Endocytic response of type I alveolar epithelial cells to hypertonic stress

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Wang S, Singh RD, Godin L, Pagano RE, Hubmayr RD. Endocytic response of type I alveolar epithelial cells to hypertonic stress. Am J Physiol Lung Cell Mol Physiol 300: L560–L568, 2011. First published January 21, 2011; doi:10.1152/ajplung.00309.2010.—We present plasma membrane (PM) internalization responses of type I alveolar epithelial cells to a 50 mosmol/l increase in tonicity. Our research is motivated by interest in ATI repair, for which endocytic retrieval of PM appears to be critical. We validated pharmacological and molecular tools to dissect the endocytic machinery of these cells and used these tools to test the hypothesis that osmotic stress triggers a pathway-specific internalization of PM domains. Validation experiments confirmed the fluorescent analog of lactosyl-ceramide, transferrin, and dextran as pathway-specific cargo of caveolar, clathrin, and fluid-phase uptake, respectively. Pulse-chase experiments indicate that hypertonic exposure causes a downregulation of clathrin and fluid-phase endocytosis while stimulating caveolar endocytosis. The toxicity-mediated increase in caveolar endocytosis was associated with the translocation of caveolin-1 from the PM and was absent in cells that had been transfected with dominant-negative dynamin constructs. In separate experiments we show that hypertonic exposure increases the probability of PM wound repair following micropuncture from 82 ± 4 to 94 ± 2% (P < 0.01) and that this effect depends on Src pathway activation-mediated caveolar endocytosis. The therapeutic and biological implications of our findings are discussed.

acute lung injury; caveolar endocytosis; cell repair; osmotic pressure

THE ALVEOLAR EPITHELIOID is composed of two cell populations, namely the very large thin type I cells (ATI) (38) and the cuboidal secretory type II cells (ATII) (41). In the acutely injured lung many ATI and to some extent ATII die by mechanisms of necrosis and apoptosis whereby the resulting alveolar wounds become coated with a fibrin-rich matrix (40). In addition, the derecruitment of lung units and the accumulation of liquid in alveolar spaces predisposes ATI and ATII to mechanical injury by overdistension and interfacial stress (4). Although physical cell wounding undoubtedly contributes to ATI necrosis, many alveolar epithelial cells repair their PM wounds and remain viable, albeit in a stressed state (37). When tested in reduced systems, this stress manifests as activation of early stress response genes and the release of proinflammatory cytokines (7).

Cell repair requires the coordinated interplay between membrane trafficking and cytoskeletal remodeling. Cell wounding-induced membrane trafficking provides the lipid substrate for plasma membrane (PM) repair and involves both exocytic and endocytic trafficking pathways (13, 20, 34). The relative contributions of each to PM repair and their coordination are poorly understood. Moreover, there is relatively little specific information on ATI repair since these cells have traditionally proven difficult to harvest and to maintain with sufficient purity in culture. To the extent to which lessons from other cell wounding models apply to ATI, one should expect that wounding will trigger the fusion and exocytosis of calcium-sensitive endomembranes, foremost lysosomes, followed by a polymerization response of the cytoskeleton near the lesion (20). Recent observations on cells exposed to pore-forming toxins or subjected to large mechanical wounds have underscored the importance of sphingomyelinase-mediated ceramide generation in facilitating cell repair (34). This mechanism links lysosomal exocytosis, the source of sphingomyelinase, with ceramide-mediated endocytosis (24). We had previously identified hypertonic cell conditioning as an intervention that prevents epithelial deformation injury and promotes PM repair in rodent lungs (25). To gain insights into osmotic stress-induced PM remodeling, specifically in ATI, we tested and validated molecular and pharmacological tools to dissect the endocytic machinery of these cells. We subsequently used these tools to test the hypothesis that osmotic stress triggers a pathway-specific internalization of PM domains and that this response is integral to PM wound repair. Our observations on ATI indicate that osmotic stress causes a transient downregulation of clathrin and fluid-phase endocytosis, while stimulating caveolar endocytosis. Moreover, we show that the toxicity-induced increase in caveolar endocytosis is mediated by Src pathway activation and is associated with improved cell repair. The biological implications of these findings will be discussed.

