Regulation of NHE3 by lysophosphatidic acid is mediated by phosphorylation of NHE3 by RSK2

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No YR, He P, Yoo BK, Yun CC. Regulation of NHE3 by lysophosphatidic acid is mediated by phosphorylation of NHE3 by RSK2. Am J Physiol Cell Physiol 309: C14–C21, 2015. First published April 8, 2015; doi:10.1152/ajpcell.00067.2015.—Na+/H+ exchange by Na+/H+ exchanger 3 (NHE3) is a major route of sodium absorption in the intestine and kidney. We have shown previously that lysophosphatidic acid (LPA), a small phospholipid produced ubiquitously by all types of cells, stimulates NHE3 via LPA1 receptor. Stimulation of NHE3 activity by LPA involves LPA1 transactivating EGF receptor (EGFR) in the apical membrane. EGFR activates proline-rich tyrosine kinase 2 (Pyk2) and ERK, both of which are necessary for NHE3 regulation. However, Pyk2 and ERK are regulated by EGFR via independent pathways and appear to converge on an unidentified intermediate that ultimately targets NHE3. The p90 ribosomal S6 kinase (RSK) family of Ser/Thr protein kinases is a known effector of EGFR and ERK. Hence, we hypothesized that RSK may be the convergent effector of Pyk2 and ERK although it is not known whether Pyk2 regulates RSK. In this study, we show that Pyk2 is necessary for the maintenance of phosphoinositide-dependent kinase 1 (PDK1) autophosphorylation, and knockdown of Pyk2 or PDK1 mitigated LPA-induced phosphorylation of RSK and stimulation of NHE3 activity. Additionally, we show that RSK2, but not RSK1, is responsible for NHE3 regulation. RSK2 interacts with NHE3 at the apical membrane domain, where it phosphorylates NHE3. Alteration of S663 of NHE3 ablated LPA-induced phosphorylation of NHE3 and stimulation of the transport activity. Our study identifies RSK2 as a new kinase that regulates NHE3 activity by direct phosphorylation.

Na+/H+ exchanger 3; lysophosphatidic acid; phosphorylation; ribosomal S6 kinase

LYSOPHOSPHATIDIC ACID (LPA) is a bioactive phospholipid that mediates a broad range of effects that alter cell fates, cytokine secretion, neural retraction, pain perception, and embryonic implantation (6). LPA acts on a family of G protein-coupled receptors, LPA1–6 (6). Most cells express multiple LPA receptors, and at least five LPA receptors are expressed in mouse intestinal epithelial cells with LPA1 and LPA5 being most abundant (20). Increasing evidence shows that oral application of LPA exerts significant effects on the gastrointestinal system (7, 17, 21). Recent studies have revealed a novel function of LPA in regulation of electrolyte transport processes. Naren and coworkers (18) have shown that LPA acting via LPA2 attenuates cholera toxin-induced Cl− secretion in mouse intestine by inhibiting the cystic fibrosis transmembrane conductance regulator (CFTR). Recently, we have shown that LPA activates Na+/H+ exchanger 3 (NHE3), which is the major Na+ transporter in the intestine (22, 45). The activity of NHE3 directly contributes to diarrhea, and the means to stimulate NHE3 is a potential treatment strategy for diarrheal diseases. In addition to the generation of LPA in situ, LPA is present in several types of foodstuff, implying that foodborne LPA may have an antidiarrheal effect by regulation of CFTR and NHE3 (2, 39).

Unlike CFTR that is inhibited by LPA2, stimulation of NHE3 by LPA is mediated by the LPA5 receptor (22). The activation of NHE3 by LPA5 requires transactivation of EGF receptor (EGFR) expressed in the apical membrane of Caco-2bbe cells (45). Transactivation of EGFR results in the activation of the RhoA-Rho-associated kinase-proline-rich tyrosine kinase 2 (Pyk2) cascade and the MEK-ERK pathway. These pathways do not appear to work in series but work in parallel because inhibition of one does not alter the other. Both Pyk2 and ERK are necessary for the regulation of NHE3 because inhibition of either pathway blocks the stimulatory effect of LPA. These results suggest that Pyk2 and ERK1/2 may converge on the same effector, but the common downstream effector of Pyk2 and ERK1/2 remains unknown.

