RhoA required for acid-induced stress fiber formation and trafficking and activation of NHE3

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Yang X, Huang H-C, Yin H, Alpern RJ, Preisig PA. RhoA required for acid-induced stress fiber formation and trafficking and activation of NHE3. Am J Physiol Renal Physiol 293: F1054–F1064, 2007. First published August 8, 2007; doi:10.1152/ajprenal.00295.2007.—Exposure to an acid load increases apical membrane Na+/H+ antiporter (NHE3) activity, a process that involves exocytotic trafficking of the transporter to the apical membrane. We have previously shown that an intact microfilament structure is required for this exocytic process (Yang X, Amemiya M, Peng Y, Moe OW, Preisig PA, Alpern RJ. Am J Physiol Cell Physiol 279: C410–C419, 2000). The present studies demonstrate that acid-induced stress fiber formation is required for stimulation of NHE3 activity. Formation of stress fibers is associated with acid-induced tyrosine phosphorylation and increases in protein abundance of two focal adhesion proteins, p125FAK and paxillin. The Rho kinase inhibitor Y27632 completely blocks acid-induced stress fiber formation and increases in apical membrane NHE3 abundance and activity, but it has no effect on acid-induced tyrosine phosphorylation of p125FAK or paxillin. Herbimycin A completely blocks acid-induced tyrosine phosphorylation of p125FAK and paxillin but only partially blocks stress fiber formation and NHE3 activation. These studies demonstrate that Rho kinase mediates acid-induced stress fiber formation, which is required for NHE3 exocytosis, and increases in NHE3 activity. Acid-induced tyrosine phosphorylation of the focal adhesion proteins p125FAK and paxillin is not Rho kinase dependent. Thus these two acid-mediated effects are associated, yet independent processes.

OKP cells; Y27632; tyrosine phosphorylation; actin

CHRONIC METABOLIC ACIDOSIS increases the activity of NHE3, the major Na+/H+ antiporter isoform on the apical membrane of the proximal tubule (43). This effect of acidosis can be reproduced in OKP cells, an opossum kidney cell line with characteristics of the renal proximal tubule (11). We have previously shown in OKP cells that media acidification leads to a time-dependent increase in NHE3 activity, apical membrane NHE3 protein abundance, and whole cell NHE3 mRNA and protein abundances (3, 26, 55). Further analysis of these time-dependent effects demonstrates that the increase in apical membrane protein abundance occurs before increases in either mRNA or whole cell protein abundance, suggesting trafficking of NHE3 to the apical membrane from a subapical pool. This was confirmed by demonstrating that the increase in apical membrane abundance is due to an increase in NHE3 exocytosis, without effects on the rate of NHE3 endocytosis (55). A role for the actin cytoskeleton in the exocytic process and in acid stimulation of NHE3 activity was suggested by studies showing that latrunculin B, an inhibitor of F-actin filament growth, blocks both acid-induced NHE3 exocytosis and the increase in activity (55).

The renal proximal tubule plasma membrane is organized into two distinct domains that are separated by the tight junction. The apical domain contains an extensive network of microvilli, stabilized by highly organized actin microfilaments. These microfilaments are attached to a dense meshwork of bundled F-actin filaments (stress fibers) and actin binding proteins that reside close to the apical pole and are anchored to the adherens junctional complex (13). In contrast, the actin cytoskeleton associated with the basolateral membrane domain is part of a larger complex that includes focal adhesions, which serve to anchor the ends of the F-actin bundles (stress fibers) to transmembrane proteins (integrins) (36, 45). Focal adhesions are large complexes of structural, enzymatic, and adaptor proteins. Stimulation of stress fiber formation is frequently associated with rapid tyrosine phosphorylation of the cytoskeletal-associated proteins p125 focal adhesion kinase (p125FAK) and paxillin and their binding to the formed focal adhesions, resulting in the formation of binding sites for other proteins that mediate the structural and signaling events (33, 45). In many cell types, stress fiber formation, focal adhesion complex assembly, and the rapid tyrosine phosphorylation of p125FAK and paxillin are RhoA dependent, effects that are mediated by Rho kinase (36, 45, 46).

