Control of intracellular trafficking of ICAM-1-targeted nanocarriers by endothelial Na\(^+\)/H\(^+\) Exchanger (NHE) proteins

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ABSTRACT

Targeting nanocarriers (NCs) loaded by antioxidant enzymes (e.g., catalase) to endothelial cell adhesion molecules (CAMs) alleviates oxidative stress in the pulmonary vasculature. However, antioxidant protection is transient, since CAM-targeted catalase is internalized, delivered to lysosomes and degraded. To design means to modulate the metabolism and longevity of endothelial cell (EC) targeted drugs we identified and manipulated cellular elements controlling the uptake and intracellular trafficking of nanocarriers targeted to InterCellular Adhesion Molecule 1 (anti-ICAM/NCs). BAPTA, thapsigargin, amiloride and EIPA inhibited anti-ICAM/NC uptake by ECs and actin rearrangements induced by anti-ICAM/NCs (required for uptake), suggesting that member(s) of Na⁺/H⁺ exchanger family proteins (NHEs) regulate these processes. Consistent with this hypothesis, a siRNA specific for the plasmalemma NHE1, but not the endosome-associated NHE6, inhibited actin remodeling induced by anti-ICAM/NCs and internalization. Anti-ICAM/NC binding to ECs stimulated formation of a transient ICAM-1/NHE1 complex. One hour after uptake, ICAM-1 dissociated from NHE1 and anti-ICAM/NCs were transported to NHE6-positive vesicles en route to lysosomes. Inhibition of PKC (an activator of intracellular NHEs) accelerated nanocarrier lysosomal trafficking. In contrast, monensin, which enhances the endosomal sodium influx and proton efflux maintained by NHE6, inhibited delivery of anti-ICAM/NCs to lysosomes by switching their trafficking to a plasma membrane recycling pathway. This markedly prolonged the protective effect of catalase-coated anti-ICAM/NCs. Therefore: i) NHE1 and NHE6 regulate distinct phases of anti-ICAM/NC uptake and trafficking; ii) pharmacological agents affecting these regulatory elements alter the itinerary of anti-ICAM/NC intracellular trafficking; and, iii) these agents modulate duration of the therapeutic effects of targeted drugs.
Keywords: Ig superfamily cell adhesion molecules, vascular immunotargeting, oxidative stress, endocytosis, sodium proton exchangers.
INTRODUCTION

Pulmonary endothelium, which is vulnerable to oxidative stress, represents a specific therapeutic target for protection by antioxidant enzymes, such as catalase and superoxide dismutase (16, 25, 42). For example, vascular immunotargeting of catalase conjugated with monoclonal antibodies to endothelial cell adhesion molecules (CAMs) protects against acute pulmonary vascular oxidative stress in animal models (2, 10, 22). In addition to the primary antioxidant effect of targeted antioxidants, a secondary effect of immunoconjugates may be to inhibit leukocyte transmigration mediated by CAMs (12, 54). This may provide an additional benefit in attenuating the inflammatory response that accompanies pulmonary oxidative stress in conditions such as ALI/ARDS, hyperoxia and ischemia-reperfusion injury (14).

An Ig-superfamily transmembrane glycoprotein, InterCellular Adhesion Molecule 1 (ICAM-1), is a good candidate for immunotargeting therapeutics to the pathologically altered endothelium, since ICAM-1 is expressed on the luminal surface of endothelial cells (ECs), and is up-regulated and functionally involved in pathological conditions, including inflammation, thrombosis and vascular oxidative stress (12, 38). Reporter cargoes and experimental therapeutics, such as catalase, conjugated with anti-ICAM or loaded onto ICAM-targeted nanocarriers: i) bind to and protect ECs against oxidants; ii) accumulate in the pulmonary endothelium after systemic injection; and, iii) undergo enhanced targeting to inflamed endothelium (2, 35-37).

The molecular mechanisms regulating cellular uptake, traffic and metabolism of targeted drugs represent key parameters that determine efficacy, duration and side effects. For example, we found that lysosomal proteolysis terminates the antioxidant effects of
catalase targeted to endothelial CAMs within a few hours after binding to cells (36, 52). This is due to the fact that multivalent anti-ICAM/NCs and protein conjugates are internalized by ECs, which occurs via a novel endocytic pathway called CAM-mediated endocytosis (41). ICAM-1 clustering by multivalent anti-ICAM/NCs triggers actin rearrangements and internalization mediated through a multi-pronged kinase cascade involving protein kinase C, src kinase, and Rho dependent kinase (ROCK) (41). CAM mediated endocytosis does not require clathrin or caveolin, however dynamin is required for internalization (41).

We also found that anti-ICAM/NC uptake and actin stress fiber formation were inhibited by amiloride (41), a pleotrophic agent which inhibits multiple classes of ion channels, including sodium proton exchangers (NHEs) and epithelial sodium channels (ENaC) (20). Amiloride suppresses macropinocytosis in some cell types (23) and inhibits actin stress fiber formation mediated by NHE1 (57), a 91 kDa transmembrane sodium-proton exchanger protein, also endowed with actin-cross-linking and other ion channel independent activities, which operates downstream of ROCK-mediated phosphorylation (11, 45). These data, together with the fact that NHE1 is ubiquitously expressed and predominantly localized to the plasma membrane (5), suggest that NHE1 may regulate the initial phases of anti-ICAM/NC endocytosis by ECs.