The p90 ribosomal S6 kinase (RSK) family of kinase is activated in response to several growth factors and mitogens, including EGF, insulin, and insulin-like growth factor-1 (1). Multiple targets, including transcription factors, glycogen synthase kinase 3β, and cyclin-dependent kinase inhibitor, whose activities are associated with regulation of cell proliferation, protein synthesis, cell mobility, and survival, are phosphorylated by RSK (1, 32). Moreover, it has been shown previously that RSK regulates NHE1 in fibroblasts and neurons (23, 38). In addition to being a target of EGFR and ERK, the diverse functions of RSK appear to correlate with a similarly broad range of effects by LPA. Therefore, we postulated that RSK is a potential kinase regulating NHE3. In this study, we demonstrate that RSK2 is the convergent effector of Pyk2 and ERK in the signaling cascade elicited by LPA5. Our study shows that RSK2 interacts with NHE3 and phosphorylates NHE3.

MATERIALS AND METHODS

Chemical and materials. LPA (18:1, 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate) was purchased from Avanti Polar Lipids (Alabaster, AL) and prepared in PBS, pH 7.2, containing 0.1% BSA. For all experiments, LPA was used at the final concentration of 1 μM unless otherwise specified, and the equal volume of PBS containing 0.1% BSA was added as a control. Rabbit anti-RSK1 and anti-p-RSK(S221) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against RSK2, p-RSK(S380), p-RSK(T573), and p-RSK(S221) were obtained from Cell Signaling Technology (Danvers, MA). A rabbit polyclonal antibody against RSK2 was prepared as described (23). Antibodies against p-RSK(S221) were purchased from Santa Cruz Biotechnology (Dallas, TX). A mouse monoclonal antibody against β-actin was purchased from Cell Signaling Technology (Danvers, MA).

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phosphoinositide-dependent kinase 1 (PKD1), and p-PKD1 were from Cell Signaling (Danvers, MA). Antibody to p-ser was from Abcam (Cambridge, MA). All other antibodies were obtained from Cell Signaling (St. Louis, MO). All chemicals were obtained from Sigma or EMD Millipore (Billerica, MA).

**RT-PCR.** RT-PCR was performed using PCR Master Mix (Roche, Indianapolis, IN) as previously described (22). The primers used are as follows: RS1: GAA GAA GGC AAC GCT GAA AG (forward) and CTC CTC CGT CAT CAC CT (reverse); RS2: CTC AGG CTA TGC TAG GC (forward) and CTC CTC CCC TGA GAA AAC CT (reverse); RS3: GGA CCG AGT GAG ATC GAA GA (forward) and CCA CAG CCT CCA GGT AGA AC (reverse); and RS5: GCC CCA ATG ATA CT CTG AA (forward) and TGG CAA CTG TCT GTG AG (reverse).

**Gene silencing.** Lentiviral vector (pLKO.1-puro) containing small hairpin (sh)RNA-targeting Pyk2 (shPyk2), RS1 (shRS1), or RS2 (shRS2) was obtained from Sigma. Lentiviral shRNA for PDK1 was obtained from Open Biosystems (Huntsville, AL). pLKO.1-puro/nontarget shRNA control (shCon) was from Sigma.

**Site-directed mutagenesis and sequencing analysis.** pcDNA with human NHE3 with a vesicular stomatitis virus glycoprotein (VSVG) epitope tag at the COOH terminus has been described previously (22). Site-directed mutagenesis of S663 or S693 of NHE3 to Ala was performed using the QuickChange site-directed mutagenesis kit according to the recommendation by the manufacturer (Stratagene, La Jolla, CA). The presence of S663A or S693A mutation was confirmed according to the recommendation by the manufacturer (Stratagene, La Jolla, CA). The presence of S663A or S693A mutation was confirmed by nucleotide sequencing.