The purpose of the present studies was to identify the signaling pathway that mediates acid-induced stress fiber formation and the role of protein tyrosine phosphorylation in this process. The results demonstrate that incubation of OKP cells in acid media × 6 h induces tyrosine phosphorylation of a group of proteins with a molecular weight pattern suggestive of focal adhesion proteins. Further studies show that two of the tyrosine-phosphorylated proteins are p125FAK (125 kDa) and paxillin (68 kDa) and that in addition to phosphorylation, media acidification increases the abundance of both proteins. Last, studies demonstrate that the Rho kinase inhibitor Y27632 blocks acid-induced stress fiber formation, NHE3 exocytosis, and stimulation of NHE3 activity but does not block acid-induced tyrosine phosphorylation or the increase in whole cell protein abundance of either p125FAK or paxillin. Herbimycin A, which blocks 50% of acid stimulation of NHE3 activity, completely blocks acid-induced tyrosine phosphorylation of p125FAK and paxillin but only partially blocks stress fiber

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formation. These studies demonstrate that acid-induced stress fiber formation is RhoA dependent but that acid-induced tyrosine phosphorylation of focal adhesion proteins is not RhoA mediated. Thus acid-induced tyrosine phosphorylation of focal adhesion proteins and stress fiber formation are associated, yet independent acid-regulated processes.

METHODS

Materials. All chemicals were purchased from Sigma unless noted as follows: penicillin and streptomycin from Whittaker Bioproducts (Walkersville, MD); culture media from Gibco (Grand Island, NY); BCECF-AM from Molecular Probes (Eugene, OR); herbimycin A and tyrphostin A47 from Alexis Biochemicals (San Diego, CA); blocking buffer (5% nonfat milk and 0.05% Tween 20), anti-focal adhesion kinase (p125FAK; mouse monoclonal IgG, H-1), and anti-phosphotyrosine (mouse monoclonal IgG; PY99) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); anti-focal adhesion kinase (p125FAK; mouse monoclonal IgG, clone 2A7) antibody from Upstate Biotechnology (Lake Placid, NY); anti-paxillin antibody (mouse monoclonal IgG, clone 349) from ICN Biomedicals (Aurora, OH); normal donkey serum and fluorescein (FITC)-conjugated, affinity-purified donkey anti-mouse IgG from Jackson ImmunoResearch Laboratories (West Grove, PA); and EZ-Link Sulfo-NHS-SS-Biotin and immunopure Immobilized Streptavidin from Pierce (Rockford, IL).

Cell culture. OKP cells were passaged in high-glucose DMEM supplemented with 100 U/ml of penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. For experimentation, cells were grown to confluence, rendered quiescent in 50:50 low-glucose DMEM/Ham’s F12 media supplemented with 10^{-9} M hydrocortisone in the absence of serum × 48 h, and then studied in quiescent media at pH 7.4 (control) or pH 6.8 (acid). Media was acidified by HCl addition, using a solution that had been equilibrated in 5% CO₂ before being added to the culture dish.

To study the effect of tyrosine kinase inhibitors, cells were pre-treated with either 1 μM herbimycin A or vehicle (DMSO) × 24 h or 20 μM tyrphostin A47 or vehicle (DMSO) × 16 h. To examine the effect of inhibiting RhoA kinase, cells were pretreated with Y27632 or vehicle (water) × 30 min before and during the 6-h acid incubation.
Na+/H+ antiporter activity. Na+/H+ antiporter activity was assayed in confluent cells grown on glass coverslips as the Na-dependent intracellular pH (pHi) recovery following acid loading using the intracellularly trapped, pH-sensitive dye BCECF, as previously described (7, 55). As media acidification does not change buffer capacity, results are expressed as dpHi/dt.