MATERIALS AND METHODS

Plasmids, Antibodies, and Reagents

These are listed in the electronic data supplement (the online version of this article contains supplemental data).

Cell Isolation and Culture

Rat ATIs were isolated by immunoselection for T1alpha (39). ATIs were seeded on glass coverslips and cultured in high-glucose DMEM supplemented with 10% FBS and 200 units (μg)/ml of penicillin and streptomycin and maintained in a 5% CO2 humidified 37°C incubator. Cells were studied 5–7 days after harvest.

Micropuncture Injury

A detailed description of the method for inducing focal PM injury and its validation can be found in Belete et al. (1). Briefly, cells were
plated on glass-bottom dishes, washed, loaded with the desired label, and mounted onto a motorized stage on an Axiosvert S100 TV fluorescence microscope (Zeiss, Thornwood, NY) equipped with a mercury short-arc lamp source. Cells were visualized with a ×100 oil immersion lens, and a Hamamatsu Orca-ER CCD digital camera was used to capture images every 0.6 to 0.8 s. A motorized microinjector (InjectMan; Eppendorf, Hauppauge, NY) was used to position a 1 μm diameter glass needle (Femtotip, Eppendorf) within the field of view above the targeted cell. The cell was then stabbed at a 45° angle with injury duration of 0.3 s. In some experiments, cells were preincubated with the Src-kinase inhibitor PP2 (10 μM) for 1 h at 37°C.

Indicators of Cell Remodeling and Repair

PM integrity was inferred from the temporal decay in calcine AM fluorescence (Molecular Probes-Invitrogen, Carlsbad, CA) following micropuncture, and was validated through exclusion of propidium iodide (5 μg/mL), which was applied 2 min after injury. Calcine AM diffuses freely throughout the cytosol and as such provides information about solute exchange across a PM wound (36). Cultured ATIs were treated with either BODIPY-LacCer (2.5 μM) and incubated with either BODIPY-LacCer (2.5 μM) and incubated with either BODIPY-LacCer (2.5 μM) or BODIPY-LacCer (5 μg/mL) at 10°C for 30 min to prelabel the PM. For experiments involving Tfn, cells were first serum starved for 2 h at 37°C. Cells were then washed in isotonic rodent Ringer solution, which contained 138 mm NaCl, 2.7 mm KCl, 1.06 mm MgCl2, 5.6 mm d-glucose, 12.4 mm HEPES, and 1.8 mM Ca2+ with a pH of 7.25 and were allowed to equilibrate for 10 min. After transfer of the cells onto the imaging system, baseline fluorescence was recorded and cells with stable baseline readings were identified. Individual cells were then injured as described above. The rate of fluorescence decay of wounded cells with stable baseline readings was analyzed. As established in preliminary experiments, we determined that a decay threshold of 0.04%/s was associated with a 4% false positive wound repair rate in ATIs (1). The osmosality of the Ringer solution used to treat the cells was 240, 290, and 340 mosM, respectively.

Pulse-Chase Experiments

BODIPY-lactosyl ceramide (LacCer), AF594/488/647-transferrin (Tfn), and AF 594/488/647-dextran (Dex) served as pathway-specific cargo in pulse-chase experiments as previously described (17). In brief, cells were washed with HEPES minimum essential medium (HMEM) and incubated with either BODIPY-LacCer (2.5 μM) or AF594/488/647-Tfn (5 μg/mL) at 10°C for 30 min to prelabel the PM. For experiments involving Tfn, cells were first serum starved for 2 h at 37°C to upregulate the Tfn receptor. To assess the rate of internalization of prelabeled endocytic markers cells were transferred to a 37°C water bath for a given time period. Excess PM-bound BODIPY-LacCer was removed by back exchange at 10°C with 5% fatty acid-free BSA-HMEM, so that internalized LacCer could be identified on epifluorescence images (2). For the same reason, PM-bound Tfn was acid stripped on ice for 15–20 s with acetic acid-HMEM (pH 3.0). Experiments involving the fluid-phase marker Dex did not require preincubation at 10°C. Cells were labeled at 37°C with 100 μg/ml prewarmed Dex and gently washed and imaged at the specified times.