**Cell cultures.** Caco-2bbe cells were grown in DMEM supplemented with 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 1 mM sodium pyruvate, 15 mM HEPES, and 1× nonessential amino acids. Caco-2bbe cells were transfected with pcDNA-NHE3, pcDNA-NHE3/S663A, or pcDNA-NHE3/S693A. Caco-2bbe/NHE3 using a Neon Transfection System (Life Technologies, Grand Island, NY) as previously described (22). Transfected cells were cultured in the presence of puromycin and subjected to acid suicide for three consecutive passages to select stable expression of NHE3 or its variant. Caco-2bbe/NHE3 cells were infected with lentiviral pCDH containing NH2-terminal hemagglutinin (HA)-tagged human LPA5 and were selected with puromycin, resulting in Caco-2bbe/NHE3/LPA5 (22). pCDH was used as a control.

**Na⁺-/H⁺-dependent intracellular pH recovery.** The Na⁺-/H⁺-dependent changes in intracellular pH (pHi) by NHE3 were determined using the ratio-fluorometric, pH-sensitive dye BCECF-AM as described previously (9). Briefer, cells were washed in Na⁺/H⁺ buffer [130 mM NaCl, 20 mM HEPES, 5 mM KCl, 1 mM tetramethylammonium-PO4 (TMA-PO4), 2 mM CaCl2, 1 mM MgSO4, and 25 mM glucose] and then were dye loaded by incubation with 6.5 µM BCECF-AM in the same solution for 10 min. The coverslips were mounted on a perfusion chamber mounted on an inverted microscope and were superfused with Na⁺/H⁺ buffer (40 mM NaCl, 5 mM HEPES, 5 mM KCl, 1 mM TMA-PO4, 2 mM CaCl2, 1 mM MgSO4, and 25 mM glucose) for 5 min, followed by perfusion with TMA⁺/H⁺ buffer (130 mM TMA-Cl, 20 mM HEPES, 5 mM KCl, 1 mM TMA-PO4, 2 mM CaCl2, 1 mM MgSO4, and 25 mM glucose) for 2 min, and subsequently with Na⁺/H⁺ buffer. When necessary, Na⁺/H⁺ buffer was supplemented with 50 µM HOE694 to inhibit NHE1 and NHE2 activities.

The rate of Na⁺-/H⁺-dependent pH recovery was calculated by determining slopes along the early stage of pH recovery by linear least-squares analysis over a minimum of 9 s. Calibration of the fluorescence signal was performed using R’/H’ ionophore nigericin as previously described (9). Comparisons of Na⁺/H⁺ exchange were made between measurements made on the same day. The microfluorometry was performed on an inverted fluorescence microscope, and photometric data were acquired using the Metafluor software ( Molecular Devices, Sunnyvale, CA).

**Immunoprecipitation and Western blot.** Immunoprecipitation was performed as previously described with a modification (10). Briefly, Caco-2/NHE3/LPA5 cells were washed twice in cold PBS, scraped, and lysed in lysis buffer (Cell Signaling) containing protease inhibitors (Roche). The crude lysate was sonicated for 2 × 15 s and spun at 14,000 × g for 15 min. Protein concentration was determined by the bicinchoninic acid assay (Sigma). Lysate (300 µg) was preclotted by incubation with 30 µl of protein G-Sepharose beads for 1 h, and the supernatant was then incubated overnight with anti-VSVG antibody. Immunocomplex was purified by incubation with 50 µl of protein G-Sepharose beads for 1.5 h, followed by three washes in lysis buffer and two washes in PBS. All the above steps were performed at 4°C or on ice. The bound immunocomplex was eluted by incubating the protein A beads in Laemmli sample buffer for 10 min at 95°C and was then separated by SDS-PAGE. The proteins were then transferred to nitrocellulose membrane for Western immunoblotting as previously described (45). For in vivo phosphorylation of NHE3, anti-p-ser antibody was used to determine NHE3 phosphorylation levels. The blot was stripped and blotted with anti-VSVG antibody to quantify the amount of immunoprecipitated NHE3. For preparation of the intestinal lysates, C57BL/6 mice were euthanized using isoflurane under approval by the Institutional Animal Care and Use Committee of Emory University and in accordance of the NIH Guide for the Care and Use of Laboratory Animals. The proximal small intestine was removed from the mouse, flushed with cold PBS, and cut open longitudinally to expose the epithelial layer. The epithelial layer was lightly scraped with a glass coverslip and lysed as described above.