**Immunoblotting.** Cells were rinsed with ice-cold PBS, scraped in RIPA buffer [150 mM NaCl, 50 mM Tris·HCl (pH 7.4), 2.5 mM EGTA, 50 mM β-glycerophosphate, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 0.5 mM DTT, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 μg/ml pepstatin, 5 μg/ml leupeptin, and 5 μg/ml aprotinin], incubated at 4°C for 45 min, and centrifuged at 12,000 rpm for 20 min. Supernatants were diluted with RIPA buffer to a concentration of 3 mg protein/ml (Bradford method, Bio-Rad, Hercules, CA), size fractionated by SDS-PAGE on a 7.5% gel, and electrophoretically transferred to nitrocellulose. After blocking with blocking buffer (5% nonfat milk and 0.05% Tween 20) for 1 h, blots were probed with PY99 (1:100 dilution), anti-p125FAK (1:1,000 dilution), or anti-paxillin (1:1,000 dilution) in 1% blocking buffer for 1 h. Blots were washed in 0.1% Tween 20 in TBS [150 mM NaCl, 10 mM Tris·HCl (pH 8.0)] × 3 for 5 min each, incubated with a 1:5,000 dilution of peroxidase-labeled anti-mouse antibody in 1% blocking buffer × 1 h, washed as above, visualized by ECL, and quantified by densitometry on a Molecular Dynamics densitometer using the ImageQuant program.

**Immunoprecipitation.** Cell extracts prepared in RIPA buffer (defined above) were diluted to 1 mg protein/ml with RIPA buffer. Monoclonal antibodies (anti-paxillin: 3 μg; anti-p125FAK: 4 μg) were added to 300 μl of cell extract, rocked × 2 h at 4°C, mixed with 25 μl protein G agarose, rocked overnight at 4°C, centrifuged at 10,000 g × 30 s, and the pellet washed × 3 with RIPA buffer. The washed pellet was suspended in 60 μl of 1× SDS loading buffer, boiled × 10 min, subjected to SDS-PAGE, and blotted with PY99 antibody, as described above.

**Immunofluorescence microscopy.** Cells grown on glass coverslips were washed × 3 with PBS and then fixed in 3.7% formaldehyde in PBS × 20 min. After fixation, cells were again washed × 3 with PBS and permeabilized with 0.2% Triton X-100 in PBS × 20 min. To visualize actin filaments (F-actin), cells were stained with 4 μg/ml FITC-phalloidin.

Fig. 2. Acid increases tyrosine phosphorylation. Anti-phosphotyrosine immunoblotting was performed on whole cell extracts. Cells were incubated at pH 6.8 for the indicated times. A: representative blot. B: summary of the data. N = 4 for each time point. *P < 0.05 vs. 0 h. **P < 0.01 vs. 0 h.

Fig. 3. Acid-stimulated antiporter is partially blocked by tyrosine kinase inhibitors. The effect of media acidification × 6 h on Na+/H+ antiporter activity was examined in the absence and presence of tyrosine kinase inhibitors. A: exposure to 20 μM tyrphostin A47 × 16 h before and during acid incubation. Vehicle: n = 5 for both groups; tyrphostin A47: n = 6 for both groups. *P < 0.01 vs. pH 7.4. #P < 0.05 vs. vehicle pH 6.8. B: exposure to 1 μM herbimycin A × 24 h before and during acid incubation. Vehicle: n = 8 for both groups; herbimycin A: n = 11 for both groups. *P < 0.05 vs. pH 7.4. #P < 0.05 vs. vehicle pH 6.8.
rhodamine-conjugated-phalloidin × 40 min at room temperature, washed × 6 with PBS × 10 min each to remove unbound phalloidin conjugate, and fluorescence visualized using an Axiosvert 135 or Axiophot Microscope (Zeiss).

Tyrosine-phosphorylated proteins and paxillin were labeled with their respective primary antibodies. Cells were blocked with 5% donkey serum in PBS at 4°C, incubated with the appropriate primary antibody [anti-phosphotyrosine PY99 (1:100 dilution) or anti-paxillin (1:1,000 dilution)] × 1 h at room temperature, washed × 3 with PBS, exposed × 45 min at room temperature to a secondary antibody solution consisting of FITC-conjugated affinity-purified donkey antiserum IgG, and washed again × 4 with PBS. Both primary and secondary antibody solutions contained 5% donkey serum. The coverslips were then mounted onto microscope slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and visualized on an Axiosvert 135 or Axioskop microscope (Zeiss).