Comparisons of pathway-specific uptake kinetics between cells exposed to isotonic and hypertonic media were carried out in parallel. At the time points of interest (1, 3, 5, and 10 min following hypertonic challenge), cells labeled with LacCer were fixed on ice with 3% paraformaldehyde and 0.02% glutaraldehyde for 15 min, and the PM-bound BODIPY-LacCer removed by back exchange. Cells labeled with Tfn were acid stripped and then fixed as described above. Cells labeled with Dex were washed with ice-cold HMEM and subsequently fixed.

Colocalization Assay

Colocalization assays were carried out to validate the specificity of pathway markers LacCer, Tfn, and Dex. To eliminate confounding “bleedover” of BODIPY-LacCer green into the red channel, we used AF647 far-red-tagged Dex or Tfn in the respective colabeling studies. Experiments were restricted to 1 min, after which cells were placed on ice, to guard against merging between pathways.

Use of Dominant-Negative Constructs

To distinguish between the responses of fluid-phase and clathrin-dependent endocytic pathways, ATI were genetically modified by overexpressing dominant-negative pathway-specific constructs. ATI were transfected by using Lipofectamine with either dominant-negative construct DN Cdc42 or DN AP180 following the manufacturer’s instruction (2). In studies of DN AP180 protein overexpression, cells were cotransfected with DN AP180 and pDsRed2-Nuc, so that the nuclei of transfected cells could be identified by their red color (32). In these studies the endocytic cargo molecules (AF488-Tfn, AF488-Dex, and BODIPY-LacCer) were labeled green. To evaluate the effects of Cdc42 on Tfn and Dex uptake, the cells were transfected with DN Cdc42-GFP. The cargo molecules (AF594-Tfn and AF594-Dex) were labeled red. In experiments with BODIPY-LacCer, which emits a green fluorescence, cells were transfected with DN Cdc42-mRed. To distinguish between caveolar and noncaveolar uptake of LacCer, a dominant-negative dynamin construct, DN dynamin, was used to cotransfect ATI with pDsRed2-Nuc. Inhibition of LacCer uptake in DN dynamin transfected cells served to eliminate osmotic stress-mediated bilayer “flipping” as confounding internalization route of this caveolar pathway marker.

mRed-Cav-1 Overexpression

Studies on several different cell lines had suggested that BODIPY-LacCer is primarily internalized by caveolar endocytosis (2, 32). To confirm this finding in ATI, cells were genetically modified to overexpress mRed-tagged caveolin-1 (Cav-1) so that colocalization with BODIPY-LacCer could be measured. The colocalization of mRed-Cav-1 with the clathrin and fluid-phase endocytosis specific cargo molecules AF488-Tfn and AF488-Dex was also analyzed to determine specificity.

Pharmacological Inhibitors of Endocytosis

To test the pathway specificity of pharmacological interventions ATI were pretreated at 37°C with HMEM containing 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2, 10 μM), Clostridium difficile toxin B (100 μM), genistein (200 μM) for 60 min preincubation with DN Cdc42 or DN AP180 following the manufacturer’s instruction (2). In studies of DN AP180 protein overexpression, cells were cotransfected with DN AP180 and pDsRed2-Nuc, so that the nuclei of transfected cells could be identified by their red color (32). In these studies the endocytic cargo molecules (AF488-Tfn, AF488-Dex, and BODIPY-LacCer) were labeled green. To evaluate the effects of Cdc42 on Tfn and Dex uptake, the cells were transfected with DN Cdc42-GFP. The cargo molecules (AF594-Tfn and AF594-Dex) were labeled red. In experiments with BODIPY-LacCer, which emits a green fluorescence, cells were transfected with DN Cdc42-mRed. To distinguish between caveolar and noncaveolar uptake of LacCer, a dominant-negative dynamin construct, DN dynamin, was used to cotransfect ATI with pDsRed2-Nuc. Inhibition of LacCer uptake in DN dynamin transfected cells served to eliminate osmotic stress-mediated bilayer “flipping” as confounding internalization route of this caveolar pathway marker.