In addition, there are NHE isoforms localized to intracellular compartments, which may intervene in anti-ICAM/NC trafficking subsequently to internalization (6, 7, 32, 43). For instance, Nhx1 (an NHE6 related yeast ortholog) is localized to late endosomes and is involved in delivery of endocytosed cargo to lysosomes (6, 7). Whether mammalian NHE isoforms (i.e., intracellular NHE6) play a similar role in regulating vesicular trafficking is not known at present, however this is plausible given that both Nhx1 and intracellular NHEs are required to maintain endosome pH, ion homeostasis and osmotic balance (5, 43).
In the present study, we used molecular and pharmacologic approaches to define potential roles for NHEs and endosome Na\(^{+}\)/H\(^{+}\) balance in actin rearrangements, endocytosis and intracellular trafficking of anti-ICAM/NCs in ECs. By altering endosome homeostasis, we were able to switch the intracellular trafficking of anti-ICAM/NCs from a lysosomal degradation pathway to a plasma membrane recycling pathway that preserved anti-ICAM/NC activity. This provides a basis to design means to prolong the therapeutic effect of ICAM-1 targeted catalase.
MATERIALS AND METHODS

Reagents. Monoclonal antibodies to human ICAM-1 were mAb R6.5 (28) and LB-2 (Santa Cruz Biotechnology; Santa Cruz, CA). Polyclonal antibodies to human EEA-1, LAMP-1, NHE1, or NHE6 were from Affinity BioReagents (Golden, CO), BD Biosciences/PharMingen (Franklin Lakes, NJ), or Chemicon International (Temecula, CA). Secondary fluorescent antibodies were from Jackson ImmunoResearch (West Grove, PA) and Molecular Probes (Eugene, OR). Green fluorescent polystyrene (latex) nanospheres, 100 nm in diameter, were from Polysciences (Warrington, PA). Unless otherwise stated, other reagents were from Sigma (St Louis, MO).

Preparation of anti-ICAM nanocarriers. Nanocarriers were prepared as described (36) by coating on fluorescently-labeled polystyrene nanospheres with either anti-ICAM alone (anti-ICAM/NCs) or anti-ICAM and biotinylated catalase at 1:0.5 molar ratio (anti-ICAM/NC/catalase). The final effective diameter of the resulting nanocarriers was determined by dynamic light scattering (56). In each case, these protocols yielded preparations with diameter ranging from 100 to 300 nm.

Cell culture. Internalization of anti-CAM/NCs occurs via a common pathway, CAM-endocytosis, in a variety of CAM-positive cell types (22, 36, 37, 40, 41). Human umbilical vein ECs (HUVEC) and an endothelial-like cell line, EAhy926 (13) were selected to study the uptake and trafficking of anti-ICAM/NCs since they provide insights that have subsequently correlated with in vivo models for endothelial targeting of anti-ICAM/NCs (37). HUVEC (Clonetics, San Diego, CA), and EAhy926 were cultured at 37°C, 5% CO₂, and 95% relative humidity in supplemented M199 or DMEM medium (GibcoBRL, Grand Island, NY), respectively (36). Cells were seeded onto 12 mm² gelatin-coated coverslips in
24-well plates and were treated overnight with TNF-α prior to experiments. TNF-α treatment up-regulates ICAM-1 expression, hence it enhances anti-ICAM/NC binding to HUVEC, yet it does not affect their internalization kinetics or trafficking (37, 41).

**Internalization, trafficking and stability of anti-ICAM/NCs.** TNF-α activated, confluent HUVEC were incubated at 4°C for 30 min with green-labeled anti-ICAM/NCs to enable binding to the cell surface. The cells were then washed, warmed to 37°C for varying periods of time, cooled to 4°C, washed and fixed with 2% paraformaldehyde at room temperature for 15 min. Cells were then treated with Texas Red goat anti-mouse IgG, which binds anti-ICAM. This protocol preferentially labels anti-ICAM/NCs bound to the cell surface vs. internalized nanocarriers that can only be labeled upon cell permeabilization with 0.2% Triton X-100 (39). Alternatively, to visualize green fluorescent anti-ICAM/NCs in ICAM-1-enriched sites at the plasma membrane, cells were permeabilized with 0.2% Triton X-100 at room temperature, washed, and labeled with red fluorescent LB-2, which recognizes the ICAM-1 cytoplasmic domain (37).

The samples were analyzed with a Nikon Eclipse TE2000-U fluorescence microscope using a 40X PlanApo objective and filters optimized for FITC, Texas Red and Alexa Fluor 350 fluorescence. Images were obtained with a Hamamatsu Orca-1 CCD camera and analyzed using ImagePro 3.0 software. Merged micrographs were scored automatically by image analysis to obtain the percentage of cell-associated particles that were internalized, as previously described (41, 56).

To examine the effect of inhibitors on uptake and/or trafficking, TNF-α activated HUVEC were pre-treated at 37°C for 30 min in the presence of either 3 mM amiloride, 20 μM ethyl-isopropyl amiloride (EIPA), 25 μM monensin, 5 μM BAPTA, 1 μM thapsigargin...
(TG), or 10 μM H7 (a PKC inhibitor). To stain filamentous actin, fixed, permeabilized cells were labeled with phalloidin conjugated to red AlexaFluor 594.

