NHE3 phosphorylation at serines 552 and 605 does not directly affect NHE3 activity

Kocinsky HS, Dynia DW, Wang T, Aronson PS. NHE3 phosphorylation at serines 552 and 605 does not directly affect NHE3 activity. Am J Physiol Renal Physiol 293: F212–F218, 2007. First published April 4, 2007; doi:10.1152/ajprenal.00042.2007.—Direct phosphorylation of sodium hydrogen exchanger type 3 (NHE3) is a well-established physiological phenomenon; however, the exact role of NHE3 phosphorylation in its regulation remains unclear. The objective of this study was to evaluate whether NHE3 phosphorylation at serines 552 and 605 is physiologically regulated in vivo and, if so, whether changes in phosphorylation at these sites are tightly coupled to changes in transport activity. To this end, we directly compared PKA-induced NHE3 inhibition with site-specific changes in NHE3 phosphorylation in vivo and in vitro. In vivo, PKA was activated using an intravenous infusion of parathyroid hormone in Sprague-Dawley rats. In vitro, PKA was activated directly in opossum kidney (OKP) cells using forskolin and IBMX. NHE3 activity was assayed in microvillar membrane vesicles in the rat model and by 22Na uptake in the OKP cell model. In both cases, NHE3 phosphorylation at serines 552 and 605 was determined using previously characterized monoclonal phosphospecific antibodies directed to these sites. In vivo, we found dramatic changes in NHE3 phosphorylation at serines 552 and 605 with PKA activation but no corresponding alteration in NHE3 activity. This dissociation between NHE3 phosphorylation and activity was further verified in OKP cells in which phosphorylation clearly preceded transport inhibition. We conclude that although phosphorylation of NHE3 at serines 552 and 605 is physiologically regulated, but alone is not sufficient to inhibit NHE3 activity.

MATERIALS AND METHODS

Materials

We purchased adult male Sprague-Dawley rats from Charles River Laboratories; goat anti-rabbit horseradish peroxidase (HRP)-conjugated and goat anti-mouse HRP-conjugated secondary antibodies from Zymed; DMEM, fetal calf serum, penicillin-streptomycin, and Na-pyruvate from Gibco; polyvinylidene fluoride (PVDF) microporous membrane and 0.65-μm filters from Millipore; enhanced chemiluminescence system from GE and Perkin-Elmer; 24-well plates for cell culture from BD Falcon. The polyclonal antibody to NaPi-IIa was a kind gift from H. Murer. The polyclonal antibody to beta-actin was purchased from Novus Biologicals. All other reagents and chem-

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icals were obtained from Sigma. NIH densitometry program (Scion Image) was provided by Scion.

Methods

Administration of PTH to Sprague-Dawley rats. All animal work was conducted according to an Institutional Animal Care and Use Committee-approved protocol at Yale School of Medicine. For each experiment, two adult male Sprague-Dawley rats were anesthetized with intraperitoneal pentobarbital sodium. One animal was given saline and the other PTH, the synthetic bovine fragment 1–34 dissolved in 4% BSA in 0.9% NaCl. PTH was given intravenously through the internal jugular vein as a bolus dose of 6.6 mg/kg followed by a continuous infusion of 0.1 mg·kg⁻¹·min⁻¹ as per the protocol of Yang et al. (39). The number of replicates of each experiment is given in the figure legends.

Preparation of total rat kidney membranes. After treatment with either saline or PTH, the kidneys were removed from the animals and placed in a sucrose buffer (250 mM sucrose, 10 mM HEPES, 5 mM leupeptin, and 0.7 μg/ml pepstatin) and phosphatase inhibitors (50 mM NaF and 15 mM sodium pyrophosphate) for homogenization. The homogenates were subjected to a low-speed centrifugation (2,400 g) for 30 min at 4°C to remove any particulate material, nuclei, and mitochondria from the supernatant. The supernatants were then subjected to a high-speed centrifugation (47,000 g for 45 min at 4°C), creating membrane pellets. The pellets were resuspended in the sucrose buffer and the protein concentrations were determined by the method of Lowry. The samples from saline and PTH rats were kept separate but handled identically and simultaneously. For experiments comparing total kidney membranes prepared from these rats, equal amounts of protein from each membrane preparation were loaded onto a gel, separated by SDS-PAGE, and then subjected to Western blotting with various antibodies.