**Biotinylation.** To assay apical membrane NHE3 abundance, surface biotinylation was performed as previously described (16, 28, 47, 55). Briefly, confluent OKP cells grown in 100-mm plates were rinsed × 3 with PBS containing 0.1 mM CaCl2 and 1.0 mM MgCl2 (PBS-Ca-Mg) at 4°C. The cells were then exposed to 1.5 mg/ml sulfo-NHS-biotin in 10 mM triethanolamine (TEA; pH 7.4), 2 mM CaCl2, and 150 mM NaCl × 1 h at 4°C with continuous rocking, rinsed with PBS-Ca-Mg containing 100 mM glycine (quenching solution) × 20 min at 4°C, lysed in modified RIPA buffer [150 mM NaCl, 50 mM Tris·HCl (pH 7.4), 5 mM EDTA, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 174 µg/ml PMSF, 5 µg/ml aprotinin, 5 µg/ml leupeptin] × 30 min at 4°C, and centrifuged at 103,000 g × 10 min. The supernatant was diluted to 3 mg protein/ml biotinylated proteins precipitated with 120 µl streptavidin-coupled agarose in a total volume of 600 ml, and the precipitate was subjected to SDS-PAGE and blotting with anti-NHE3 antibodies, as previously described (55).

**Statistics.** Data are reported as means ± SE. Statistical significance was determined using unpaired or paired Student t-tests as appropriate and set at P < 0.05.

## RESULTS

**Acid causes stress fiber and focal adhesion formation.** Incubation of OKP cells in acid (pH 6.8) media × 6 h leads to extensive stress fiber formation compared with cells incubated in control (pH 7.4) media (Fig. 1, A and B). Acid-induced stress fiber formation is associated with tyrosine phosphorylation and formation of focal adhesions, assessed by anti-phosphotyrosine (Fig. 1, C and D) and anti-paxillin (Fig. 1, E and F) immunofluorescence, respectively.

**Acid increases protein tyrosine phosphorylation.** To confirm that exposure to acid media leads to protein tyrosine phosphorylation, OKP cells were incubated in pH 6.8 media for 0, 2, 4, or 6 h, and protein tyrosine phosphorylation was assessed on whole cell lysate by immunoblotting using the monoclonal anti-phosphotyrosine antibody PY99. As shown in Fig. 2, acid incubation leads to a time-dependent increase in tyrosine phosphorylation of proteins of 68, 105, 125, and 192 kDa, the trend being apparent after 2 h of exposure to acid media.

**Acid increases p125FAK tyrosine phosphorylation and protein abundance.** Cells were exposed to acid media for the indicated times. A: top: p125FAK was immunoprecipitated, and then immunoblotting was performed with PY99; bottom, immunoblotting was performed on whole cell lysate using anti-p125FAK antibody.

![Fig. 4. Acid increases p125FAK tyrosine phosphorylation and protein abundance.](image-url)

![Fig. 5. Acid increases paxillin tyrosine phosphorylation and protein abundance.](image-url)
Tyrosine kinase pathways mediate acid-induced increase in NHE3 activity. We previously demonstrated that media acidification significantly increases NHE3 activity at 6 h and that acid-induced increases in NHE3 activity at 24 h require tyrosine kinases (51). The above studies suggest that acid media increases tyrosine phosphorylation of selected proteins within 2 h. To determine whether tyrosine kinase pathways also contribute to the acid stimulation of NHE3 activity at 6 h, cells were exposed to the tyrosine kinase inhibitors tyrphostin A47 and herbimycin A. As shown in Fig. 3A, tyrphostin A47 causes a small, nonsignificant increase in baseline NHE3 activity and inhibits 66% of acid-induced NHE3 activation. Similarly, herbimycin A, which has no effect on baseline NHE3 activity, inhibits 41% of acid-induced NHE3 activation (Fig. 3B). Thus, while it is unclear why tryphostin A47 has a small effect on baseline NHE3 activity, it is clear from both the tyrphostin A47 and herbimycin A studies that inhibition of tyrosine phosphorylation decreases the magnitude of the 6-h acid effect by ~50%.

