Intercellular Adhesion Molecule 1 Engagement Modulates Sphingomyelinase and Ceramide, Supporting Uptake of Drug Carriers by the Vascular Endothelium

Daniel Serrano, Tridib Bhowmick, Rishi Chadha, Carmen Garnacho, Silvia Muro

Objective—Engagement of intercellular adhesion molecule 1 (ICAM-1) on endothelial cells by ICAM-1-targeted carriers induces cell adhesion molecule–mediated endocytosis, providing intraendothelial delivery of therapeutics. This pathway differs from classical endocytic mechanisms and invokes aspects of endothelial signaling during inflammation. ICAM-1 interacts with Na\(^+\)/H\(^+\) exchanger NHE1 during endocytosis, but it is unclear how this regulates plasmalemma and cytoskeletal changes. We studied such aspects in this work.

Methods and Results—We used fluorescence and electron microscopy, inhibitors and knockout tools, cell culture, and mouse models. ICAM-1 engagement by anti-ICAM carriers induced sphingomyelin-enriched engulfment structures. Acid sphingomyelinase (ASM), an acidic enzyme that hydrolyzes sphingomyelin into ceramide (involved in plasmalemma deformability and cytoskeletal reorganization), redistributed to ICAM-1-engagement sites at ceramide-enriched areas. This induced actin stress fibers and carrier endocytosis. Inhibiting ASM impaired ceramide enrichment, engulfment structures, cytoskeletal reorganization, and carrier uptake, which was rescued by supplying this enzyme activity exogenously. Interfering with NHE1 rendered similar outcomes, suggesting that Na\(^+\)/H\(^+\) exchange might provide an acidic microenvironment for ASM at the plasmalemma.

Conclusion—These findings are consistent with the ability of endothelial cells to internalize relatively large ICAM-1-targeted drug carriers and expand our knowledge on the regulation of the sphingomyelin/ceramide pathway by the vascular endothelium. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: acid sphingomyelinase ■ actin cytoskeleton ■ CAM-mediated endocytosis ■ ceramide/sphingomyelin pathway ■ ICAM-1
cles. This contributes to formation of membrane engulfing structures and invaginations, leading to internalization of objects ranging from ~180 nm to ~5 μm in diameter, providing a remarkable flexibility for design of drug carriers. It is likely that uptake of these drug carriers by ECs requires not only specialized cytoskeletal structures but also lipid platforms providing reduced diffusion of the involved elements (ICAM-1, NHE1, the cytoskeleton, and signaling molecules). This has been observed during engagement of ICAM-1 by leukocyte β2 integrins, which also involves protein kinase C and Src kinase, actin stress fibers, and specialized lipid domains on the EC surface.

However, CAM-mediated endocytosis remains relatively uncharacterized. In this work, we studied the regulatory link between plasmalemma and cytoskeletal elements involved in endocytosis via ICAM-1. We found an unforeseen role for acid sphingomyelinase (ASM) and the sphingomyelin/ceramide pathway in this process, with NHE1 providing a ground for spatial regulation of these events. Our data are consistent with the ability of ECs to internalize relatively large drug carriers bound to ICAM-1, and they also expand the knowledge of the role of the sphingomyelin/ceramide pathway on the function of the vascular endothelium.

Methods

Antibodies and Reagents
Antibodies to human and mouse ICAM-1 were R6.5 and phycoerythrin-LB-2 (Santa Cruz Biotechnology, Santa Cruz, CA) for human and YN1 for mouse. Antibodies to platelet-EC adhesion molecule 1, NHE1, VCAM-1, clathrin, heavy chain, mannose 6-phosphate receptor (M6PR), ASM, ganglioside GM1, and ceramide were from BD Biosciences (Franklin Lakes, NJ); EMD Chemicals (Gibbstown, NJ), Millipore (Billerica, MA), and Sigma-Aldrich (Saint Louis, MO). Secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Polystyrene-latex beads were from Polysciences (Warrington, PA). BODIPY FL C12-phosphatidylcholine and Texas Red–labeled phalloidin were from Molecular Probes (Eugene, OR). Human recombinant ASM was provided by Dr Edward Schuchman (Mount Sinai School of Medicine, New York, NY), and neutral sphingomyelinase (SM) was from Sigma-Aldrich. All other reagents were from Sigma-Aldrich.

Cell Cultures
Human umbilical vein endothelial cells (HUVECs) from Lonza (Walkersville, MD) were cultured in supplemented M-199 medium. Mouse lung ECs were isolated from wild-type C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) or ASM−/− mice (provided by Dr Schuchman) using magnetic beads coated with anti-platelet-EC adhesion molecule 1, and cultured in supplemented Dulbecco’s modified Eagle’s medium. Cells were seeded on 1% gelatin-covered coverslips and treated overnight with 10 ng/mL tumor necrosis factor-α to induce ICAM-1 overexpression.

Preparation of Polymer Carriers Targeted to Cell Surface Markers
Model carriers were prepared by adsorbing antibodies to ICAM-1, VCAM-1, or M6PR on the surface of 4.5-μm-diameter (or 100 nm, when indicated) polystyrene, as described.6,9,27,28 rendering ~5000 (or ~7000) antibody molecules/μm2, equivalent to ~320000 (or ~250) antibody molecules/particle. Alternatively (when indicated), 4.5-μm carriers were prepared using a 50:50 mass-ratio mix of anti-ICAM and IgG or recombinant ASM (~2500 anti-ICAM molecules/μm2).