SDS-PAGE and immunoblotting. Rat kidney membranes (75 μg), microvillar membrane vesicles (15–20 μg), or OKP cells were solubilized in sample buffer (10% SDS, 20% glycerol, 2% β-mercaptoethanol, 2.5 mM Tris, pH 6.8), and then subjected to SDS-PAGE using 7.5% polyacrylamide gels. Proteins were then transferred from the polyacrylamide gel to a PVDF microporous membrane that was used for immunoblotting. The PVDF membrane was incubated with blocking solution (5% nonfat dry milk and 0.1% Tween 20 in PBS, pH 7.4) for 1 h at room temperature to block nonspecific binding. The PVDF membrane was subsequently incubated overnight at 4°C with the primary antibody in blocking solution at the following concentrations: anti-PS552 [monoclonal 14D5 (18)] at 1:1,000, anti-PS605 [monoclonal 10A8 (18)] at 1:1,000 for rat kidney membranes, and 1:500 for OKP cells, anti-NHE3 [monoclonal 3H3 (18)] at 1:1,000, rabbit polyclonal anti-NaPi-IIa (23) at 1:10,000, anti-beta-actin at 1:20,000 for OKP cells, and 1:40,000 for MMV. After the membrane was washed with blocking solution, appropriate secondary antibody was added at a concentration of 1:2,000 and allowed to incubate for 1 h at room temperature. Goat anti-mouse HRP-conjugated secondary antibody was used for blots probed with anti-PS552, anti-PS605, and anti-NHE3. Goat anti-rabbit HRP-conjugated secondary antibody from Zymed was used for blots probed with anti-NaPi-IIa. Membranes were again washed with Blotto and then rinsed with PBS. Antibody was visualized using an enhanced chemiluminescence system.

Microvillar membrane vesicle preparation. After treatment with either saline or PTH, the kidneys were removed and placed in K-HEPES buffer (200 mM mannitol, 80 mM HEPES, 41 mM KCl, pH 7.5) on ice. Rat kidney cortices were isolated and homogenized in this same buffer. The technique of differential centrifugation and magnesium precipitation described previously by our lab was used to isolate microvillar membrane vesicles (MMVs) from these rat cortices (2). The samples from saline and PTH rats were kept separate but handled identically and simultaneously. Protein concentrations were determined by the method of Lowry, and samples were stored at −80°C. For experiments comparing MMV prepared from saline and PTH rats, equal amounts of protein from each membrane preparation were used.

Measurement of 22Na uptake in MMV. 22Na uptake was measured in triplicate or quadruplicate at room temperature using the rapid filtration technique described previously (3). MMVs prepared simultaneously from saline- and PTH-treated rats were thawed and allowed to equilibrate in 105 mM mannitol, 52 mM KCl, 42 mM HEPES, 52 mM MES, 22 mM KOH, pH 6.1, for 2 h at room temperature. The uptake of 22Na was then assayed after mixing 10 μl of the preincubated membrane vesicles (100–200 μg microvillar membrane protein) with 40 μl of a “hot” solution containing 0.1 μCi of 22Na, resulting in final medium composition 136 mM mannitol, 51 mM KCl, 1 mM NaCl, 42 mM HEPES, 10 mM Mes, 32 mM KOH, pH 7.5. The uptake reaction was stopped after 10 s by addition of 3 ml ice-cold solution containing 100 mM KCl, 42 mM HEPES, pH 7.5. Vesicles were collected on 0.65-μm Millipore filters. After washing the filters with an additional 6 ml of stop solution, 22Na radioactivity was measured using a liquid scintillation counter. Final protein concentration was determined using the method of Lowry.