Acid increases p125FAK and paxillin tyrosine phosphorylation and protein abundances. The molecular weight pattern of the proteins tyrosine phosphorylated by media acidification (Fig. 2) suggests that they are focal adhesion proteins. To confirm this, studies were performed examining the effect of acid media on two specific focal adhesion proteins, p125FAK (focal adhesion kinase) and paxillin. Immunoprecipitation with an anti-p125FAK antibody followed by immunoblotting with PY99 demonstrates a time-dependent increase in acid-induced p125FAK tyrosine phosphorylation (Fig. 4). Immunoblotting with anti-p125FAK antibody on whole cell lysate also demonstrates an increase in p125FAK protein abundance. Similar results were obtained when an anti-paxillin antibody was used for immunoprecipitating and immunoblotting (Fig. 5).

Role of RhoA in acid activation of NHE3. RhoA is a member of the Rho family of small GTPases (22). Downstream targets of RhoA include Rho-kinase or ROCK, which mediates RhoA reorganization of the actin cytoskeleton, including stress fiber and focal adhesion formation (22). To determine whether RhoA is involved in acid-induced reorganization of the cytoskeleton and stimulation of NHE3 activity, the effects of RhoA were inhibited by exposure to a specific ROCK inhibitor, Y27632 (19). Addition of 10 or 20 μM Y27632 × 30 min before and during acid incubation partially blocks, while 50 μM Y27632 completely blocks, acid-induced stress fiber formation (Fig. 6).

To determine whether stress fiber formation is required for acid stimulation of NHE3, NHE3 activity was assayed in the presence and absence of Y27632. Y27632 has no effect on baseline (pH 7.4) NHE3 activity at any concentration (Fig. 6, A-C, filled bars). However, in the presence of Y27632, acid stimulation of NHE3 activity is inhibited 46 (10 μM) (Fig. 7A), 59 (20 μM) (Fig. 7B), and 63% (50 μM) (Fig. 7C). Compared with vehicle, the effect of acid in the presence of Y27632 is significantly decreased when cells are exposed to 20 or 50 μM inhibitor (P < 0.05) (Fig. 7, B and C) and almost statisti-
cally different in the presence of 10 μM Y27632 (P = 0.068) (Fig. 7A).

We have previously shown that acid stimulation of NHE3 activity is due to exocytic insertion of NHE3 into the apical membrane (55). To determine whether the failure of acid to stimulate NHE3 activity when stress fiber formation is blocked is due to decreased apical membrane NHE3 abundance, apical membrane NHE3 abundance was measured using surface biotinylation, as previously described (55). As shown in Fig. 8A and summarized in Fig. 8B, in the absence of Y27632, media acidification × 6 h increases apical membrane NHE3 abundance by 58%, while in the presence of 50 μM Y27632, apical membrane NHE3 abundance is not different in cells exposed to pH 7.4 or 6.8 media.

Interaction between tyrosine phosphorylation and RhoA. RhoA and ROCK are required for stress fiber formation and NHE3 trafficking to the apical membrane. To determine whether RhoA is involved in acid-induced tyrosine phosphorylation of focal adhesion proteins, cells were exposed to acid media × 6 h in the presence or absence of 50 μM Y27632. p125FAK and paxillin were immunoprecipitated, and tyrosine phosphorylation was assayed with PY99 or protein abundance was assayed with the appropriate antibody on whole cell lysate. As shown in Figs. 9 (p125FAK) and 10 (paxillin), while the ROCK inhibitor causes a decrease in baseline tyrosine phosphorylation of both p125FAK and paxillin that is not statistically significant, it has no effect on acid-induced tyrosine phosphorylation, or on baseline or acid-induced increases in protein abundance. The lack of an effect on p125FAK and paxillin

Fig. 7. Acid-induced antiporter stimulation is blocked by ROCK inhibitor Y27632. Y27632 or vehicle was applied for 30 min before and then during the 6-h acid incubation; 10 (n = 13 for each group; A), 20 (n = 12 for each group; B), or 50 (n = 12 for each group; C) μM Y27632 was applied. *P < 0.05 vs. pH 7.4.