Given that the fluorophore is embedded in polystyrene particles, green fluorescence emitted by beads used in these protocols is not significantly affected by pH changes. Hence, to identify compartments containing internalized anti-ICAM/NCs, TNF-α activated HUVEC were incubated with anti-ICAM/NCs as described above. After surface labeling of non-permeabilized cells with blue AlexaFluor 350 goat anti-mouse IgG, the cells were permeabilized by 15 min incubation with 0.2% Triton X-100 at room temperature, washed, and labeled with polyclonal rabbit anti-NHE1, anti-NHE6 or anti-EEA-1 followed by Texas Red goat anti-rabbit IgG. Alternatively, HUVEC lysosomes were labeled with Texas Red dextran (10,000 MW) internalized by fluid phase endocytosis (36).

To determine the intracellular stability of anti-ICAM/NCs, internalized particles were counterstained with Texas Red goat anti-mouse IgG that recognize non-degraded anti-ICAM. Co-localization and particle stability were quantified using the same image analysis utilized to measure anti-ICAM/NC uptake described above.

**Co-immunoprecipitation.** Cells on 60 mm dishes were washed twice with PBS at 4°C, then scraped and resuspended in cold PBS and centrifuged at 200 x g for 5 min. The cells were then resuspended in PBS containing 0.5% Triton X-100, 0.02% SDS, 1 mM phenylmethylsulfonylfluoride (PMSF), 1:100 protease inhibitor cocktail (Sigma) and lysed for 30 min. The cell lysate was precleared by treating for 1 h with protein A agarose (Invitrogen, Carlsbad, CA) followed by microcentrifugation (16,000 x g for 5 min at 4°C). The cleared lysate was incubated with primary antibody at 4°C for 1 h, and subsequently incubated with protein A agarose for 1 h and then microfuged. The pellet was washed 3
times with PBS, resuspended in SDS-PAGE sample buffer and then analyzed by PAGE and immunoblot.

**siRNA treatment.** Pre-designed, single stranded siRNA oligonucleotides to human NHE1 and NHE6 and control oligonucleotides were from Ambion (Auston, TX). Sense and anti-sense oligonucleotides were resuspended to 100 μM final concentration in annealing buffer and annealed at 37°C for 1 h. For each dsRNA oligo, 2 μg were added to 50 μl serum-free medium containing 5 μl GTS Gene Silencer Reagent (Gene Therapy Systems, San Diego, CA), incubated for 5 min at room temperature and then added to EAhy926 cells plated on 60 mm dishes in 1 ml medium. After 16 h incubation, the medium was changed to medium containing TNF-α and the cells were further incubated at 37°C for 16-24 h prior to further experimental manipulation.

**Antioxidant protection by anti-ICAM/NC/catalase.** The antioxidant effect of anti-ICAM/NC/catalase was tested at different periods of time after their internalization within control HUVEC or cells treated with 25 μM monensin. Following internalization, the cells were incubated for 15 min at room temperature with 5 mM H₂O₂ in Phenol Red-free RPMI. The cells were washed after H₂O₂ treatment, incubated with 0.1 mM calcein AM and 1 mM ethidium bromide (Live/Dead kit, Molecular Probes, Eugene, OR) for 15 min at 37°C, and finally scored as percentage of surviving (calcein positive/ethidium negative) cells.

**Statistics.** Unless otherwise stated, the data were calculated as the mean ± standard deviation, from a minimum of 70 cells from two independent experiments. Statistical significance was determined by Student’s *t* test.
RESULTS

**NHE1 regulates endocytosis of anti-ICAM/NCs.** Inhibition of anti-ICAM/NC endocytosis and concomitant actin stress fiber formation by amiloride suggested a role for NHE1 in CAM-mediated endocytosis (41). However, amiloride is a potent inhibitor of other ion channels, such as ENaC (20). Thus, we examined the effect of other agents which alter Na⁺/H⁺ exchange in cells (EIPA and monensin) on anti-ICAM/NC endocytosis by HUVEC. EIPA, but not monensin, significantly inhibited anti-ICAM/NC uptake to a level comparable to amiloride (Figure 1). Inhibitors of Ca²⁺ signaling (BAPTA and thapsigargin (TG)) also suppressed anti-ICAM/NC internalization by HUVEC. This suggests a possible role for calmodulin/Ca²⁺ signaling in actin reorganization induced by the anti-ICAM/NCs. Calmodulin binds to the NHE1 cytosolic domain upon activation at sites where actin polymerization occurs (45). In agreement with this hypothesis, EIPA inhibited actin filament reorganization induced by binding of anti-ICAM/NCs to the cells. To further confirm these results we used an endothelial-like cell line, EAhy926, transfected with siRNA corresponding to either NHE1 or NHE6, which specifically decreased the expression of these proteins (Figure 2). Disruption of NHE1, but not NHE6, inhibited anti-ICAM/NC uptake and impaired stress fiber formation.