OKP cell culture. OKP cells were grown in DMEM with 10% fetal calf serum, 50 U/ml penicillin, 50 mg/ml streptomycin, and 1 mM Na-pyruvate at 37°C in 5% CO2-95% air. Cells were transferred to 24-well culture plates, serum-starved for 24 h, and then used at 95–100% confluency for Western blotting or transport assays.

Radioactive sodium uptake in OKP cells. For 22Na uptake assay, cells in a 24-well plate were used at 90–100% confluency. NHE3 activity was measured after acid-loading by the NH4Cl prepulse technique (14). After aspiration of the culture medium, the cells were incubated in an isotonic NH4Cl solution (30 mM NH4Cl, 90 mM choline chloride, 5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 5 mM glucose, and 20 mM HEPES-Tris, pH 7.4) at room temperature for 30 min. This solution was then aspirated and an isotonic choline chloride solution containing 1 mM NaCl, 120 mM choline chloride, 5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 5 mM glucose, 1 mM NaCl, and 20 mM HEPES-Tris, pH 7.4, was added for 5 min. After aspiration of the “cold” choline Cl solution, a “hot” choline chloride solution with 2 μCi/ml of 22Na was added for 60 s. The influx of radioactive sodium was terminated after 60 s by three rapid washes of the cell monolayers with ice-cold choline chloride solution. Cells were then solubilized using 0.2 M NaOH and then neutralized with addition of an equal amount of 0.2 N HCl. The solubilized cells were then transferred to vials and the radioactivity was measured using a liquid scintillation counter.

Forskolin/IBMX treatment of OKP cells. Cells were treated with either vehicle (Ctrl) or forskolin/IBMX (F/I) for 5, 10, or 30 min before solubilization of cells or assay of transport activity. For all conditions, the cells were exposed to an NH4Cl preincubation solution for 30 min followed by a choline chloride solution for 5 min. For the 30-min exposure, forskolin (10−4 M) + IBMX (10−3 M) was added for the last 25 min of the 30-min preincubation with the NH4Cl solution, and then in the choline chloride solution for 5 min. The 10-min exposure was carried out as follows: preincubation with NH4Cl solution for 25 min, continued preincubation for another 5 min with forskolin (10−4 M) + IBMX (10−3 M) added to the NH4Cl solution, followed by the addition of the choline chloride solution with forskolin (10−4 M) + IBMX (10−3 M) for 5 min. For the 5-min exposure, forskolin (10−3 M) + IBMX (10−3 M) were added only to the choline chloride solution for 5 min. At the end of the treatment period, cells were either solubilized immediately with sample buffer for SDS-PAGE and immunoblotting or exposed immediately to radioactive sodium for a 60-s assay of transport activity.
RESULTS

In Vivo Effect of PKA Activation on Renal NHE3 Expression, Phosphorylation, and Activity in Sprague-Dawley Rats

As a means of activating PKA in vivo (1, 34), intravenous PTH was administered to Sprague-Dawley rats. Total NHE3 expression was compared in equal amounts (75 μg) of kidney membranes prepared from rats administered PTH and rats administered saline. In Fig. 1A, bottom, these kidney membranes were probed with anti-NHE3, a general NHE3 antibody. No difference in total NHE3 expression was seen between the control and treatment animals. These same kidney membranes were then probed with anti-PS552 and anti-PS605, monoclonal phosphospecific antibodies which recognize NHE3 only when phosphorylated at either serine 552 or 605, respectively (18). As illustrated in Fig. 1A, top and middle, NHE3 phosphorylation at serine 552 and serine 605 was dramatically increased in the kidney membranes prepared from PTH-administered animals compared with controls. These findings were confirmed by densitometric analysis of Western blotting data for four pairs of animals (Fig. 1B). These findings prove that phosphorylation of endogenously expressed NHE3 in vivo can be induced at serines 552 and 605, two PKA phosphorylation sites.