Enrichment of Lipids at Sites of ICAM-1 Engagement on the Endothelial Plasmalemma
Anti-ICAM carriers were incubated for 15 minutes at 37°C with control HUVECs or HUVECs treated with 5 mmol/L methyl-β-cyclodextrin (to remove cholesterol), 50 μmol/L imipramine (to inhibit ASM), or a mix of 50 μmol/L imipramine and 500 μU/mL neutral SM (to inhibit ASM while providing SM activity). Nonbound carriers were washed and cells were fixed. Carrier engulfment by ECs was verified by scanning electron microscopy. In parallel, cholesterol was stained using 50 μg/mL filipin, and sphingomyelin was visualized by preincubating HUVECs with 0.2 μg/mL BODIPY-sphingomyelin.9 Ganglioside GM1 and ceramide were immunostained. Cell-bound carriers were identified by phase-contrast microscopy, and enrichment of molecules at these sites was quantified as the fold increase of fluorescence intensity at engulfment structures (~2 μm above the cell surface) over that of adjacent regions, as described in the online-only Data Supplement.

Recruitment of Proteins at Sites of ICAM-1 Engagement on the Endothelial Plasmalemma
Anti-ICAM, anti-VCAM, or anti-M6PR carriers were incubated for 15 or 30 minutes at 37°C with control HUVECs or HUVECs treated with 5 mmol/L methyl-β-cyclodextrin (to remove cholesterol), 50 μmol/L imipramine (to inhibit ASM), and 500 μU/mL neutral SM (to inhibit ASM while providing SM activity), 3 mmol/L amiloride or 20 μmol/L EIPA (to inhibit NHE1), 0.5 μmol/L wortmannin (to inhibit phosphatidylinositol 3-kinase), or 10 μmol/L 1–(5-isquinolinylsulfonyl)-2-methyl-piperazine (H-7, to inhibit protein kinase C). Anti-ICAM-coat on carriers was detected by immunofluorescence. ASM, ICAM-1, NHE1, M6PR, and VCAM-1 were also immunostained for fluorescence microscopy. Semiquantitative analysis of the enrichment of these molecules at sites of carrier binding was performed as described above.

The intracellular distribution of ASM was assessed by computing the total number of ASM-positive vesicles (~100–300 nm diameter) and those located within 5 μm of the nucleus (perinuclear). Perinuclear intracellular ASM was calculated as the number of total minus perinuclear ASM-positive vesicles. Coprecipitation of ASM, α-actinin, and moesin with ICAM-1 was assessed by incubating HUVECs with anti-ICAM magnetic beads for 15 minutes at 37°C as described, followed recovery of bound beads, protein elution and separation by electrophoresis, and liquid chromatography/mass spectrometry.

Actin Remodeling and CAM-Mediated Endocytosis
Formation of filamentosous actin (F-actin) on binding of anti-ICAM carriers to HUVECs over a period of 15 minutes at 37°C, was visualized by fluorescence microscopy using Alexa Fluor 594–phalloidin.

For endocytosis in cell cultures, HUVECs, wild-type mouse lung ECs, or ASM−/− mouse lung ECs were incubated at 37°C with anti-ICAM carriers for 30 minutes to allow binding, followed by washing of nonbound carriers and incubation at 37°C for 1 hour to allow endocytosis. Similar tests were performed in 3 mmol/L amiloride, 50 μmol/L imipramine, 0.5 μmol/L wortmannin, 10 μmol/L H-7, or Na+–depleted solution (138 mmol/L choline chloride, 5.4 mM KCl, 1 mM CaCl2, 1 mL/L MgCl2). Endocytosis of carriers displaying 50% anti-ICAM surface density was also tested, as well as the effect of coocating carriers with both anti-ICAM and recombinant ASM, to rescue this activity. After cell fixation, carriers accessible at the cell surface were counterstained using Texas Red–labeled secondary antibody, and the number of carriers internalized per cell was quantified by phase-contrast (total carriers) and fluorescence (surface-located carriers) microscopy, as described.

C57BL/6, caveolin−/−, and ASM−/− mice were anesthetized intraperitoneally with 100/10 mg/kg body weight ketamine/xylazine and injected intravenously with ~180 nm anti-ICAM carriers (in-
stead of 4.5-μm counterparts that may cause embolization). Intracardial perfusion 3 hours after injection was used to remove circulating carriers and carriers loosely bound the vasculature, and lungs were processed into 80- to 90-nm-thick resin-embedded sections to visualize the pulmonary endothelium by transmission electron microscopy. Endocytosis of anti-ICAM carriers by pulmonary ECs in vivo was semiquantitatively assessed as the number of EC internalized carriers per field, as described. All animal studies conformed to institutional animal care and use committee regulations.

Statistics

Data are means±SEM. Statistical significance was determined by the Student t test. Additional descriptions of methods are provided in the online-only Data Supplement.