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and chlorpromazine (CPZ, 8 μg/ml), nystatin (25 μg/ml), or methyl β-cyclodextrin (mβ-CD, 200 μM) for 30 min, respectively (2, 29). Inhibitors were then present throughout all subsequent steps of the experiments.

**Cav-1 Translocation**

PM-associated Cav-1 was identified by immunostaining of methanol cold (−20°C) fixed cells with the antibody (M0TL43420), which has a PM Cav-1 pool preference, as described by Pol et al. (27). To identify total Cav-1, cells were fixed with 4% paraformaldehyde at room temperature for 15–30 min, permeabilized with 0.1% saponin, and the antibody (RbTL) with affinity to all fractions of Cav-1 was added. PM labeling was quantified with fluorescence microscopy, whereas total Cav-1 labeling was assessed by total internal reflection fluorescence (TIRF) microscopy. In some experiments, cells were pretreated with the Src-kinase inhibitor PP2 (10 μM) for 1 h at 37°C.

**Src Pathway Activation**

ATI cells were pretreated with the Src-kinase inhibitor PP2 (10 μM) or placebo for 1 h at 37°C and subsequently challenged with hypertonic saline (340 mosM) for 30, 60, and 120 s, respectively. Cell lysate was collected with RIPA lysis buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 0.01 M Na₂HPO₄, 1 mM NaF, 5 mM Na₃VO₄, 1 mM PMSF, 10 μg/ml of each aprotinin and leupeptin, pH 7.2). Bound phospho Src (P-Src) and total Src (T-Src) were analyzed by SDS-PAGE separation on a 10% polyacrylamide gel, followed by immunoblotting with a polyclonal antibody against P-Src (at phosphorylation site pY418) and a monoclonal antibody against T-Src at the size of 59 kDa. The phosphorylation of Src was quantified by use of Quantity One 1-D Analysis Software (Bio-Rad) and normalized by T-Src and β-actin loading controls.

**Image Analysis and Statistics**

Epifluorescence images were acquired with a Olympus AX70 microscope and analyzed via the MetaMorph image processing program (Molecular Devices, Sunnyvale, CA) (2). TIRF imaging was carried out by use of an Olympus IX70 microscope with an Olympus TIRF module. More than 10 image fields and 30–50 cells were analyzed per experiment. All photomicrographs in a given experiment were exposed and processed identically for a given fluorophore. For colocalization studies, no bleedover between microscope channels was observed at the concentration and exposure setting used. The data presented in each treatment group represent the average fluorescence values normalized by the values of the corresponding matched controls. Data are displayed as means and standard deviations. Statistical significance of differences between treatments, individual labels, and time from challenge was tested via a one-way ANOVA with the

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**Fig. 2.**

**A**: effects of 6 pharmacological agents on the endocytic uptake of transferrin (Tfn), lactosyl ceramide (LacCer), and dextran (Dex) in rat ATI. Data are the results of pulse-chase experiments at the 5 min time point. CTL, placebo control; CPZ, chlorpromazine; MB-CD, methyl β-cyclodextrin; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine. *P < 0.01 compared with controls. **B**: effects of dominant-negative (DN) AP180 and Cdc42 gene overexpression on endocytic uptake of Tfn, Dex, and LacCer in rat ATI. The cells in left panels were cotransfected with DN AP180 and pDsRed2-Nuc, so that the nuclei of transfected cells (also marked by white dashed margins) could be identified by their red color. In contrast, the endocytic cargo molecules (AF488-Tfn, AF488-Dex, and BODIPY-LacCer) appear green. To evaluate the effects of DN Cdc42 overexpression on Tfn and Dex uptake the cells in the top right panels were transfected with DN-Cdc42-GFP. They may be identified by their green cytoplasmic fluorescence (outlined by white broken lines). The cargo molecules (AF594-Tfn and AF594-Dex) appear in red. Because BODIPY-LacCer emits a green fluorescence, the cells at bottom right were transfected with DN Cdc42-mRed, i.e., transfected cells (outlined by white broken lines) may be identified by their red fluorescence. Note that overexpression of DN AP180 resulted in selective inhibition of Tfn uptake (top left) whereas overexpression of DN Cdc42 resulted in selective inhibition of Dex uptake (middle right). Scale bar, 50 μM. **C**: averages ± SD of results from experiments shown in B (derived from ≥8 cells in each of 3 separate isolations/experiments). *P < 0.01 compared with controls.
Tukey-Kramer multiple-comparison test on a JMP program (SAS, Cary, NC).