The Western blotting results shown in Fig. 1 represent changes in NHE3 phosphorylation in total kidney membranes. However, NHE3-mediated Na+/H+ exchange activity occurs in the apical membrane of the proximal tubule. To test for changes in total NHE3 content and NHE3 phosphorylation at serines 552 and 605 specifically within the apical membrane of the proximal tubule, MMVs were isolated from control and treatment rats. As shown in Fig. 2, Western blotting of these MMVs confirmed a similar pattern of total NHE3 expression and phosphorylation as was seen in the total kidney membranes. Specifically, in the MMVs, total NHE3 expression was unchanged while NHE3 phosphorylation at both serines 552 and 605 was increased in PTH-administered animals compared with controls (Fig. 2A). The Western blotting data for the MMVs were confirmed by densitometry and are presented in Fig. 2B. When the densitometry data for total NHE3 were normalized to beta-actin as a loading control, similar results were obtained and there was no statistically significant difference in total NHE3 expression between control and PTH-treated rats (data not shown). Therefore, in the microvillar domain, where NHE3 normally functions, phosphorylation changes are clearly present, while total NHE3 expression remains unchanged.

Next, we sought to evaluate the relationship between NHE3 phosphorylation and NHE3 activity in the brush-border membrane after PKA activation induced by intravenous PTH administration. To this end, Na+/H+ exchange activity was assayed in renal MMVs prepared from both control and treatment rats. When Na+/H+ exchange activity was analyzed in the MMVs, surprisingly, there was no significant difference in activity between saline- and PTH-administered animals (Fig. 3). The remarkable increase in NHE3 phosphorylation seen in Fig. 2 with no corresponding change in NHE3 activity demonstrates dissociation between NHE3 phosphorylation and activity.

Given this unexpected result, we felt it was essential to establish a normal physiological response of the proximal
tubule to PTH in our animal model. In this regard, we examined the effect of PTH administration on NaPi-IIa expression in renal MMVs. Renal brush-border membrane expression of NaPi-IIa has been well documented to decrease in response to PTH administration in multiple animal models (17, 29, 39). To evaluate the effect of PTH on NaPi-IIa expression in the apical membrane of our animal model, Western blots were prepared from the same MMVs used for the Na\(^+\)/H\(^+\) exchange assay and probed with a polyclonal antibody to NaPi-IIa. Figure 4A demonstrates the expected decrease in NaPi-IIa expression seen in animals who received PTH compared with controls. This finding is confirmed by densitometric analysis of three independent experiments (Fig. 4B) and establishes a normal physiological response of the proximal tubule to PTH in our animal model. Therefore, we have identified a dose and time point after in vivo PTH administration at which there is a robust decrease in NaPi-IIa expression, a significant increase in NHE3 phosphorylation at serines 552 and 605, yet no detectable change in NHE3 activity.

**In Vitro Effect of PKA Activation on Endogenous NHE3 Expression, Phosphorylation, and Activity in OKP Cells**

To better characterize the dissociation between NHE3 phosphorylation and NHE3 activity seen in the rat PTH model, we studied their relationship in a proximal tubule cell model. Use of a proximal tubule cell culture model afforded several advantages over further study in rats: 1) ability to activate PKA directly, 2) assay of transport activity in intact live cells rather than isolated membrane vesicles, 3) a more facile analysis of the temporal relationship between phosphorylation and transport inhibition, and 4) verification of our findings in an entirely different system. In a previous publication, we examined NHE3 phosphorylation and activity in OKP cells after a 30-min exposure to forskolin and IBMX (drugs which increase intracellular cAMP) (18). In that study, we documented an increase in NHE3 phosphorylation at both serines 552 and 605, no change in total NHE3 content, and a decrease in NHE3 activity compared with control. Here, we use the same cellular model but investigate the temporal relationship between NHE3 activity and phosphorylation in more detail. Figure 5 shows that \(^{22}\)Na uptake is essentially unchanged compared with control after a 5-min exposure to forskolin/IBMX; however, 10- and 30-min exposures to forskolin/IBMX decrease \(^{22}\)Na uptake by 40–50%. Thus, there is a significant delay in the onset of transport inhibition after drug addition.