Results

Visualization of Lipid Components at Sites of Engagement of Anti-ICAM Carriers by ECs

To visualize the cell surface and enrichment of particular molecules during the initial steps of CAM-mediated endocytosis, we looked at the interaction of 4.5-μm anti-ICAM carriers with the EC plasmalemma. Fluorescence microscopy showed that within 15 minutes, anti-ICAM carriers bound to ECs and were engulfed (confirmed by scanning electron microscopy; Figure 1A) by ICAM-1-enriched membrane protrusions (Video I in the online-only Data Supplement; Figure 1B).

Next, we visualized lipid-raft components, because ICAM-1 cross-linking with antibodies elicits its association with detergent-resistant membrane fractions. Cholesterol, sphingomyelin, and ganglioside GM1 were enriched by 1.5±0.05-fold, 2.5±0.1-fold, and 3.2±0.1-fold, respectively, in regions of carrier engulfment versus adjacent “background” areas (Figure 1C; Figure IA in the online-only Data Supplement). Cholesterol removal by methyl-β-cyclodextrin (Figure 1D, top panels; Figure IB in the online-only Data Supplement) impaired engulfment, reflected by reduced ICAM-1 signal around carriers (39.8% reduction; Figure 1D, top panels; Figure III in the online-only Data Supplement). NHE1 (a linker between CAM-mediated endocytosis and the cytoskeleton) in the first stages of anti-ICAM carrier engulfment. Neither methyl-β-cyclodextrin nor amiloride inhibited ICAM-1 signal at carrier-binding sites. This finding was validated in vivo after intravenous injection of anti-ICAM carriers (180-nm, to avoid potential embolization by 4.5-μm carriers) in wild-type, caveolin-1−/−, or ASM−/− mice, followed by visualization and semi-quantitative estimation of carrier endocytosis by pulmonary ECs (a preferential target for anti-ICAM carriers) using transmission electron microscopy, as recently described.

In agreement with cell culture experiments, endocytosis of...
anti-ICAM carriers was inhibited in ASM−/− mice but not in caveolin-1−/− mice (12.2% and 92.8% of wild-type mice, respectively; Figure 2B). This verifies that ASM is required for endocytosis of anti-ICAM carriers by the vascular endothelium.

**Recruitment of ASM, Ceramide Generation, and Role of NHE1 at Sites of Engulfment of Anti-ICAM Carriers by ECs**

We then determined whether ASM is involved in CAM-mediated endocytosis through ceramide generation at sites of carrier engulfment by ECs. Immunofluorescence showed that in the absence of anti-ICAM carriers, most ASM localized in the perinuclear region of cells, with a fraction located to vesicular-like structures (41.8±4.7 vesicles/cell; Figure IV in the online-only Data Supplement) and few ASM-positive vesicles found outside this perinuclear area (23.7±3.3 vesicles/cell). ICAM-1 engagement by anti-ICAM carriers induced appearance of ASM-positive vesicles at the cell periphery (2.3-fold increase at 30 minutes), and carrier engulfment areas became enriched in ASM (Figure 3A). Confirming the specificity of this, ASM was not recruited to sites of carrier binding to VCAM-1, another CAM involved in inflammation, or M6PR, involved in clathrin-mediated transport of ASM (Figure 3A). Anti-M6PR carriers induced recruitment neither ICAM-1 nor NHE1, but they did recruit clathrin heavy chain (Figure V in the online-only Data Supplement), validating this model.

ASM staining colocalized with ICAM-1 at areas of anti-ICAM carrier engulfment (85.1±2.9% of carriers; Figure 3B), where ASM appeared within ICAM-1-lined vesicular structures (Figure 3B, 16×). However, opposite to α-actinin or moesin, ASM did not communoprecipitate with ICAM-1 (Table II in the online-only Data Supplement). This suggests that on release from intracellular vesicles, the enzyme simply interacts with sphingomyelin at neighboring sites of the plasmalemma rather than ICAM-1.

Importantly, ceramide also became enriched at these sites (3.5±0.01-fold over adjacent regions; Figure 4A; Figure VIA in the online-only Data Supplement), which was impaired by inhibiting ASM with imipramine (23.6% decrease), and rescued by exogenously added neutral SM (92% of control). Similarly, EPA (an amiloride derivative that specifically inhibits NHE1) also reduced ceramide enrichment (35.6% decrease), suggesting that this ion exchanger, which mediates H+ efflux, may provide the acidic environment for ASM activity at the plasmalemma. Indeed, ASM staining also colocalized with NHE1 (85.3±3.4% of carriers; Figure 4B), and both ASM inhibition with imipramine and NHE1 inhibition with EPA affected similarly carrier engulfment (37.9% and 36.6% decrease, respectively; Figure 4C; Figure VIB in the online-only Data Supplement), which was rescued by exogenous neutral SM (87.8% of control). Furthermore, Na+ depletion (to impair Na+/H+ transport) led to a 93% reduction in CAM-mediated endocytosis (Figure 2A). This suggests that NHE1 may connect the ASM-mediated sphingomyelin/ceramide pathway to CAM-mediated endocytosis.

**Effect of Impairing ASM on CAM-Mediated Actin Rearrangement**

As described for other systems, ASM-mediated production of ceramide on ICAM-1 engagement may contribute to formation of lipid domains (Figures 1 and 4) and vesiculization, resulting in endocytosis (Figure 2). This pathway is also known to promote cytoskeletal rearrangement, a requirement for CAM-mediated uptake.