**Surface biotinylation.** Surface biotinylation of NHE3 was performed as previously described (10). Briefly, cells were rinsed twice in PBS and incubated in borate buffer (154 mM NaCl, 7.2 mM KCl, 1.8 mM CaCl2, and 10 mM H3BO3, pH 9.0) for 10 min. Cells were then incubated for 40 min with 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL) in borate buffer. Unbound sulfo-NHS-LC-biotin was quenched with Tris buffer (20 mM Tris and 120 mM NaCl, pH 7.4). Cells were lysed in lysis buffer and sonicated for 2 × 15 s. Lysate was agitated for 30 min and spun at 14,000 × g for 30 min at 4°C to remove the insoluble cell debris. An aliquot was retained as the total fraction representing the total cellular NHE3. Protein concentration was determined, and 1 mg of lysate was then incubated with streptavidin-agarose beads (Pierce) for 2 h. The streptavidin-agarose beads were washed three times in lysis buffer and twice in PBS. All the above procedures were performed at 4°C or on ice. Biotinylated surface proteins were then eluted by boiling the beads at 95°C for 10 min. Dilutions of the total and surface NHE3 were resolved by SDS-PAGE and immunoblotting with an anti-VSVG antibody. Denaturation analysis was performed using ImageJ software (National Institutes of Health).

**Confocal immunofluorescence microscopy.** Caco-2bbe cells grown 7 days postconfluence on Transwells were washed twice with cold PBS, fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized in 0.2% Triton X-100 in PBS for 5 min, and blocked in PBS containing 5% normal goat serum for 30 min at room temperature. Cells were then stained with anti-VSVG or anti-p-RSK antibody for 1 h at room temperature. Following three washes, 10 min each, with PBS, the cells were incubated with Alexa 488-conjugated donkey anti-mouse IgG or Alexa 555-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) for 1 h at room temperature. After 3 × 10 min washes with PBS, the excised Transwells were mounted with ProLong Gold Antifade Reagent (Invitrogen) and observed under a Zeiss LSM510 laser confocal microscope (Zeiss Microimaging, Thornwood, NY) coupled to a Zeiss Axiosplane2 with ×63 Pan-Apochromat oil lenses.

**Statistical analysis.** Statistical analyses were performed by Student’s t-test for paired comparison. Results were presented as means ± SE. A P value of <0.05 was considered significant.
RESULTS

RSK is activated by LPA. There are four RSK isoforms (1), and we initially assessed the expression levels of RSK isoforms in Caco-2bbe cells by RT-PCR. RSK1 and RSK2 mRNA was abundant compared with RSK3 or RSK4, suggesting that RSK1 and RSK2 are the major RSK isoforms in these cells (Fig. 1A). The presence of RSK1 and RSK2 was confirmed by Western blot (Fig. 1B), which showed the presence of RSK1 and RSK2 in mouse ileal epithelium and several other intestinal cell lines, although the relative expression levels of RSK1 and RSK2 vary. Henceforth, we assume that intestinal epithelial cells mainly express RSK1 and RSK2. We next sought to determine whether LPA regulates RSK by determining RSK phosphorylation. RSK is characterized by phosphorylation at multiple sites (1, 32). LPA increased phosphorylation of RSK at S221 (a.a. numbering is based on RSK1) and S380 in Caco-2bbe cells stably transfected with LPA5 (Fig. 1C). However, no change in RSK phosphorylation was observed in control transfected Caco-2bbe cells, consistent with the previous studies that LPA5 expression in Caco-2 cells is low (22, 45). The time course of the effect shows that LPA acutely phosphorylates RSK without altering RSK expression (Fig. 1D).