Consistent with a role for NHE1 in regulating uptake by CAM-mediated endocytosis, HUVEC incubated for 15 min at 37°C in the presence of anti-ICAM/NCs showed a redistribution of NHE1, which co-localized with both membrane-bound and internalized nanocarriers, as determined by immunofluorescence microscopy (Figure 3). In contrast, there was significantly less co-localization of anti-ICAM/NCs with NHE6 at this time point. The change in NHE1 distribution and co-localization with anti-ICAM/NCs suggested that ICAM-1 clustering induced the formation of a NHE1/ICAM-1 complex, which was confirmed by
co-immunoprecipitation. Cells incubated with anti-ICAM/NCs showed an increased amount of NHE1 that co-immunoprecipitated with ICAM-1. Interestingly, pre-treatment of cells with EIPA did not inhibit NHE1/ICAM-1 complex formation, despite the inhibitory effect of EIPA on uptake and cytoskeletal rearrangement, suggesting that NHE1/ICAM-1 interaction was a distinct event upstream from these processes.

**Sorting of internalized anti-ICAM/NCs to NHE6-positive endosomes.** Anti-ICAM/NCs remained in NHE1-positive compartments ~30 min after internalization, but both co-immunoprecipitation of NHE1 and ICAM-1 and co-localization of NHE1 and anti-ICAM/NCs were markedly decreased 1 h after internalization (Figure 4). The dissociation of NHE1, ICAM-1 and anti-ICAM/NCs coincided with an increase in co-localization of anti-ICAM/NCs with NHE6: 1 h after internalization, ~60% of anti-ICAM/NCs were localized in an NHE6 positive compartment. At this time point, >80% of internalized anti-ICAM/NCs were in EEA-1 positive endosomes (see Figure 6, below), suggesting partial remodeling of the NHE1 and NHE6 composition of endocytic vesicles containing anti-ICAM/NCs. Although nanocarriers localized to NHE6-positive vesicles 1 h after internalization, we found that ICAM-1 did not form complexes retrievable with anti-NHE6 (S. Muro, V. R. Muzykantov, and M. Koval, unpublished observations). Consistent with the lack of ICAM-1 binding to NHE6, we previously found that ICAM-1 had recycled to the plasma membrane 1 h after internalization (37).

NHE6 mediates Na⁺/H⁺ exchange in early and recycling endosomes, which has the potential to regulate acidification and osmolarity in these compartments and, by analogy to the yeast ortholog Nhx1, lysosomal biogenesis (6, 7). Although monensin does not inhibit anti-ICAM/NC internalization (Figure 1), it enhances Na⁺ influx and H⁺ efflux from endosomes and other intracellular compartments, which mimics the activity of a
constitutively activated intracellular NHE. Given this, we examined the effect of monensin on the intracellular trafficking of anti-ICAM/NCs. Monensin markedly altered anti-ICAM/NC trafficking. Three hours following internalization, fewer than 15% of internalized anti-ICAM/NCs were transported to lysosomes in monensin treated cells as compared to ~75% of internalized anti-ICAM/NCs transported to lysosomes in untreated control cells (Figure 5). In contrast to the monensin effect, EIPA did not inhibit lysosomal trafficking of anti-ICAM/NCs (Figure 5), which is consistent with a reported low sensitivity of intracellular NHEs to this drug as opposed to NHE1 (45, 47). In addition, pharmacologic inhibition of PKC by H7 resulted in accelerated anti-ICAM/NC trafficking to lysosomes (Figure 5). Since PKC is required for NHE activation (45), inhibiting PKC should inhibit NHE6 activity. The contrasting effects of monensin and H7 on lysosomal delivery of anti-ICAM/NC are consistent with a potential role for NHE6 in regulating this process.

In contrast to the effect of monensin on lysosomal trafficking of internalized anti-ICAM/NCs, there was little effect of monensin on anti-ICAM/NCs sorting to EEA-1 positive endosomes (Figure 6). Since we recently found that internalized ICAM-1 is recycled to the plasma membrane after dissociating from anti-ICAM/NCs (37), we tested whether monensin stimulated recycling of anti-ICAM/NCs to the plasma membrane. Consistent with this, cells treated with monensin recycled over 70% of the internalized anti-ICAM/NCs to the plasma membrane compared to control cells which showed very little anti-ICAM/NC recycling (Figure 6). Moreover, the majority of recycled nanocarriers resided in ICAM-1-enriched sites at the plasma membrane (80 ± 9% of recycled nanocarriers), suggesting that monensin inhibits anti-ICAM/NC dissociation from ICAM-1. Therefore, monensin induced the recycling of ICAM-1 complexed to anti-ICAM/NCs, which ordinarily does not occur unless ICAM-1 dissociates from the immunoconjugate (37).
Given that lysosomal degradation is the major mechanism that inactivates anti-ICAM/NC based therapeutic agents (36), the results described above suggested that a "monensin switch" from lysosome delivery to plasma membrane recycling would decelerate degradation of drugs delivered by anti-ICAM/NC. As shown in Figure 7, this was the case according to two criteria. First, using an immunofluorescence assay for degradation of antibodies coating anti-ICAM/NCs, we found that internalized anti-ICAM was preserved in monensin-treated cells (Figure 7). Second, the ability of anti-ICAM/NCs coated with catalase (anti-ICAM/NC/catalase) to protect HUVEC from H2O2-induced injury was markedly prolonged by monensin, where monensin-treated cells showed more than a 3-fold prolongation of the duration of antioxidant protection by anti-ICAM/NC/catalase as compared to control cells. Thus, monensin treatment, by diverting anti-ICAM/NC/catalase from lysosomes to a recycling pathway, decreased their degradation and prolonged the duration of their antioxidant effect.
DISCUSSION