RESULTS

Hypertonic Exposure Increases the Probability of PM Wound Repair in ATI

ATI were injured with a 1-μm-diameter glass pipette. Subsequent PM repair was inferred from the fluorescence decay patterns of the cytosolic label calcein. Figure 1 shows the probability of repair, i.e., the percentage of cells that successfully repaired micropuncture wounds, when exposed to media of different tonicities, namely, hypotonic (240 mosM), isotonic (290 mosM), and hypertonic (340 mosM). The probability of repair was significantly greater for ATI exposed to hypertonic media (101 of 108 cells, 94 ± 2%; \( P < 0.01 \)) than those exposed to either isotonic (92 of 112 cells; 82 ± 4%) or hypotonic media (85/112; 76 ± 4%), respectively.

Molecular Specificity of Endocytic Pathways in ATI

Pathway selectivity of pharmacological inhibitors. BODIPY-LacCer uptake was inhibited by the cholesterol sequestering agent mβ-CD as well as by nystatin, by the nonspecific tyrosine kinase inhibitor genistein, and by the Src kinase inhibitor PP2 (Fig. 2A). However, BODIPY-LacCer uptake was unaffected by pharmacological inhibitors of the clathrin pathway such as CPZ or inhibitors of the fluid-phase pathway such as toxin B. As expected, the uptake of Tfn was inhibited by CPZ but was unaffected by inhibitors of caveolar endocytosis (mβ-CD and PP2) or of fluid-phase uptake such as toxin B. In turn, Dex uptake was inhibited by toxin B but not by PP2, mβ-CD, nor CPZ. In aggregate, manipulating key elements of the three endocytosis pathways under consideration by molecular and pharmacological means established Tfn, Dex, and LacCer as pathway-specific cargo.

Overexpression of dominant-negative constructs acting on clathrin and fluid-phase pathways. The overexpression of the dominant-negative construct DN AP180, an adaptor protein and key mediator of clathrin-dependent endocytosis, inhibited 68% of Tfn uptake but had no effect on Dex uptake (Fig. 2, B and C). Overexpression of the dominant-negative construct DN Cdc42, a cytoskeleton-associated protein and key regulator of fluid-phase endocytosis, inhibited 80% of Dex uptake but had no effect on the internalization of Tfn (Fig. 2, B and C). Not surprising, these data confirm that Tfn and Dex may be used to quantitate clathrin and fluid-phase uptake mechanisms in ATI. Neither DN AP180 nor DN Cdc42 altered the uptake of

![Fig. 3](http://ajplung.physiology.org/)

**Fig. 3.** Relative distributions (colocalization) of caveolin-1 (Cav-1) with LacCer, Tfn, and Dex in mRed-tagged Cav-1 overexpressing ATI. A and B: there is a 70% concordance between the itineraries of LacCer (G, green) and Cav-1 (R, red), but only a 20 and 25% colocalization between Dex and Tfn, respectively. *\( P < 0.01 \) compared with LacCer/Cav-1. C and D: colocalization studies between LacCer-G (BODIPY-green)/Dex-R (AF647-far red) and LacCer-G (BODIPY-green)/Tfn-R (AF647-far red), with corresponding magnification of a small region. The lack of significant colocalization (red and green labels remain for the most part confined to different membrane compartments) is consistent with Dex and Tfn’s preference for different, i.e., noncaveolar uptake routes. Scale bar, 20 μM.
BODIPY-LacCer, suggesting that this sphingolipid is internalized by an alternative endocytic pathway.

**Overexpression of Cav-1 constructs and LacCer itinerary.** As shown in Fig. 3, A and B, 70% of internalized BODIPY-LacCer colocalized with mRed-Cav-1, whereas only 20% of internalized Dex and 25% of internalized Tf colocalized with mRed Cav-1. BODIPY-LacCer colocalized only 25% and 20% with the clathrin and fluid-phase markers AF647-Tf and AF647-Dex, respectively (Fig. 3, C and D). These findings suggest that caveolar endocytosis is the preferred uptake route for the sphingolipid LacCer (31).