In accord with this, ASM inhibition by imipramine hindered formation of actin stress fibers induced by anti-ICAM
carriers (Figure 5A), as previously observed in the case of NHE1 inhibition by amiloride or EIPA.21 Imipramine did not affect ASM recruitment to carrier-binding sites (93.4±5% of control), yet it inhibited uptake of anti-ICAM carriers (Figure 5A), suggesting that ASM recruitment preceded ICAM-1-dependent cytoskeletal rearrangement. Confirming this, inhibition of protein kinase C, which regulates actin stress fiber formation by CAM-mediated endocytosis, did not affect ASM recruitment to ICAM-1 binding sites, but inhibited carrier uptake (Figure 5B). In contrast, these events were independent of phosphatidylinositol 3-kinase (Figure 5B), previously shown to play no role in CAM-mediated uptake.6

Discussion

Binding of anti-ICAM carriers to endothelial ICAM-1 leads to intracellular transport of said carriers via CAM-mediated endocytosis (Table I in the online-only Data Supplement).6,9 This pathway supports delivery of therapeutics into the vascular endothelium by means of carrier particles ranging from ~180-nm to several micrometers in diameter6,9 and shows regulatory elements (signaling molecules, cytoskeletal reorganization6,21) reminiscent of those elicited on ICAM-1 engagement by leukocytes during inflammation.40–42 Here, we found that this process occurs at specialized lipid domains on the EC plasmalemma, where anti-ICAM carriers colocalized with ICAM-1-positive engulfment structures. This occurred in areas enriched in cholesterol, sphingomyelin, and gangliosides and was inhibited by disrupting said domains by cholesterol chelation. Although this was expected, we found an unforeseen contribution by ASM and the sphingomyelin/ceramide pathway toward the formation of ICAM-1-enriched membrane protrusions and CAM-mediated uptake.
Sphingomyelinase-mediated formation of ceramide domains at the plasmalemma is associated with phosphorylation and redistribution of actin-adapters of the ezrin/radixin/moesin family, followed by conversion of ceramide into sphingosine-1-phosphate and hyperphosphorylation of ezrin/radixin/moesins, finally culminating in the formation of membrane protrusions. ICAM-1 redistributes to detergent-resistant membrane fractions on cross-linking with antibodies, and it coprecipitates with ezrin/radixin/moesin proteins, as also observed here. Thus, it is possible that CAM-mediated endocytosis proceeds through a similar mechanism.

NHE1 might provide a bridge among ICAM-1 engaged by anti-ICAM carriers, ASM activity and the sphingomyelin/ceramide pathway, and the reorganization of the actin cytoskeleton supporting carrier engulfment and endocytosis. NHE1 is involved in cytoskeletal rearrangement and may cross-link actin filaments to the ICAM-1 cytosolic domain.

Its ion exchange activity also regulates elasticity at the endothelial surface, explaining engulfment and endocytosis of micrometer-sized objects targeted to ICAM-1. Because ICAM-1 interacts with NHE1 on binding of anti-ICAM carriers, and because of the directionality of NHE1 ion flux, NHE1 might create an acidic microenvironment at ICAM-1 engagement regions. Although there is controversy concerning the pH range at which exocytosed ASM can exert activity, NHE1 contribution would explain how this acidic enzyme can display activity at the otherwise neutral extracellular environment and how ECs might regulate ceramide production with spatial precision. A similar function of NHE1 has been shown for other pH-sensitive enzymes. Particularly, NHE1 inhibition with carpiporide ameliorates cisplatin-induced, ASM-dependent generation of ceramide.

Altogether, these data (complemented by data reported previously) suggest a model by which engagement of ICAM-1 in lipid domains enriched in sphingomyelin induces exocytosis of ASM to these areas of the endothelial plasmalemma (Figure 6). Engaged ICAM-1 forms a complex with NHE1, resulting in local acidification and ASM-driven hydrolysis of sphingomyelin into ceramide. This favors actin polymerization and cytoskeleton remodeling, stabilizes the engagement platform by restricting molecular diffusion and providing cytoskeletal anchorage, regulates membrane deformability, and favors formation of engulfment structures and endocytic vesicles. These events support the ability of ECs to internalize relatively large drug carriers targeted to ICAM-1, as opposed to classical clathrin- or caveolamediated endocytosis. Results shown here and previously also indicate that multivalent binding to ICAM-1 is required to induce these events, yet this is still to be elucidated, several ceramides affect the molecular and biophysical features of the plasmalemma, for instance, by promoting the formation of large lipid domains, affecting membrane function. Ceramide production by ASM at the outer leaflet of the plasma membrane also favors vesiculization and promotes cytoskeletal rearrangement, suggesting that a similar pathway may contribute to formation of engulfment structures and vesicles supporting uptake of anti-ICAM carriers by ECs, as observed in this work.
achievable with carriers displaying different antibody surface densities, emphasizing the design flexibility of this strategy.