Targeting therapeutic cargoes to endothelial cell adhesion molecules (CAMs) provides a platform for the targeted delivery of drugs to the vascular endothelium (2, 10, 22, 35, 38, 42). There are several approaches to the production of therapeutic agents targeted to CAMs, including pharmacologically active nanocarriers in the 100-300 nm diameter range conjugated to anti-CAM antibodies (anti-CAM/NCs). Nanocarriers offer several advantages for drug delivery, including size permitting intravascular administration, multifunctionality, modularity, and the ability to be targeted to specific cells (24). The efficacy of this drug delivery system has been demonstrated by its capacity to protect endothelial cells from oxidant induced toxicity both in vitro and in vivo (2, 22, 36, 37, 56). Results of this study indicate that the endothelial Na+/H+ exchanger proteins, NHE1 and NHE6, regulate the uptake, intracellular trafficking and subsequent metabolism of anti-ICAM/NCs (Figure 8). Our data further support a differential role for plasma membrane NHE1 vs. intracellular NHE6 regulating early vs. late stages of CAM-mediated endocytosis, i.e., internalization vs. vesicular trafficking.

Clustering of ICAM-1 by multivalent anti-ICAM/NCs stimulated the formation of an ICAM-1/NHE1 complex, which was a key event required for remodeling of the actin cytoskeleton and CAM-mediated endocytosis, and thus responsible for intracellular delivery of anti-ICAM/NCs. Co-clustering of ICAM-1 and NHE1 was upstream of CAM-mediated endocytosis and stress fiber formation since ICAM-1/NHE1 complexes formed in the presence of EIPA, which inhibited the latter two processes. Previous studies have demonstrated interaction of ICAM-1 with α-actinin and ERMs, as well as stimulation of actin filament formation by ICAM-1 clustering (1, 3, 9). In light of our results, it seems likely that NHE1 may function as a docking element mediating cytoskeletal rearrangements.
via recruiting ERM scaffold proteins (11, 57). The RhoA-ROCK pathway (which can be activated by ICAM-1 clustering (41)) is known to promote NHE1 activation via serine phosphorylation (45). This prompts conformational changes which can allow Ca\(^{2+}\)-dependent binding of calmodulin to NHE1 at sites of actin reorganization (45). Given that both ROCK (41) and Ca\(^{2+}\) inhibitors attenuate anti-ICAM/NC uptake (Fig. 1), it is plausible that additional signal cascades initiated by ICAM-1 clustering activate NHE1. The pathway by which ICAM-1 clustering (induced by anti-ICAM/NCs) activates NHE1 remains to be studied. One possibility is that this pathway may be comparable to the plasma membrane and actin reorganization observed by EC upon ICAM-1 engagement by leukocyte LFA-1, which regulates inflammation and endothelial permeability (3, 9, 17).

Although roles for intracellular NHEs in regulating endocytosis and membrane trafficking are just beginning to be elucidated, some insights can be gained from studies of the yeast ortholog Nhx1 (6, 7). Yeast deficient in Nhx1 expression, so-called E type VPS mutants, are incapable of sorting endocytosed material to the vacuole (6). Nhx1 and mammalian NHE isoforms (such as NHE6 in ECs) play a role in maintaining lumen pH balance in endosomes by mediating the exchange of Na\(^+\) and K\(^+\) for H\(^+\) ions (6, 7, 43). While changes in luminal acidity are known to regulate some elements of intracellular trafficking, such as ligand-receptor dissociation and lysosome enzyme function (29, 31, 34), regulation of vesicle formation, budding and targeting by changes in luminal pH are not well understood.

By analogy to its yeast ortholog Nhx1, Na\(^+\)/H\(^+\) exchange by NHE6 (which has been localized to early and recycling endosomes positive for EEA-1 and rab11) is believed to maintain a moderately low pH within the endosomal lumen, and to regulate ion balance and osmolarity, required for ligand/receptor dissociation and vesicular trafficking (7, 43). This is
likely to be the case for intracellular trafficking following CAM-mediated endocytosis. For instance, the kinetics of transport of anti-ICAM/NCs to NHE6-enriched compartments, which was relatively slow (~1 h), is consistent with our previous observation that internalized anti-ICAM/NCs resided in EEA-1 positive endosomes for 1-2 h before delivery to lysosomes and degradation (36). Therefore, disruption of ICAM/NHE1 and anti-ICAM/NC complex after internalization may be mediated by NHE6 in endosomal compartments, likely followed by rapid recycling of ICAM-1, and possibly NHE1, to the plasma membrane (Figure 8). It has also been postulated that further inactivation of NHE6 may prompt acidification of the vesicular lumen and lysosomal biogenesis (15), which is consistent with lysosomal delivery of anti-ICAM/NCs (36).