**Endocytic PM Remodeling in Response to Hypertonic Stress**

The relatively small increase in medium tonicity (50 mosM) caused divergent PM retrieval responses, enhancing caveolar endocytosis on the one hand and inhibiting clathrin-dependent and fluid-phase endocytosis on the other (Fig. 4). ATI transfected with a dominant-negative construct of dynamin failed to internalize LacCer under either control or osmotic stress conditions (Fig. 5). This observation indicates that the hypertonic stress-induced increase in LacCer uptake was indeed-mediated by caveolar uptake mechanisms as opposed to a PM translocase. Consistent with this interpretation, immunostaining of antibodies with high affinity to the PM-associated Cav-1 indicated its translocation to a cytosolic compartment (Fig. 6).

**Tonicity-Induced Stimulation of Caveolar Endocytosis and Enhanced PM Wound Repair are Dependent on Src Pathway Activation**

Consistent with the interpretation of Fig. 6, the epifluorescence and TIRF images of Fig. 7A show that a 50 mosM increase in medium tonicity caused the internalization of PM associated Cav-1 (note the decrease in PM fluorescence in both epifluorescence and TIRF images labeled Hyper in Fig. 7A). Cav-1 internalization was blocked by pretreatment of ATI with the Src-kinase inhibitor PP2 (Fig. 7, B and C). This observation is in agreement with the PP2-related inhibition of Lac-Cer uptake shown in Fig. 2A. By preventing the osmotic stress-induced activation of the Src pathway (Fig. 7D), PP2 not only inhibited caveolar endocytosis but also abolished the salutary effect of hypertonic exposure on PM wound repair (Fig. 7E).

**DISCUSSION**

We have shown that a relatively small increase in extracellular tonicity of 50 mosM inhibits PM retrieval in ATI via the receptor-mediated and fluid-phase endocytic pathways. Our observations also suggest that this inhibition is cargo and uptake pathway specific and does not apply to caveolar endocytosis, which in fact was transiently enhanced. We have shown that ATI exposed to hypertonic media are more likely to repair PM wounds and that tonicity-mediated facilitation of

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**Fig. 4.** Pulse-chase experiments of Tf, LacCer, and Dex in ATI exposed to a 50 mosM increase in extracellular tonicity for 10 min. A: representative 5 min snap shots of Tf (red)-, LacCer (green)-, and Dex (red)-labeled ATI after exposure to an isotonic (Iso; top) vs. hypertonic (Hyper, bottom) environment. Note that, compared with isotonic controls cells exposed to a hypertonic challenge lack the punctate Tf and Dex structures, indicative of an endosomal staining pattern. In contrast, osmotically challenged cells show a more intense granular LacCer staining pattern consistent with enhanced endocytosis of this caveolar uptake label. Scale bar, 50 μM. B: average results of osmotic challenge responses shown as % of time-matched isotonic controls. Exposure of ATI to a 50 mosM increase in medium tonicity was associated with a transient inhibition of clathrin and fluid-phase marker uptake (Tf and Dex) and an increase in caveolar endocytosis (LacCer). *P < 0.01 between LacCer and either Dex or Tf at the 3-, 5-, and 10-min time points.
Cell repair is critically dependent on Src pathway activation and associated caveolar endocytosis. Our interest in the topic “osmotic stress response of ATI” originated with the observation that exposing rodent lungs to a hypertonic environment resulted in alveolar epithelial cell protection against deformation injury (25). We had initially postulated that this mechanoprotective effect reflected hypertonic stress-induced increases in the cells’ surface-to-volume ratio. A disproportionate decrease in cell volume relative to the surface area of the PM envelope would allow excess PM to unfold and thereby maintain sublytic tension in the face of deforming stress. Given this hypothesis we needed to understand the mechanisms/molecular pathways by which ATI regulate PM surface area in response to environmental stress (21, 22). In reviewing the large literature on the topic it became readily apparent that osmotic stress responses as well as the cargo selectivity of endocytic pathways varied greatly among cell types (9, 12, 18). Furthermore, most studies to date had employed much larger stresses (hundreds of mosM) than were tested here. Since no ATI-specific data were available, we felt it important to validate the tools and approaches that are commonly used to characterize endocytic uptake pathways in other mammalian systems. Consequently, Tfn, Dex, and Lac-Cer proved to be pathway-specific cargo of receptor-mediated, fluid-phase, and caveolar endocytosis mechanisms in ATI, respectively. The overexpression of the dominant-negative construct DN AP180, a key mediator of clathrin-dependent endocytosis, inhibited 68% of Tfn uptake, whereas overexpression of the dominant-negative construct DN Cdc42, a key regulator of fluid-phase endocytosis, inhibited 80% of Dex uptake (Fig. 2). Importantly, inhibition of these pathways did not shunt the respective cargo molecules to alternative uptake routes (16). The results of pharmacological inhibition of the clathrin, fluid-phase, and caveolar pathways, respectively, were consistent with responses in other mammalian cells (18). Accordingly, CPZ inhibited Tfn/receptor-mediated uptake, whereas toxin B was relatively selective for Dex/fluid-phase endocytosis. Moreover, cholesterol depletion of the PM with the sequestering agent mβ-CD was associated with a profound inhibition of LacCer uptake, presumably reflecting destruction of caveolar microdomains. In contrast to some mammalian cells, reducing the cholesterol content of the PM had no effect on Cdc42-dependent fluid-phase endocytosis mechanisms in ATI (2).