These results hold relevance regarding inflammation and vascular pathologies. ECs constitutively secrete ASM, whose activity increases in inflammation and accelerates progression of atherosclerotic lesions.\(^{49-51}\) Ceramide production by sphingomyelinesinases has also been associated with increased redox signaling and leukocyte attachment to brain endothelium.\(^{52,53}\) Leukocyte influx into alveoli is decreased on ASM inhibition,\(^{54}\) and metabolic imbalances causing high levels of ceramide can lead to higher leukocyte levels in tissue.\(^{55}\) Leukocyte transmigration across the endothelium has been linked to cholesterol-, sphingomyelin-, or ganglioside-rich areas in lipid rafts.\(^{30}\) Hence, our data showing redistribution of ASM to endothelial structures where ICAM-1 is engaged, along with enrichment of ceramide in these regions, are consistent with this literature.

These results advance current knowledge on the regulation of CAM-mediated endocytosis, which may provide new tools for modulation of intraendothelial therapies, and also underscore the implication of sphingomyelin/ceramide signaling in the vascular function.

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Disclosures

None.

References


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ICAM-1 engagement modulates sphingomyelinase and ceramide, supporting uptake of drug carriers by the vascular endothelium

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Running title: Role of sphingomyelinase in endocytosis via ICAM-1
Extended Methods

Antibodies and reagents

Monoclonal antibodies to the extracellular domain of human or mouse ICAM-1 were R6.5 and phycoerythrin-conjugated LB-2 (Santa Cruz Biotechnology, Santa Cruz, CA), or YN1, respectively. Monoclonal antibodies to mouse platelet-endothelial cell adhesion molecule 1 (PECAM-1), human clathrin heavy chain, human vascular cell adhesion molecule 1 (VCAM-1), human mannose 6-phosphate receptor (M6PR), or ceramide were from BD Biosciences (Franklin Lakes, NJ), EMD Chemicals (Gibbstown, NJ), Millipore (Billerica, MA), or Sigma-Aldrich (Saint Louis, MO). Polyclonal antibodies to human acid sphingomyelinase (ASM), Na⁺/H⁺ exchanger protein 1 (NHE1) or ganglioside GM1 were from Santa Cruz Biotechnology or EMD Chemicals. Secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Polystyrene-latex beads were from Polysciences (Warrington, PA). BODIPY® FL C12-sphingomyelin and Texas Red-labeled phalloidin were from Molecular Probes, Inc. (Eugene, OR). Human recombinant ASM was provided by Dr. Edward Schuchman (Mount Sinai School of Medicine, New York, NY) and neutral SM was from Sigma-Aldrich. All other reagents were from Sigma-Aldrich.

Cell cultures

Human umbilical vein endothelial cells (HUVECs) purchased from Lonza Walkersville (Walkersville, MD) were cultured in M-199 medium supplemented as described. Mouse lung endothelial cells (MLECs) were isolated from wild-type C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) or ASM−/− mice (kindly provided by Dr. Edward Schuchman, Mount Sinai School of Medicine, New York, NY). Isolation of lungs from mice was done after anesthesia by intraperitoneal injection with 100 mg/kg body-weight ketamine and 10 mg/kg body-weight xylazine, and adjusted to IACUC regulations. Mouse lungs were cut into 1-2 mm fragments, followed by digestion overnight at 4°C in 1 mg/mL collagenase and filtration through a 40 μm nylon mesh. Cells were isolated by incubation with anti-PECAM Mec13.3-coated Dynabeads® (Invitrogen Corporation, Carlsbad, CA) for 30 min at 4°C, and cultured in supplemented DMEM. For experiments, ECs were seeded on 1%-gelatin-coated glass coverslips. Cells
were treated for 16 h with 10 ng/mL TNFα (BD Biosciences, Franklin Lakes, NJ) to induce endothelial activation and up-regulation of ICAM-1 expression.

**Preparation of model polymer carriers targeted to cell surface markers**

Model polymer carriers targeted to ICAM-1 to induce CAM-mediated endocytosis, or to VCAM-1 or M6PR used as controls, were prepared by adsorbing the corresponding antibodies on the surface of 100 nm- or 4.5 μm-diameter polystyrene particles (anti-ICAM, anti-VCAM, or anti-M6PR carriers), as described. Non-coated antibodies were separated by centrifugation and the resulting coated carriers were resuspended in phosphate buffered saline (PBS) containing 1% bovine serum albumin, and sonicated at low power for 20 s to avoid aggregation. As measured in parallel using 125Iodinated antibody counterparts, the antibody surface density on 4.5 μm-diameter carriers was ~5,000 antibody molecules/μm² (~320,000 antibody molecules/carrier), while the antibody density on 100 nm-diameter carriers (which exhibited a final diameter of ~180 nm after coating, determined by dynamic light scattering) was ~7,000 antibody molecules/μm² (~250 antibody molecules/carrier).

Alternatively, for experiments to rescue CAM-endocytosis by coating recombinant ASM on carrier articles, 4.5 μm carriers were prepared using a 50:50 mass-ratio mix of anti-ICAM and either control IgG or ASM, rendering ~2,500 anti-ICAM molecules/μm².