Fig. 8. Y27632 inhibits acid-induced increase in apical membrane NHE3 protein abundance. Thirty minutes before and then during the 6-h acid incubation, 50 μM Y27632 or vehicle was applied. Apical membrane NHE3 was then biotinylated, precipitated with agarose-bound streptavidin, and identified by immunoblot with anti-NHE3 antiserum. A: typical blot. B: summary of results. N = 9 for each group. *P < 0.05 vs. pH 7.4.
phosphorylation is consistent with immunofluorescence studies showing that Y27632 has no effect on acid-induced PY99 staining (right). However, herbimycin A only partially blocks stress fiber formation (left). These observations are consistent with the hypothesis that stress fiber formation is integral to NHE3 activation in that herbimycin A decreases the magnitude of the acid effect on NHE3 activity by only ~50%.

Thus, taking the Rho kinase inhibitor and herbimycin data together, stress fiber, but not tyrosine phosphorylation of p125FAK and paxillin, is Rho kinase dependent. However, acid-induced tyrosine phosphorylation of cell proteins is required for the full effect of acid on stress fiber formation and NHE3 activity. Whether p125FAK and/or paxillin is (are) the required tyrosine-phosphorylated protein(s) is not clear.

Fig. 12. As shown in Fig. 12, as a positive control, herbimycin A completely blocks acid-induced PY99 staining (right). However, herbimycin A only partially blocks stress fiber formation (left).

These observations are consistent with the hypothesis that stress fiber formation is integral to NHE3 activation in that herbimycin A decreases the magnitude of the acid effect on NHE3 activity by only ~50%.

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Given the apparent dissociation between tyrosine phosphorylation and stress fiber formation, studies were done to examine the effect of herbimycin A on stress fiber formation. As shown in Fig. 12, as a positive control, herbimycin A completely blocks acid-induced PY99 staining (right). However, herbimycin A only partially blocks stress fiber formation (left).

These observations are consistent with the hypothesis that stress fiber formation is integral to NHE3 activation in that herbimycin A decreases the magnitude of the acid effect on NHE3 activity by only ~50%.

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DISCUSSION

In both in vivo (acid feeding) and in vitro (media acidification) models of chronic metabolic acidosis, apical membrane NHE3 activity is increased, despite a less well developed brush-border membrane in OKP cells (27, 32, 39, 56, 57). Using cultured OKP cells, we previously showed that the increase in antiporter activity seen after 6 h of media acidification is mediated by exocytic insertion of NHE3 into the apical membrane (3, 26, 55, 55). In addition to the acid-induced increase in apical membrane expression, data from the OKP cell model have been congruent with in vivo results with respect to signaling intermediates activated by acid (27, 32, 39, 56, 57).

 Trafficking of NHE3 into and out of the apical microvillus-enriched membrane mediates regulation of NHE3 activity by a number of other physiological regulators, including endothelin-1, pressure (hypertension or renal injury-induced), sympathetic nervous system activity, cell volume, parathyroid hormone, glucocorticoids, dopamine, angiotensin II, albumin, PKCα, PKA/cAMP, intracellular Ca\(^{2+}\), EGF, and bFGF (4, 5, 12, 14, 15, 17, 18, 23, 24, 26, 29, 30, 37, 39, 42, 48, 49, 53–55). RhoA, Rho kinase, and the actin cytoskeleton may play a
critical role in the physiological regulation of NHE3, as disruption of the actin cytoskeleton alters NHE3, but not NHE1 activity (8, 17, 21, 25, 48, 49). The mechanism whereby the cytoskeleton modulates NHE3 activity has not been elucidated, but recent studies suggest that RhoA, which is abundant in the apical membrane of the brush border, and its downstream mediator Rho kinase are involved by regulating the phosphorylation of myosin light chain kinase, organization of the actin cytoskeleton, and the mobility of NHE3 within the apical membrane environment (2, 6, 41, 44, 45, 50). This latter effect may determine the abundance of functional exchangers on the apical membrane, but there is presently no evidence for a role for RhoA in the physiological regulation of NHE3 (2). In the present study, we demonstrate a role for the RhoA signaling pathway in acid stimulation of NHE3 activity. Exposure of OKP cells to a specific Rho kinase inhibitor, Y27632, for 6 h blocks acid-induced formation of stress fibers and increases in NHE3 apical membrane protein abundance and NHE3 activity.