Acute exposure of ATI to a 50 mosM increase in medium tonicity caused a divergent cargo- and pathway-specific membrane internalization response. The uptake of clathrin- and fluid-phase-dependent endocytosis markers was inhibited, whereas that of LacCer, a glycosphingolipid and cargo with preference for the caveolar pathway, was enhanced. Selective inhibition of receptor-mediated endocytosis by hypertonic media had been first reported in polymorphonuclear leukocytes (3). This inhibition occurred irrespective of the ionic strength of the hypertonic solution but did not extend to the uptake of the fluid-phase marker Dex. Inhibition of clathrin-mediated

Fig. 5. Internalization of LacCer (green) in normal ATI and ATI, which overexpress a DN dynamin construct (red nuclear label). ATI were cotransfected with DN dynamin and pDsRed2-Nuc to overexpress a dominant-negative dynamin construct. DN dynamin-expressing cells were outlined by dashed lines and compared with the neighboring untransfected cells in the same image field. A representative example (A) and summary statistics (B) indicate an 80% reduction of LacCer uptake in DN dynamin transfected cells. Scale bar, 50 μM. *P < 0.01 compared with control.

Fig. 6. Representative ATI cell images indicating the abundance of PM caveolin-1 staining (labeled with an antibody with particular affinity to PM-associated Cav-1) in ATI under isotonic conditions (top; note the intense fluorescence at the cell border). Contrast the paucity/absence of PM-associated Cav-1 in cells 5 min after a hypertonic challenge (note the absence of fluorescence at the cell border). Scale bar, 50 μM.
Endocytosis was later confirmed in chick as well as human fibroblasts and was attributed to a cytoplasm acidification-mediated clathrin precipitation into microcages (11). Although cytosolic acidification consequent to hypertonic shock was associated with inhibition of Dex uptake in Dictyostelium cells (26), in mammalian cells hypertonic inhibition of fluid-phase endocytosis had not been reported before.

Previous observations on fibroblasts (NIH3T3) had suggested that a large hypertonic stress (600 mM sucrose) initiates a Src kinase-mediated phosphorylation and translocation of Cav-1 from the PM to the cytosol (15, 38). Our measurements of osmotic stress-induced changes in intracellular Cav-1 distributions in ATI and their dependence on Src pathway activation are consistent with such a mechanism (Figs. 6 and 7). Moreover, the enhanced LacCer uptake following the hypertonic challenge suggests a concomitant upregulation of caveolar endocytosis in ATI. The literature suggests that the activation and translocation of caveolar scaffolding proteins is a required, but not a sufficient, step in the endocytosis of caveolar cargo molecules (18). In fibroblasts, activated Cav-1 had been shown to translocate to focal adhesion complexes raising the possibility of a mechanotransduction mechanism (38). Since Cav-1 translocation could be enhanced by actin network-disrupting agents, it was proposed that Cav-1 activation and translocation are nonspecific manifestation of actin network disruptions in response to a variety of cell stressors. It is generally assumed that the depolymerization of the subcortical cytoskeleton facilitates membrane trafficking to and from the PM. Accordingly, interventions favoring G actin over F actin formation also enhance exocytic responses such as surfactant secretion in type II cells (30).