To examine the time course of NHE3 phosphorylation, and its relationship to NHE3 inhibition, we used anti-NHE3, anti-PS552, and anti-PS605 to probe membranes prepared from OKP cells exposed to either vehicle (Ctrl) or forskolin/IBMX for 5, 10, or 30 min. As seen in the representative Western blot in Fig. 6, and confirmed by densitometry in Fig. 7, there is no significant delay in the onset of transport inhibition after drug addition.
change in total NHE3 content at any of the time points after forskolin/IBMX exposure compared with control. Normalization of total NHE3 expression to beta-actin (as a loading control) confirmed these findings (data not shown). The representative Western blot in Fig. 6 demonstrates the increase in NHE3 phosphorylation at both serines 552 and 605 seen at all time points after forskolin/IBMX exposure compared with control. In particular, it should be noted that a similar increase in NHE3 phosphorylation is seen 5 min after drug exposure (when no transport inhibition was detected) and at 30 min (when transport inhibition was actually observed). Densitometric analysis of five independent experiments revealed approximately a twofold increase in phosphorylation at the 5-min time point with no substantial additional increase in phosphorylation at either residue at later time points. These data are presented in Fig. 7. Therefore, we uncovered a temporal dissociation between NHE3 phosphorylation and inhibition of transport activity after forskolin/IBMX administration. These results confirm the lack of direct association between NHE3 phosphorylation and NHE3 activity as seen in the rats after PTH administration. Although NHE3 phosphorylation may well be required for transport inhibition as suggested by previous studies (19, 41), these findings demonstrate that NHE3 phosphorylation per se does not directly inhibit transport.

DISCUSSION

We found that activation of PKA in vivo, via intravenous PTH administration, markedly increased renal NHE3 phosphorylation at serines 552 and 605 in Sprague-Dawley rats. These findings demonstrate that changes in phosphorylation can occur in vivo at specific residues of endogenously expressed NHE3. Despite these dramatic changes in NHE3 phosphorylation, however, there was no change in NHE3 activity in this animal model of PKA activation. To substantiate the newly discovered dissociation between NHE3 phosphorylation and activity, we studied their relationship more thoroughly in a cell model with endogenous NHE3 expression (OKP cells). Further study in OKP cells allowed verification of our findings in a completely different system and facilitated analysis of the temporal relationship between phosphorylation and transport inhibition. In the OKP cell model, no transport inhibition was observed at an early time point (5 min) after PKA activation despite an increase in NHE3 phosphorylation. This increase in NHE3 phosphorylation was essentially identical to that observed at later time points when NHE3 activity was reduced. Taken together, these data demonstrate that phosphorylation of NHE3 must not directly cause transport inhibition.

The goal of our study was to evaluate the relationship between NHE3 phosphorylation and activity, and our findings should not be taken to cast doubt on multiple previous studies that have established the ability of PTH to inhibit proximal tubule bicarbonate reabsorption in vivo (6, 7, 13, 15, 28). In fact, there are multiple technical differences between our study and earlier studies to explain this apparent disparity in findings. For example, we assayed NHE3 activity in isolated MMVs to make a direct correlation with phosphorylation in the same preparation. PTH-induced inhibition of bicarbonate reabsorption, as observed at the level of the intact tubule, could result from alteration of other parameters affecting net transtubular bicarbonate reabsorption besides brush-border Na\(^+\)/H\(^+\) exchange. Indeed, Na\(^+\)-K\(^+\)-ATPase activity, Na\(^+\)-HCO\(_3\) \(^{-}\) cotransport activity, and paracellular permeability, all of which affect transtubular bicarbonate reabsorption, have been reported to be regulated by PTH (24, 30, 32, 40). Furthermore, early PTH-mediated inhibition of NHE3 may depend on factors which may not be present or functional in MMVs.

