reduce or even reverse the absorptive oncotic pressure gradient, thus promoting fluid extravasation and edema formation. This notion is in line with clinical data demonstrating that albumin resuscitation may impair respiratory function in patients with severe hypovolemic shock (81) and may explain the failure of albumin therapy to improve survival in critically ill patients despite its apparent advantages over simple saline infusion (11, 40).

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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