**Enrichment of lipids at sites of ICAM-1 engagement on the endothelial plasmalemma**

Anti-ICAM carriers (4.5 μm in diameter) were incubated for 15 min at 37ºC with control HUVECs or HUVECs treated with 5 mM methyl-β-cyclodextrin (Cdx, a cholesterol chelator), 20 μM 5-(N-ethyl-N-isopropyl)amiloride (EIPA, which inhibits NHE1 involved in CAM-mediated endocytosis), 50 μM imipramine (an inhibitor of ASM), or a mix of 50 μM imipramine and 500 mU/mL neutral SM (to inhibit endogenous ASM while rescuing this enzyme activity). Carriers that were not firmly bound to HUVECs were washed, and cells were fixed with cold 2% paraformaldehyde. Binding and/or engulfment of anti-ICAM carriers by ECs were verified by scanning electron microscopy (SEM). In parallel, cholesterol was stained using 50 μg/mL filipin and sphingomyelin was visualized by incubating HUVECs with 0.2 μg/mL
BODIPY®-sphingomyelin for 16 h prior to experiments. Ganglioside GM1 and ceramide were detected by immunostaining.

For analysis, carriers were first located using phase-contrast microscopy (Olympus IX81, Olympus, Inc., Center Valley, PA) with a 40x oil immersion objective (UPlanApo, Olympus, Inc., Center Valley, PA). Fluorescence micrographs at the identified positions were then obtained in the z-axis every 0.5 μm, using an ORCA-ER camera (Hamamatsu Corporation, Bridgewater, NJ) and SlideBook™ 4.2 software (Intelligent Imaging Innovations, Denver, CO), and images were analyzed using Image-Pro 6.3 (Media Cybernetics, Bethesda, MD). Enrichment of lipid molecules in areas of the EC plasmalemma where carriers were bound was visualized using pseudocolored fluorescence-intensity surface plots, which were obtained at the focal plane of the plasmalemma surface versus the carrier mid cross-section. The mid cross-section of carriers is raised ~2 μm above the plasmalemma level; hence, enrichment of cellular molecules at this focal plane shows EC membrane actively engulfing said carriers. For semi-quantitative analysis of these experiments, fluorescence intensity profiles were averaged from ≥ 32 carriers and enrichment of a particular lipid was calculated as fold increase (Δ) in the average intensity at the mid cross-section region of engulfed carriers divided by the average intensity of ~2 μm surrounding areas where the plasmalemma is not raised engulfing carriers ("background").

**Recruitment of proteins at sites of ICAM-1 engagement on the endothelial plasmalemma**

Anti-ICAM, anti-VCAM, or anti-M6PR carriers (4.5 μm in diameter) were incubated for 15 min or 30 min at 37°C with control HUVECs or HUVECs treated with 5 mM Chol (to chelate cholesterol), 50 μM imipramine (to inhibit ASM), a mix containing 50 μM imipramine and 500 mU/mL neutral SM (to inhibit endogenous ASM while rescuing this enzyme activity), 3 mM amiloride or 20 μM EIPA (to inhibit NHE1-dependent CAM-mediated endocytosis), 0.5 μM wortmannin (to inhibit PI3 kinase), or 10 μM 1-(5-isoquinolinylsulfonyl)-2-methyl-piperzine (H-7, to inhibit PKC). Anti-ICAM on the surface of carriers was detected by immunofluorescence using a secondary antibody. ICAM-1, VCAM-1, or M6PR were immunostained using antibodies that recognize extracellular domains of these antigens and do not require cell permeabilization. Immunostainings of ASM, NHE1, or clathrin heavy chain were performed after permeabilization with cold 0.2% Triton X-100.
For visualization and semi-quantitative analysis of the enrichment of molecules at sites of carrier binding, we used the protocol described above. In addition, the intracellular distribution of ASM was assessed by computing the total number of ASM-positive vesicles (~100-300 nm fluorescent objects) and those located within 5 μm distance around the nucleus (perinuclear). Peripheral intracellular ASM was calculated as the number of total – perinuclear ASM-positive vesicles.

Co-precipitation of ASM, α-actinin, and moesin with ICAM-1 upon ICAM-1 crosslinking was assessed by incubating HUVECs with anti-ICAM Protein A-coated magnetic Dynabeads® (Invitrogen, Grand Island, NY) for 15 min at 37°C as described\(^8\), followed by isolation of the magnetic beads bound to cells. Bead-bound proteins were then eluted and separated using SDS polyacrylamide gel electrophoresis. Coomassie-stained protein bands at the expected molecular weight of ASM, α-actinin, moesin, and ICAM-1 were excised from the gel, digested with trypsin, and analyzed by liquid chromatography/mass spectrometry for protein identification using Scaffold™ (Proteome Software Inc., Portland, OR).

**Actin remodeling and CAM-mediated endocytosis of anti-ICAM carriers**

For visualization of filamentous actin (F-actin), TNFα-activated HUVECs were incubated with 4.5 μm anti-ICAM carriers for 15 min at 37°C, followed by washing, fixation, permeabilization, and fluorescent labeling of F-actin using Texas Red-conjugated phalloidin.