It has long been appreciated that monensin, an ionophore that effectively enhances Na\(^+\)/H\(^+\) exchange in endosomes and mimics constitutively activated NHE6, has the capacity to interfere with vesicle sorting (33). In particular, monensin inhibits trafficking from the cis to medial aspects of the Golgi apparatus (27, 55) and has also been shown to inhibit recycling of many plasma membrane receptors, such as transferrin receptors, LDL receptors, and \(\beta\)2 adrenergic receptors (4, 26, 49, 53). However, this effect is not universal, since recycling of CCR5 chemokine receptors and \(\beta\)1 adrenergic receptors is not inhibited by monensin (26, 48). In most cases, the effect of monensin on receptor recycling is due to the component of receptor traffic that is transported through the Golgi, as opposed to the rab11-dependent recycling pool which bypasses the Golgi apparatus (21, 49, 53), although it remains to be determined whether there are also monensin-sensitive plasma membrane recycling pathways that bypass this compartment.

We found that monensin stimulated the recycling of anti-ICAM/NCs to the plasma membrane (Figure 8). According to our knowledge, this is the first demonstration that
monensin can stimulate a plasma membrane recycling pathway, which may occur as a reminiscence of the ICAM-1 recycling pathway (37). Although one effect of monensin is to alkalize the endosome lumen, enhanced anti-ICAM/NC recycling was not simply due to increased endosomal pH, since we previously found that chloroquine is also effective at increasing endosome pH, however endocytosed anti-ICAM/NCs are still transported to lysosomes in chloroquine treated cells (36). Since monensin also enhances ion transport into endosomes (18, 43), one possibility is that endosome ion content may help regulate sorting of membrane trafficking. For instance, unrepressed Na⁺/H⁺ exchange by monensin may secondarily lead to Cl⁻ influx to the endosomal lumen to maintain ion balance, accompanied by H₂O influx, which may cause aberrant engorgement of endosomes (Figure 8), favoring exocytosis (15). However, the precise role for endosome ion content in regulating transport remains obscure at present.

Monensin treatment acted as a "switch" that both inhibited anti-ICAM/NC delivery to lysosomes and enhanced plasma membrane recycling of internalized anti-ICAM/NCs. This in turn prevented degradation and prolonged the duration of anti-ICAM/catalase/NCs to protect against oxidant-mediated toxicity. This offers another method to modulate the duration and, perhaps, efficacy of nanocarrier-based therapeutic agents that complements other approaches including: i) modifying nanocarrier geometry to influence internalization rate and traffic, ii) prolonging nanocarrier retention time in endosomes, by inhibiting delivery to lysosomes using nocodazole, iii) inhibiting nanocarrier degradation within lysosomes by increasing lysosome pH using weak bases, and iv) through the use of sustained treatment by the administration of multiple doses of nanocarriers (36, 37, 56). Monensin treatment differs from these methods in that it provides a means to enable anti-ICAM/NCs to be simultaneously and stably localized to the plasma membrane and recycling endosomal
compartments. In the case of anti-ICAM/NCs containing catalase, this strategy may help maximize the interception of both extracellular and intracellular oxidants. Although toxicity should be taken into consideration as a limiting factor in terms of in vivo therapies (8, 44), pharmacologic agents with the ability to inhibit trafficking of endocytosed anti-ICAM/NCs to lysosomes and to enhance plasma membrane recycling are likely to provide a useful adjunct to enhance the duration and efficacy of nanocarrier-based drug delivery systems. Our findings suggest that understanding roles for NHEs in regulating vesicle targeting is key to the successful implementation of auxiliary pharmacologic agents that optimize the application of targeted nanocarriers as a drug delivery platform.

From a more general perspective, results of this study are of interest for endothelial biology and transport fields. For example, NHE1-mediated ICAM-1-dependent cytoskeletal reorganization, similar to that observed in this work, may be involved in regulation of vascular permeability during pathological events. Indeed, actin filament formation controls endothelial barrier properties (46). Furthermore, microvascular endothelial cell NHEs have been implicated in ischemia/reperfusion injury by enhancing ICAM-1 surface density via Ca\(^{2+}\) signaling (17), a mediator which is known to increase microvessel permeability during lung injury (30, 50, 51). Whether NHEs also regulate protein transport across the lung epithelial barrier, as in the case of transcytosis involving caveoli or clathrin-coated pits (19), remains to be determined.

In conclusion, the Na\(^+\)/H\(^+\) exchanger proteins NHE1 and NHE6 regulate anti-ICAM/NCs-induced actin reorganization and subsequent internalization and vesicular trafficking by ECs, which may play physiological and pathophysiological role in a variety of processes including endothelial drug delivery. Pharmacological manipulations of these
endothelial NHEs may help modulate the sub-cellular delivery, longevity and effects of therapeutics targeted to endothelium by anti-ICAM/NCs.
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FIGURE LEGENDS

**Figure 1. NHE inhibitors and Ca^{2+} blockers suppress CAM-mediated endocytosis and actin remodeling.** a-f. TNF-α activated HUVEC were incubated with fluorescent anti-ICAM/NCs for 1 h at 37°C in either control medium (a) or medium containing amiloride (b), EIPA (c), monensin (d), BAPTA (e), or thapsigargin (TG) (f), fixed and counterstained with TexasRed-labeled goat anti-mouse IgG. Double-labeled yellow particles represent anti-ICAM/NCs bound to the cell surface (arrows) and single-labeled green particles are anti-ICAM/NCs internalized within the cells (arrowheads). Bar = 10 μM. g. Quantification of CAM-mediated endocytosis, calculated as mean ± SD (n ≥ 70 cells from 2 independent experiments). h-o. Cells were incubated with anti-ICAM/NCs in either the absence (h-k) or presence of EIPA (l-o) for 0 min (h,l), 5 min (i,m), 1 h (j, n) or 3 h (k, o), then fixed and F-actin was labeled using AlexaFluor 594-conjugated phalloidin. Control cells showed formation of stress fibers (arrows) induced by anti-ICAM/NCs, as opposed to EIPA-treated cells. Bar = 10 μM.