The hypothesis that the enhanced Cav-1 translocation that had been observed in fibroblasts reflected a stress-related disruption of the actin network is at odds with an overwhelming...
ing body of evidence that hypertonic stress causes the accumulation of F actin and a concomitant polymerization of the subcortical actin network (12, 35). Notwithstanding cell-specific variability in the enzymes that influence both spatial and temporal control of cytoskeletal remodeling, molecular crowding, and reduced availability of free water have profound effects on nucleotide-dependent filament stability, lowering the critical concentration of ADP actin for assembly (5). It is therefore more appropriate to think in terms of osmotic stress-related actin remodeling as opposed to disruption. The uncritical acceptance of a binary state, in which the actin network either facilitates or inhibits membrane trafficking, would also be hard to reconcile with pathway- and cargo-specific effects of osmotic stress, i.e., enhancing some while inhibiting others. A spatially regulated reorganization of the actin cytoskeleton (CSK) can certainly propel vesicular membranes to target sites even without the involvement of conventional molecular motors (14, 28).

Having placed our endocytic PM remodeling results of ATI in the context of published data from nonpulmonary cells and tissues, we will now consider hypotheses that link osmotic stress-mediated PM remodeling with PM wound repair. Initial research on the determinants of PM wound repair underscored the importance of exocytic transport of calcium-sensitive endomembranes, notably of lysosomes, as prerequisite for successful lipid bilayer rescaling (19, 33). It has only become clear recently that the accumulation of lysosome-derived lipids and acid sphingomyelinase at the wound site also triggers an endocytic response, without which bilayer repair is retarded or inhibited (34). The consequences of this series of trafficking events on PM biophysical properties have yet to be fully characterized and understood. On the basis of a detailed account of the temporal and spatial coordination between cytoskeletal remodeling and the accumulation of endomembrane-derived lipids at the injury site, Godin et al. (6) recently concluded that wound edge energy and the fluidity of the subcortical cytoskeleton and of the lipid bilayer were critical for successful repair of PM lesions. However, in the absence of compensatory mechanisms exposure to a hypertonic stress is expected to solidify, i.e., decrease the fluidity of either structure (42). The selective enhancement of caveolar endocytosis and the consequent removal of liquid-ordered PM domains may be one such compensatory mechanism. The osmotic stress-triggered synthesis of ectoines, zwitterionic water-binding organic molecules that have been shown to hydrate model membranes, may be another (10). Godin et al. demonstrated that wounding fluidizes the actin CSK near micropuncture sites irrespective of the actin polymerization state prior to wounding. To the extent to which CSK fluidity is a determinant of the adhesive interactions between PM lipids and the subcortical CSK (23), a fluid CSK network allows greater lateral mobility of PM lipids and thereby promotes bilayer defect closure.

In conclusion, we have shown that the membrane-retrieval response of primary rat ATI to a modest hypertonic stress is nuanced. It is associated with a transient inhibition of cargo uptake via the clathrin and fluid-phase pathways, whereas caveolar endocytosis appears to be enhanced. Activation of the Src pathway appears to be critical for this PM remodeling response. Our study was primarily motivated by interest in cell repair and by a search for lung-protective adjuncts in the care of mechanically ventilated patients. However, the endocytosis pathway-specific osmotic stress response of ATI, which are rich in caveolae, also suggests a means to enhance pulmonary drug or gene delivery through the use of hypertonic aerosols (8). Finally, the surprising finding that a hypertonic milieu enhances as opposed to retards alveolar epithelial repair, raises a number of testable mechanistic hypotheses about the biophysical determinants of cell repair and will further motivate efficacy studies in preclinical lung injury models.

REFERENCES