NHE3 regulation by LPA requires PDK1. Full activation of RSK involves sequential phosphorylation of RSK by ERK and PDK1 (1). However, to our best knowledge there is no precedence where Pyk2 is involved in RSK phosphorylation. Hence, we assessed whether LPA-induced phosphorylation of RSK is dependent on Pyk2. The present model of RSK activation depicts that ERK phosphorylates RSK at T573 and S363, which is followed by autophosphorylation at S380. Phosphorylation at S380 creates a docking site for PDK1, which phosphorylates S221, leading to full activation of RSK (1, 32). Inhibition of MEK by U0126 ablated LPA-induced phosphorylation of RSK at S221 and at T573 (Fig. 2A). Similarly, knockdown of Pyk2 blocked phosphorylation at S221 but did not affect phosphorylation at T573, consistent with previous studies that show that phosphorylation of T573 is dependent on ERK. These results suggest that Pyk2 modulates phosphorylation of RSK and also confirm that Pyk2 and ERK differentially regulates RSK. Although the role of Pyk2 in RSK regulation is uncertain, it has been shown that Pyk2 mediates activation of PDK1 by angiotensin II (40). Hence, we examined whether LPA activates PDK1 in Caco-2bbe cells. Figure 2B shows that LPA slightly increased phosphorylation of PDK1 at S241, which is the site of autophosphorylation within the catalytic domain. The small effect was not surprising because PDK1 is often thought to be constitutively active (25). On the contrary, knockdown of Pyk2 markedly decreased the phosphorylation level of PDK1 under basal and LPA-treated conditions without altering PDK1 expression. These data suggest that Pyk2 is necessary for the maintenance of PDK1 autophosphorylation at S241.

To ascertain that PDK1 is involved in LPA-mediated regulation of NHE3, we assessed RSK phosphorylation and NHE3 activity in cells with PDK1 knockdown. In line with the finding that Pyk2 regulates PDK1, silencing of PDK1 abated LPA-induced phosphorylation of RSK (Fig. 2C). In addition, LPA-mediated stimulation of NHE3 activity was blocked by PDK1 knockdown (Fig. 2D). These results indicate that Pyk2-PDK1 and ERK cooperatively regulate RSK, which in turn stimulates NHE3 activation.

RSK2 interacts with NHE3. Although our results suggest that RSK is the converging point of the RhoA-Pyk2-PDK1 and MEK-ERK pathways, how RSK regulates NHE3 remains to be determined. NHE3 regulation is mostly mediated by trafficking of NHE3 proteins between the brush-border membrane and intracellular pools in intestinal cells although not in kidney in vivo (24). As we have reported previously (22, 45), LPA increased NHE3 abundance in the surface membrane as evidenced by the increased NHE3 fluorescence level at the apical membrane of Caco-2bbe cells (Fig. 3A). RSK is normally present in the cytoplasm of quiescent cells, but upon activation p-RSK translocates to the plasma membrane (30). Consistently, we observed an increased level of p-RSK in the apical membrane, where it colocalized with NHE3. These results were corroborated by surface biotinylation, which depicted increased surface abundance of NHE3 and p-RSK in Caco-2bbe/NHE3 cells only when LPA5 was coexpressed (Fig. 3B). Interestingly, RSK2 was increased in the surface fraction. On the contrary, the abundance of RSK1 at the cell surface was low, and no change in RSK1 was observed in response to LPA.
To further ascertain the possibility that RSK directly targets NHE3, we performed coimmunoprecipitation of NHE3 and RSK from cell lysates. Figure 3C shows that an increased amount of p-RSK2 coimmunoprecipitated with NHE3 in response to LPA. Moreover, we found RSK2 coimmunoprecipitated with NHE3 was significantly increased in Caco-2be/NHE3/LPA5 cells treated with LPA. On the contrary, RSK1 presence was not observed under all conditions tested. These results imply that LPA induces interaction of RSK2 with NHE3.