**Figure 2. NHE1-specific siRNA inhibits internalization and actin reorganization induced by anti-ICAM/NCs.** a,b. EAhy926 cells were transfected with siRNA targeted to either a non-represented sequence (control), NHE1 or NHE6, then incubated for 2 days, harvested and the amount of NHE1 or NHE6 protein was determined by immunoblot. By densitometry (b), siRNA significantly reduced protein expression to ~40 % of control values (p < 0.05, n=3). c. Cells treated with control, NHE1 or NHE6 siRNA were incubated for 24 h and then assayed for the amount of anti-ICAM/NCs internalized during 1 h incubation at 37°C. Data are mean ± SE (n ≥ 70 cells from 2 independent experiments) normalized to the level of internalization for control transfected cells (* p<0.1). d-f. Cells
treated with control (d), NHE1 (e) or NHE6 (f) siRNA were incubated for 24 h and then assayed for actin stress fibers (arrows) induced by anti-ICAM/NCs using by AlexaFluor 594-conjugated phalloidin. Bar - 10 μm.

**Figure 3. Clustering by anti-ICAM/NCs induces complex formation between ICAM-1 and NHE1.** a,b. TNF-α activated HUVEC incubated in the absence (a) or presence (b) of anti-ICAM/NCs, fixed and immunostained for NHE1. Anti-ICAM/NCs induced clustering of NHE1 (arrowheads). Bar = 10 μm. c. Cells were incubated with anti-ICAM/NCs for 15 min at 37°C, fixed and then triple labeled to show surface-localized anti-ICAM/NCs (AlexaFluor 350, blue), nanocarriers (green) and NHE1 or NHE6 (Texas Red). White particles represent sites of NHE1 clustering by anti-ICAM/NCs at the cell surface (arrow in high magnification inset), whereas yellow color represents colocalization within intracellular vesicles (arrowhead in inset). Bar = 10 μm. Inset bar = 1 μm. d. Quantification of cells labeled as in c, showing the percentage co-localization of markers (NHE1 or NHE6) with anti-ICAM/NCs. e. Control cells or cells treated with anti-ICAM/NCs for 30 min at 4°C were harvested, solubilized and then immunoprecipitated with antibodies against the cytosolic domain of ICAM-1. By immunoblot, cells incubated with anti-ICAM/NCs formed co-immunoprecipitatable NHE1/ICAM-1 complexes, as quantified in panel f, where data are mean ± SE (n = 3). Pre-treatment of cells with EIPA had little effect on NHE1/ICAM-1 complex formation. g. At steady state, in the absence of anti-ICAM/NCs, few ICAM-1 molecules form a complex with NHE1. NHE1/ICAM-1 complex formation is induced by binding of anti-ICAM/NCs, which clusters ICAM-1 in the plasma membrane. In this model, the signal cascade initiated by anti-ICAM/NC binding to ICAM-1 triggers the formation of actin fibers through interactions with the
cytosolic domain of NHE1. Uptake and stress fiber formation were inhibited by EIPA, however, NHE1/ICAM-1 complex formation was not.

Figure 4. Transport of anti-ICAM/NCs from NHE1 to NHE6 positive endosomes. a-d. HUVEC were incubated with anti-ICAM/NCs at 37°C for either 30 min (a,c) or 60 min (b,d), then fixed and immunostained for either NHE1 (a,b) or NHE6 (c,d). Internalized anti-ICAM/NCs co-localizing with either NHE1 or NHE6 are denoted by arrowheads in high magnification insets. Bar = 10 μm. Inset bar = 1 μm. Quantification of anti-ICAM/NCs co-localized with either NHE1 or NHE6 at 30 or 60 min after internalization (mean ± SD, n ≥ 70 cells from 2 independent experiments). f,g. Cells treated with anti-ICAM/NCs at 37°C for 15 or 60 min, harvested, solubilized and immunoprecipitated with anti-ICAM or anti-NHE1 antibodies. By immunoblot, the amount of co-immunoprecipitable NHE1/ICAM-1 complexes decreased with increasing time at 37°C, as quantified in g, where data are mean ± SE (n = 3) and the dashed line corresponds to the amount of NHE1/ICAM complex formed at the plasma membrane (Figure 3 e,f).