Both the apical and basolateral membranes of epithelial cells are associated with an actin cytoskeleton and stress fibers (13, 36, 45). On the basolateral membrane, the formed stress fibers are associated with tyrosine-phosphorylated p125FAK and paxillin, whose presence is a marker for the presence of focal adhesions that anchor the F-actin filaments to transmembrane proteins and mediate communication with the basolateral external environment (33, 34, 45). On apical membranes with...
microvilli, the actin cytoskeleton serves to stabilize the microvillus structure and interacts with membrane proteins through scaffolding and/or actin-binding proteins (1, 9, 20, 35, 38). A role for focal adhesion proteins in the formation of apical membrane stress fibers has not been shown. In many cell types, both stress fiber and focal adhesion formation and tyrosine phosphorylation of p125FAK and paxillin are dependent on Rho kinase (22, 34, 36, 40, 45, 46). While these studies have focused on the role of Rho kinases in the formation and regulation of stress fibers associated with focal adhesions and communication with the extracellular environment, studies have also confirmed a role for Rho kinases in regulating cytoskeletal organization and its association with the scaffolding ezrin/radixin/moesin proteins that are associated with the apical membrane actin cytoskeleton in epithelial cells (20).

The present studies demonstrate stress fiber formation and a pattern of tyrosine-phosphorylated proteins typical of focal adhesion proteins following 6-h exposure to media acidification. The latter was confirmed by demonstrating acid-induced increases in both p125FAK and paxillin phosphorylation and whole cell protein abundances. However, while Rho kinases are required for acid-induced stress fiber formation and stimulation of NHE3 activity, they are not required for acid-induced tyrosine phosphorylation or increased protein abundance of either p125FAK or paxillin. Consistent with the dissociation between acid-induced stress fiber formation and tyrosine phosphorylation of focal adhesion proteins, herbinycin A, which blocks acid-induced tyrosine phosphorylation of all cells proteins, only partially blocks stress fiber formation. Thus acid-induced tyrosine phosphorylation of cell protein is required for the full effect of acid on stress fiber formation and NHE3 activity.

Involvement of stress fibers in acid regulation of NHE3 apical membrane protein abundance and activity is consistent with our previous studies demonstrating that latrunculin B, an inhibitor of F-actin filament growth, blocks acid-induced exocytic insertion of NHE3 into the apical membrane and stimulation of NHE3 activity (55).

We previously demonstrated that acid incubation activates c-Src at 30 and 90 s (52). While p125FAK can activate c-Src, we showed that neither p125FAK or paxillin is phosphorylated in response to acid at these early time points (52). Acid activation of c-Src is mediated by Pyk2, a FAK family member (31).

Prior studies from our laboratory have demonstrated that acid-stimulated NHE3 activity is mediated by endothelin-1 (ET-1) signaling through the endothelin B (ETB) receptor in both whole animals and cultured cells (27, 57). As with acid stimulation of NHE3, ET-1 stimulation of NHE3 involves exocytic insertion of NHE3 into the apical membrane and requires an intact cytoskeleton for both the increase in apical membrane NHE3 abundance and transporter activity (10, 42). It is also associated with stress fiber formation and tyrosine phosphorylation of paxillin and p125FAK (10, 42). Consistent with ET-1/ETB mediating the effect of acid on NHE3 activity, preliminary studies in our laboratory suggest that ET-1/ETB stimulation of NHE3 activity is also inhibited by Rho kinase inhibition with Y27632 (unpublished observations).

Based on prior published data from our laboratory and the present observations, we conclude that media acidification increases ET-1 secretion in proximal tubule cells, which binds the ETB receptor in an autocrine or paracrine manner. Ligand binding leads to RhoA and ROCK activation, resulting in the formation of stress fibers that mediate exocytic insertion of NHE3 into the apical membrane and increased NHE3 activity. Associated, but independent of stress fiber formation, is acid-induced tyrosine phosphorylation of a number of cell proteins, including two focal adhesion proteins p125FAK and paxillin. The role of these proteins remains to be defined.

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Rho KINASES MEDIATE NHE3 REGULATION