Antibodies to p-RSK cannot distinguish p-RSK1 from p-RSK2. To determine whether LPA activates RSK1, RSK2, or both, we knocked down RSK1 or RSK2 before LPA treatment. Silencing of RSK1 did not appear to have a significant effect on LPA-induced phosphorylation at S221 or S380 (Fig. 4A). On the contrary, RSK2 knockdown lessened the RSK phosphorylation level under basal conditions and importantly ablated LPA-induced change. These results suggest that RSK2, but not RSK1, is activated by the LPA-LPA5 signaling cascade. To ascertain the role of RSK2 in regulation of NHE3, we compared the effect of RSK1 or RSK2 depletion on NHE3 surface expression and activity. Silencing of RSK1 did not alter LPA-induced changes in NHE3 surface expression or Na⁺/H⁺ transport activity (Fig. 4, B and C). RSK2 depletion, however, completely blocked the changes by LPA, further demonstrating the critical role of RSK2 in the regulation of NHE3 by LPA.

LPA increases NHE3 phosphorylation at S663 through RSK2. The minimum consensus phosphorylation target sequence of RSK is R/K-X-R-X-X-pS/T, which is also shared by other kinases of the AGC (PKA, PKG, and PKC) family (1, 28). NHE3 contains the R/K-X-R-X-X-pS/T motif at S663 (RRKRLGS) and S693 (KRRNSS). To determine whether NHE3 is phosphorylated by LPA-mediated signaling, we immunoprecipitated NHE3 in Caco-2be/NHE3/HA-LPA5 cells treated or untreated with LPA, followed by immunoblotting using anti-p-Ser antibody. Figure 5A demonstrates that LPA increased NHE3 phosphorylation in vivo. On the other hand, LPA did not alter phosphorylation of NHE3-S663A, whereas S693A mutation did not affect LPA-mediated phosphorylation of NHE3. Consistent with the phosphorylation pattern, S663A, but not S693A, ablated LPA-induced NHE3 activation (Fig. 5B). We showed above that LPA specifically increases RSK2 phosphorylation. To determine that RSK2 is responsible for NHE3 phosphorylation, NHE3 phosphorylation was determined in cells with RSK2 knockdown. Unlike control cells, LPA-induced phosphorylation of NHE3 was abrogated by knockdown of RSK2 (Fig. 5C). These results indicate that the LPA-induced phosphorylation of NHE3 is mediated by RSK2.

It has been anticipated that NHE3 is phosphorylated by a number of protein kinases, but it remains to be determined how phosphorylation of NHE3 protein regulates NHE3 activity. In this context, we questioned whether phosphorylation of NHE3 at S663 by RSK2 is required for trafficking of NHE3 to the plasma membrane. To address this problem, we performed surface biotinylation of NHE3/S663A and NHE3/S693A. Figure 5D shows that LPA increased wild-type and S693A NHE3 abundance in the plasma membrane. However, no significant change was observed with NHE3/S663A. These results suggest that phosphorylation at S663 is critical for apical trafficking of NHE3 by LPA.

DISCUSSION

Recent studies have shown that LPA inhibits secretory diarrhea by inhibition of CFTR and stimulation of NHE3 in the intestine (18, 22, 37). Stimulation of NHE3 by LPA is dependent on the LPA5 receptor that is highly expressed in the intestinal tract (16, 22). NHE3 regulation is dependent on both Pyk2 and ERK, but how these two kinases ultimately regulate NHE3 is unclear. The study reported here identifies RSK2 as the signaling node where Pyk2 and ERK intersect to regulate NHE3. We also show that RSK2 phosphorylates NHE3 and that the phosphorylation of NHE3 by RSK2 is essential for apical trafficking of NHE3 (Fig. 6).
that RSK is a known effector of ERK. RSK is activated by sequential phosphorylation by ERK and PDK1 (1, 32). PDK1 is a pivotal regulator involved in the activation of multiple members of the AGC superfamily of Ser/Thr protein kinases, including Akt, PKC, SGK, and RSK (26). Our previous study showed that PDK1 is involved in stimulation of NHE3 by SGK1 (9). In addition, the PDK1 hypomorphic mice display a marked decrease in intestinal NHE3 activity without changing NHE3 expression, demonstrating that PDK1 is a pivotal kinase for NHE3 regulation (33). Although a role of Pyk2 in activation of RSK has not been reported, a previous study by Taniyama et al. (40) has shown that Pyk2 acts as a scaffold for phosphorylation of PDK1 by Src. Similarly, IGF-1 forms a complex consisting of SHP2, Pyk2, Src, and Grb2 that recruits and phosphorylates PDK1 (35). Although PDK1 is thought to be constitutively active with autophosphorylation at S241 located in the catalytic domain (3), previous studies have shown that PDK1 activity can be regulated by phosphorylation at sites other than S241 (3, 27). For instance, angiotensin II stimulates phosphorylation of PDK1 at Tyr-373/376 in vascular smooth muscle cells (40). In addition, insulin can further increase phosphorylation levels at S244 in mouse hypothalamic neuronal cells while phosphorylating PDK1 at other sites, suggesting that PDK1 can be regulated in certain cell types (31). The effect of LPA on PDK1 phosphorylation at S241 was not robust, but knockdown of Pyk2 markedly decreased autophosphorylation of PDK1 at S241. Our results indicate that Pyk2 plays a critical role in maintenance of PDK1 autophosphorylation and demonstrate that PDK1 is necessary for the regulation of NHE3 by LPA. However, whether Pyk2 phosphorylates PDK1 at Tyr and the mechanistic basis for PDK1 regulation by Pyk2 are not known.