Figure 5. Monensin inhibits delivery of anti-ICAM/NCs to lysosomes. HUVEC were pre-incubated with Texas Red dextran to label lysosomes, then further incubated with fluorescent anti-ICAM/NCs (green) for 1 h (a,d,g,j), 2 h (b,e,h,k) or 3 h (c,f,I,l) in control medium (a-c), or medium containing EIPA (d-f), H7 (g-i) or monensin (j-l), fixed and imaged by fluorescence microscopy. Control cells transported anti-ICAM/NCs to Texas Red-labeled lysosomes (arrows), which was accelerated by H7. In contrast, anti-ICAM/NCs internalized by monensin treated cells showed little co-localization with lysosomes (arrowheads). Bar = 10 μm. m. Quantification of anti-ICAM/NC delivery to Texas Red-
labeled lysosomes. Shown is the mean ± SD of n ≥ 70 cells from 2 independent experiments. By 3 h, the majority of internalized anti-ICAM/NCs were transported to lysosomes by control cells (black bars) and EIPA treated cells (hatched bars) with comparable kinetics. In contrast, cells treated with H7 (white bars) had more rapid transport of nanocarriers to lysosomes and cells treated with monensin showed little, if any nanocarriers delivery to lysosomes (gray bars).

Figure 6. Monensin enhances anti-ICAM/NC recycling to the plasma membrane. HUVEC were incubated with anti-ICAM/NCs (green) for 1 h (a,d), 2 h (b,e) or 3 h (c,f), in control (a-c) or monensin containing medium (d-f), fixed, immunolabeled for surface-bound anti-ICAM/NCs (blue), permeabilized and finally immunostained for an early endosome marker, EEA-1 (red). Arrowheads denote anti-ICAM/NCs localized to EEA-1 positive endosomes. Arrows denote anti-ICAM/NCs localized to the plasma membrane. Bar = 10 µm. g. Transport of anti-ICAM/NCs through early endosomes was comparable for control cells (black bars) and monensin-treated cells (gray bars). h. Cells treated with monensin show the majority of anti-ICAM/NCs localized to the plasma membrane 3 h after internalization. In contrast, control cells showed few anti-ICAM/NCs localized to the plasma membrane, instead, anti-ICAM/NCs internalized by these cells were delivered to lysosomes (Figure 5). Mean ± SD of n ≥ 70 cells from 2 independent experiments.

Figure 7. Monensin delays anti-ICAM/NC degradation and prolongs the anti-oxidant effect of anti-ICAM/NC/catalase. a-f. Control or monensin-treated HUVEC were incubated with fluorescent anti-ICAM/NCs for 1 h (a,d), 2 h (b, e) or 3 h (c,f), fixed,
permeabilized and immunostained for intact anti-ICAM using Texas Red labeled anti-mouse IgG. Intact, yellow anti-ICAM/NCs are denoted by arrows, nanocarriers with degraded anti-ICAM are denoted by arrowheads. Bar = 10 µm. g. Quantification of anti-ICAM/NCs degradation. Relative stability was determined as the percent of yellow anti-ICAM/NCs, determined as mean ± SD of n ≥ 70 cells from 2 independent experiments. Nearly all of the anti-ICAM coating was degraded by control cells (black bars) after 3 h at 37 °C. In contrast, most of the anti-ICAM/NCs were intact at this time point in the case of monensin treated cells (gray bars). h. Control HUVEC (black bars) or HUVEC treated with monensin (gray bars) were incubated with anti-ICAM/NC/catalase for varying periods of time and then challenged with H₂O₂ to induce oxidative injury. Percent of cell survival was quantified by fluorescence microscopy using the Live/Dead assay from at least 500 cells per condition and represent mean ± SD. Monensin treatment significantly enhanced antioxidant protection of anti-ICAM/NC/catalase.

**Figure 8. Model for endocytosis and sorting of anti-ICAM/NCs.** In control cells, NHE6 exchanges Na⁺ for H⁺ to help regulate endosome acidification by vacuolar H⁺/ATPase. Acidification in the early endosome favors dissociation of anti-ICAM/NCs from ICAM-1 and NHE1, thus permitting delivery of anti-ICAM/NCs to lysosomes and recycling of ICAM-1 to the plasma membrane. Monensin enhances Na⁺/H⁺ exchange independently of NHE6 and thus inhibits endosome acidification and increases endosome Na⁺ content, resulting in a net influx of H₂O into the endosome lumen. This inhibits anti-ICAM/NC dissociation from ICAM-1, prevents lysosomal maturation and favors recycling of internalized anti-ICAM/NCs to the plasma membrane.
Figure 1
Figure 2
Figure 3

(a) Control
(b) anti-ICAM/NC

(c) NHE1
(d) NHE6

(e) IP: ICAM-1
(f) NHE1/ICAM-1

(g) steady state, binding/clustering, uptake/actin binding
Figure 4

(a) and (b) show immunofluorescence images of NHE1 at 30 min and 60 min, respectively. (c) and (d) show NHE6 images at the same time points. (e) Graph showing the percentage of colocalization between NHE1 and NHE6 at 30 min and 60 min. (f) shows IP: ICAM-1 with IB: NHE1 and IB: ICAM-1 images at 15 and 60 minutes. (g) Graph showing the relative protein co-IP with IP: ICAM-1 and NHE1 images at 15 and 60 minutes.
Figure 5

![Image showing the effects of different treatments (control, EIPA, H7, monensin) on lysosomal localization over time (1h, 2h, 3h)].

A bar graph below the images shows the percentage of lysosomal localization for each treatment at 1h, 2h, and 3h time points, with error bars indicating variation.
Figure 6

(a, b, c) Control

(d, e, f) Monensin

(g, h) Graph showing endosomal localization and recycling percentage over time.
Figure 7
Figure 8