We are aware of only one previous study in which PTH was acutely administered in vivo and its effects on Na\(^+\)/H\(^+\) exchange activity assayed in brush-border vesicles. In that study, Fan et al. (13) administered a single intravenous bolus dose of 100 \(\mu\)g/kg to parathyroidectomized Sprague-Dawley rats. Using this protocol, NHE3 activity was inhibited <20% at 1 h and reached maximal inhibition of 40% at 4 h after PTH administration. This contrasts with the far lower bolus PTH dose (6.6 \(\mu\)g/kg) used in our study in which no change in Na\(^+\)/H\(^+\) activity was detected after 1 h. Since the central conclusion of our study is the dissociation of NHE3 phosphorylation from the inhibition of transport activity, we did not perform additional experiments using larger PTH doses and/or later time points that may have allowed detection of NHE3 inhibition. Rather, we conducted additional studies in an in vitro proximal tubule model to confirm the dissociation between NHE3 phosphorylation and transport activity seen in vivo.

Our study clearly showed that phosphorylation of serines 552 and 605 of NHE3 precedes transport inhibition and therefore cannot be directly responsible for altering NHE3 activity. Nevertheless, phosphorylation of one or both of these residues may be required for PKA-mediated transport inhibition. In fact,
previous mutagenesis studies have clearly established that serine 605 and probably serine 552 are essential for PKA-induced inhibition of Na\(^+/\)H\(^-\) exchange activity (20, 41). Therefore, rather than imposing a direct effect, phosphorylation of these sites must initiate subsequent regulatory events that ultimately lead to inhibition of NHE3 activity. In fact, our findings of dissociation between NHE3 phosphorylation and transport activity in isolated MMVs provide further evidence that phosphorylation-induced inhibition of NHE3 activity must be dependent on additional factors or processes that are absent in MMVs but are functional in intact living cells, such as OKP cells. Thus our findings do not at all refute the concept that phosphorylation of NHE3 at residues 552 and/or 605 may be required for inhibition of transport activity in response to PKA activation. However, our findings do establish clearly that phosphorylation alone does not cause direct inhibition of NHE3 activity, for example by an allosteric effect, since phosphorylated NHE3 is capable of normal transport activity.

One mechanism by which NHE3 phosphorylation at serines 552 and 605 may result in subsequent inhibition of transport activity is by affecting NHE3 subcellular trafficking, or modulating the interaction of NHE3 with other regulatory proteins. There is some published evidence to suggest a role for NHE3 phosphorylation in its subcellular trafficking. For instance, dopamine has been shown to stimulate endocytosis of NHE3 that is PKA dependent and blocked by mutation of PKA phosphorylation sites (16). PTH itself induces changes in the localization of NHE3 within the brush-border membrane (39). Additionally, it has been demonstrated that NHE3 phosphorylated at serine 552 has a distinct subcellular localization compared with total NHE3 at baseline in vivo (18). It has been proposed that phosphorylation-induced changes in NHE3 subcellular localization are part of a biphasic effect of phosphorylation on NHE3 activity: an early phosphorylation-induced change in subcellular localization (11, 13). However, our data establish that phosphorylation cannot lead to a direct inhibition of NHE3 activity. Future studies will be necessary to determine the mechanism(s) by which NHE3 phosphorylation may contribute to the modulation of Na\(^+/\)H\(^-\) exchange activity.

**GRANTS**

This work was supported by National Institutes of Health (NIH) Grants DK-17433 and DK-33793 to P. S. Aronson and NIH Grant K08-DK-072073-02 to H. S. Kocinsky.

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