RSK1 and RSK2 are closely related with 78% identity, and the isoform selectivity of RSKs is incompletely understood (1, 32). RSK1 knockout mice appear viable although no phenotype has been reported (8). On the other hand, human mutation in RSK2 is linked to Coffin-Lowry syndrome, an X-linked mental retardation, and RSK2-deficient mice exhibit impaired learning and coordination (29). Other studies have supported the specificity of RSK1 and RSK2. RSK1 but not RSK2 induces neurite outgrowth, whereas RSK2 uniquely induces IL-2 and IL-15 in T lymphocytes (19, 36). These studies demonstrate that RSK1 and RSK2 do not have redundant functions. In the present study, we focused on RSK1 and RSK2 based on their relative abundant mRNA expression. Both RSK1 and RSK2 proteins were present in mouse enterocytes and several intestinal epithelial cell lines. Because the efficacy of anti-RSK1 and anti-RSK2 antibody is expected to differ, it is not possible to directly compare the abundance of these two RSK isoforms. Nonetheless, several lines of evidence show that RSK2, but not RSK1, is involved in NHE3 regulation. First, RSK2 interacts with NHE3 in response to LPA. Second, LPA induced translocation of RSK2 to the brush-border membrane of Caco-2bbe cells where NHE3 is located, and lastly knockdown of RSK2, but not RSK1, ablated stimulation of NHE3 by LPA. A question remains whether RSK3 or RSK4 may take part in NHE3 regulation. However, knockdown of RSK2 abolished the effects of LPA on NHE3 phosphorylation, trafficking, and activity. Hence, the possibility of RSK3 or RSK4 altering NHE3 is low.