For experiments of endocytosis in cell culture, TNFα-activated HUVECs, wild-type MLECs or ASM\(^{-/-}\) MLECs were incubated at 37°C with 4.5 μm anti-ICAM carriers for 30 min to allow carrier binding, followed by washing non-bound carriers and incubation at 37°C for 1 h to allow full endocytosis. Inhibition experiments were performed in the presence of 3 mM amiloride, 50 μM imipramine, 0.5 μM wortmannin, 10 μM H-7, or in Na\(^+\)-depleted ionic solution (138 mM choline chloride, 5.4 mM KCl, 1 mM CaCl2, 1 mM MgCl2). Endocytosis of carriers displaying 50% anti-ICAM surface-density was also tested, as well as the effect of co-coating carriers with both anti-ICAM and with recombinant ASM, to rescue this activity in the presence of inhibitors. After cell fixation with cold 2% paraformaldehyde, samples were stained using Texas Red-labeled goat anti-mouse IgG, which binds to the anti-ICAM coat on non-internalized carriers. We have previously shown that, due to absence of a permeabilization step, this secondary antibody does
not gain access to internalized carriers. Uptake can then be determined by fluorescence microscopy using Image-Pro 6.3 by counting total carriers (phase contrast) and surface-bound Texas Red-fluorescent carriers.

For in vivo experiments, C57BL/6, caveolin-1−/− (Jackson Laboratory, Bar Harbor, ME) or ASM−/− mice (provided by Dr. Edward Schuchman, Mount Sinai School of Medicine, New York, NY) were anesthetized by intraperitoneal injection with 100 mg/kg body-weight ketamine and 10 mg/kg body-weight xylazine, and then were injected intravenously with ~180 nm anti-ICAM carriers (instead of 4.5 μm carriers that can cause capillary embolization due to their size). Endocytosis of anti-ICAM carriers by ECs was assessed in the lungs because this organ represents a first-pass area for materials injected intravenously, it receives the entire cardiac input, and its endothelium expresses relative high levels of ICAM-1, altogether providing good endothelial binding of anti-ICAM carriers, as previously shown. Mice were subjected to intracardial perfusion with saline 3 h after carrier injection to remove circulating carriers and carriers loosely bound to the vasculature. Lungs were then isolated, fixed in 2.5% glutaraldehyde and 0.1 M sodium cacodilate buffer, and processed into 80-90 nm-thin resin-embedded sections for transmission electron microscopy. Endocytosis of anti-ICAM carriers by pulmonary ECs was semi-quantitatively assessed as the number of internalized carriers per field, as previously described. Animal studies adjusted to IACUC regulations.

Statistics

Data are means ± standard error of the mean (s.e.m.). Statistical significance was determined by Student's t-test.
References


Table I. Molecular components associated with CAM-mediated endocytosis induced by anti-ICAM carriers.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Abbreviation</th>
<th>Roles</th>
<th>Inhibitors</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercellular adhesion molecule 1</td>
<td>ICAM-1</td>
<td>Co-receptor of leukocyte β2 integrins targeted by anti-ICAM carriers used for endothelial drug delivery</td>
<td>-</td>
<td>1, 2, 3, 6</td>
</tr>
<tr>
<td>Na⁺/H⁺ exchanger 1</td>
<td>NHE1</td>
<td>H⁺ efflux and Na⁺ influx; crosslink to the actin cytoskeleton; interacts with ICAM-1 upon binding of anti-ICAM carriers</td>
<td>Amiloride and its derivative EIPA</td>
<td>21, 22, 45, 46</td>
</tr>
<tr>
<td>Acid sphingomyelinase</td>
<td>ASM</td>
<td>Hydrolizes sphingomyelin into ceramide</td>
<td>Imipramine</td>
<td>31, 36, 39, 49</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>-</td>
<td>Regulates lateral diffusion of molecules in the plasmalemma; rich in specialized membrane lipid domains</td>
<td>Chelated by cyclodextrin (Cdx)</td>
<td>-</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>SM</td>
<td>Sphingolipid abundant in specialized membrane lipid domains; serves as a substrate for sphingomyelinases</td>
<td>-</td>
<td>33, 38</td>
</tr>
<tr>
<td>Ceramide</td>
<td>-</td>
<td>Sphingolipid that facilitates formation of large membrane domains, vesicles, and cytoskeletal rearrangement</td>
<td>-</td>
<td>32, 33, 34, 38</td>
</tr>
<tr>
<td>Dynamin</td>
<td>-</td>
<td>Large GTPase involved in vesicular scission</td>
<td>Dominant negative dynamin</td>
<td>6</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td>PKC</td>
<td>Signaling molecule; acts upstream of CAM-mediated actin remodeling</td>
<td>BIM-1, H-7</td>
<td>6</td>
</tr>
<tr>
<td>Src kinase</td>
<td>-</td>
<td>Signaling molecule; acts upstream of CAM-mediated actin remodeling</td>
<td>Radicicol</td>
<td>6</td>
</tr>
<tr>
<td>Rho-dendent kinase</td>
<td>ROCK</td>
<td>Signaling molecule; acts upstream of CAM-mediated actin remodeling</td>
<td>Y27632</td>
<td>6</td>
</tr>
<tr>
<td>Filamentous actin</td>
<td>F-actin</td>
<td>Forms stress fibers associated to uptake of ICAM-1-targeted carriers</td>
<td>Depolymerized by latrunculin A</td>
<td>6, 21</td>
</tr>
</tbody>
</table>
Table II. Liquid chromatography/mass spectrometry analysis of endothelial proteins immunoprecipitated with anti-ICAM carriers.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein identification probability</th>
<th>Percent of total spectra*</th>
<th>Number of assigned spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>100%</td>
<td>2.1%</td>
<td>11</td>
</tr>
<tr>
<td>α-actinin</td>
<td>100%</td>
<td>2.5%</td>
<td>13</td>
</tr>
<tr>
<td>Moesin</td>
<td>100%</td>
<td>1.1%</td>
<td>6</td>
</tr>
<tr>
<td>ASM</td>
<td>-</td>
<td>Not observed</td>
<td>Not observed</td>
</tr>
</tbody>
</table>