The classical paradigm of acute regulation of NHE3 models around reversible phosphorylation of NHE3. Previous studies have shown that phosphorylation of NHE3 at S552 and S605 is essential for the inhibition of NHE3 by PKA (14, 48). Additionally, phosphorylation at S719 by casein kinase 2 is important for the basal NHE3 activity (34). On the other hand, phorbol ester inhibits NHE3 in PS120 fibroblasts without a change in NHE3 phosphorylation although this finding has been disputed in AP-1 cells (43, 44). Our results demonstrate phosphorylation of NHE3 at S663, which is critical for the stimulation of NHE3 activity. The S663 residue was previously shown to be essential for acute stimulation of NHE3 by...
glucocorticoids and SGK (9, 42). Hence, S663 emerges as an important phosphorylation site associated with elevated NHE3 activity. However, how phosphorylation alters NHE3 is not yet clear. A recent study by Kocinsky et al. (13) has shown that increased phosphorylation of NHE3 at S552 and S605 was not accompanied by an immediate decrease in NHE3 activity, but rather the phosphorylation of NHE3 preceded functional inhibition. Although the mechanism by which phosphorylation contributes to NHE3 regulation remains unclear, it is generally accepted that phosphorylation of NHE3 is a functionally important step. In the present study, we found that the S663A mutation was sufficient to block trafficking of NHE3 to the apical membrane. Therefore, the primary role of the post-translational modification appears to be the translocation of NHE3 to the cell surface rather than NHE3 protein turnover. However, it is not apparent how phosphorylation at S663 modulates apical trafficking of NHE3 by LPA. In the case of NHE3 regulation by PKA or SGK1, phosphorylation of NHE3 is reported to require the presence of Na+/H+-exchanger regulator factor 1 (NHERF1) or NHERF2, which is linked to the actin cytoskeleton via ezrin (9, 15). In this context, it is noteworthy that RSK contains a PDZ binding motif in the COOH terminus, and the interaction of RSK2 with the PDZ domain proteins of the Shank family has been shown to modulate the synaptic transmission by amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (41). We have shown previously that LPA-dependent regulation is dependent on NHERF2, such that NHERF2 interaction is important for the optimal signaling by LPA (22). Because NHERF2 also interacts with NHE3 (47), NHERF2 may have a dual function in direct regulation of NHE3. However, NHERF2 alone is insufficient to address the specificity of RSK2, and it is likely that phosphorylation of NHE3 by RSK2 may enhance its interaction with other protein, such as ezrin, that can link NHE3 to the apical membrane (5). Additional studies are needed to determine whether phosphorylation at S663 affects NHE3 interaction with NHERF2 or the cytoskeletal network.

![Fig. 4. Activation of NHE3 by LPA is RSK2 dependent. A: Caco-2-bbe/NHE3/HA-LPA5 cells transfected with shCon, shRSK1, or shRSK2 were treated with LPA to determine RSK phosphorylation at S221 and S380. RSK phosphorylation levels were quantified relative to the control condition (-) for each cell line. Bottom: knockdown efficiency of RSK1 and RSK2. B: surface expression of NHE3 in cells with knockdown of RSK1 or RSK2 was determined as described in MATERIALS AND METHODS. The amounts of surface NHE3 were quantified relative to control conditions. C: effect of RSK knockdown on NHE3 activity was determined. NHE3 activities are expressed as the rate of Na+/H+-dependent intracellular pH recovery. Results are presented as means ± SE; n ≥ 6. *P < 0.05.

![Fig. 5. Phosphorylation at S663 is necessary for the activation of NHE3 by LPA. A: NHE3 and NHE3 mutants were immunoprecipitated from cells treated with or without LPA for 5 min. Immunocomplex was resolved by SDS-PAGE, and immunoblotting was performed using anti-p-Ser antibody to determine phosphorylation levels of NHE3 (p-Ser). Bottom: amount of immunoprecipitated NHE3. RC, p-Ser normalized to NHE3; WT, wild-type. *P < 0.05 compared with PBS-treated control. Western blots are representative of 3 independent experiments. B: NHE3 activity was determined in cells expressing NHE3, NHE3/S663A, or NHE3/S693A. NHE3 activities are expressed as the rate of Na+/H+-dependent pH recovery; n ≥ 6. *P < 0.01. C: NHE3 phosphorylation was determined in cells transfected with shCon or shRSK2. D: surface expression of NHE3 variants was determined by surface biotinylation. *P < 0.05 compared with PBS-treated control.

![Fig. 6. Activation of NHE3 by LPA is mediated by RSK2. This schematic model depicts how LPA regulates NHE3 in Caco-2bbe cells. Transactivation of EGF receptor (EGFR) by LPA5 activates RhoA-RhoA-Rho associated kinase (ROCK)-Pyk2 and ERK (45). The present study shows that Pyk2 and ERK phosphorylate RSK2, which in turn phosphorylates NHE3 at S663. This phosphorylation of NHE3 by RSK2 appears to be necessary for trafficking NHE3 to the apical membrane.](http://ajpcell.physiology.org/)

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In summary, this study identifies RSK2 as a new protein kinase that regulates NHE3. This study also shows the role of Pyk2 in stimulation of RSK2 through its effect on PDK1. Stimulation of NHE3 by LPA requires phosphorylation of NHE3 at S663, which is important for trafficking of NHE3 to the apical membrane.

REFERENCES