* Total spectra include trypsin peptides and non-peptide charged molecule contaminants

Analysis used an identification filter of: Minimum 2 peptides, with 95% minimum peptide identification probability
Video I. Engulfment of anti-ICAM carriers by ICAM-1-rich areas of the endothelial plasmalemma.

Activated HUVECs were incubated with 4.5 μm anti-ICAM carriers for 15 min at 37°C to engage ICAM-1 on ECs, followed by washing and fixation. Fluorescence micrographs were obtained at different focal planes along the z-axis (167 nm steps), after staining anti-ICAM on the surface of carriers using a FITC-labeled secondary antibody and ICAM-1 on the EC surface using a Texas Red-labeled antibody. Frames run through the z-axis from the top of the carrier to the endothelial plasmalemma.
Figure I. Quantification of enrichment of lipids at sites of anti-ICAM carrier engulfment by ECs.

Activated HUVECs were incubated with 4.5 μm anti-ICAM carriers for 15 min at 37°C to engage ICAM-1 on ECs, followed by washing and fixation. (A) Cholesterol, sphingomyelin or ganglioside GM1 were stained using fluorescent blue filipin, green BODIPY-sphingomyelin, or anti-GM1 and a Texas Red-conjugated secondary antibody, respectively. (B) Effect of methyl-β-cyclodextrin (Cdx) on enrichment of
cholesterol labeled with blue filipin (upper panel) or ICAM-1 immunostained with a Texas Red-labeled antibody (middle and bottom panels) in regions of anti-ICAM-carrier binding. In all cases, graphs show fluorescence intensity plots at the mid cross-section plane of anti-ICAM carriers bound on ECs, and enrichment of molecules in these engulfment areas compared to adjacent areas of the endothelial plasmalemma (Δ, which indicates fold increase). Data represent mean and s.e.m. (n ≥ 65 carriers).
Figure II. Effect of methyl-β-cyclodextrin on engulfment of anti-ICAM carriers by ECs. Activated HUVECs were incubated with 4.5 μm anti-ICAM carriers for 15 min at 37°C to engage ICAM-1 on ECs under control conditions or in the presence of methyl-β-cyclodextrin (to remove cholesterol), following by processing for SEM visualization. Arrows indicate membrane-engulfed carriers and arrowheads indicate carriers bound to the cell surface but non-associated with engulfment structures. Scale bar = 10 μm.
Figure III. Effect of amiloride on the engulfment of anti-ICAM carriers by ICAM-1-rich structures in ECs. Activated HUVECs were incubated for 15 min at 37°C with 4.5 μm anti-ICAM carriers to engage ICAM-1 on ECs, under control conditions or in the presence of amiloride. Cells were washed and fixed, ICAM-1 was immunostained using a Texas Red-labeled antibody, and samples were observed by phase-contrast (left panels) and fluorescence (right panels) microscopy. Presence or absence of carrier engulfment is marked with arrows or arrowheads, respectively. Scale bar = 10 μm.
Figure IV. Redistribution of endothelial ASM upon ICAM-1 engagement by anti-ICAM carriers.

Activated HUVECs were incubated in the absence (Control) or presence of anti-ICAM carriers for 30 min at 37°C. Cells were fixed and permeabilized, and ASM was stained with a Texas Red-labeled secondary antibody. Arrowheads mark ASM at the perinuclear region of cells. Arrows mark ASM at the cell periphery. Dashed lines mark the cell borders, as observed by phase contrast. Scale bar = 10 μm.
Figure V. Recruitment of molecules at sites of M6PR engagement by anti-M6PR carriers. Activated HUVECs were incubated with 4.5 μm anti-mannose-6-phosphate receptor (M6PR) carriers for 15 min at 37°C to engage M6PR on ECs, followed by washing and fixation. Phase contrast (left panels) and fluorescence micrographs (right panels) were obtained after immunostaining ICAM-1, NHE1, or clathrin heavy chain with Texas Red. Arrowheads indicate lack of enrichment of the corresponding marker around carriers. Arrows indicate enrichment of the corresponding marker around carriers. Scale bar = 10 μm.
Figure VI. Quantification of ceramide enrichment and contribution of ASM and NHE1 to engulfment of anti-ICAM carriers by ECs. Activated HUVECs were incubated with 4.5 μm anti-ICAM carriers for 15 min at 37°C to engage ICAM-1 on ECs under control conditions or in the presence of EIPA (to inhibit NHE1) or imipramine (to inhibit ASM), followed by washing and fixation. Ceramide (top) or ICAM-1 (bottom) were immunostained. Graphs show fluorescence intensity plots at the mid cross-section plane of anti-ICAM carriers bound on ECs. Data represent mean and s.e.m. (n ≥ 150 carriers).