Defective PTH regulation of sodium-dependent phosphate transport in NHERF-1$^{-/-}$ renal proximal tubule cells and wild-type cells adapted to low-phosphate media

Rochelle Cunningham,1 Xiaofei E,1 Deborah Steplock,1 Shirish Shenolikar,2 and Edward J. Weinman1,3

1Department of Medicine, Department of Physiology, University of Maryland School of Medicine, and 2Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina

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Cunningham, Rochelle, Xiaofei E, Deborah Steplock, Shirish Shenolikar, and Edward J. Weinman. Defective PTH regulation of sodium-dependent phosphate transport in NHERF-1$^{-/-}$ renal proximal tubule cells and wild-type cells adapted to low-phosphate media. Am J Physiol Renal Physiol 289: F933–F938, 2005. First published June 7, 2005; doi:10.1152/ajprenal.00005.2005.—The present experiments were designed to address two questions related to Npt2a trafficking and phosphate transport in response to PTH. In OK cells using an ezrin binding domain-deficient NHERF-1 as a dominant-negative reagent, we found that basal phosphate transport was decreased but that the response to PTH remained intact (17). On the other hand, Mahon and co-workers (21), using a subclone of OK cells that had reduced NHERF-1, found a striking decrease in the inhibitory effect of PTH on phosphate transport. OK cells, in contrast to proximal renal tubules of mice, express only NHERF-1. Accordingly, the first objective of the present experiments was to study the role of NHERF-1 in modulating the effect of PTH on phosphate transport and membrane expression of Npt2a using primary proximal tubule cell cultures from wild-type and NHERF-1$^{-/-}$ mice. The second goal of the present experiments was to determine the effect of PTH on phosphate transport in proximal tubule cells from wild-type mice adapted to growth in low-phosphate media. A number of prior studies in intact animals have suggested that adaptation to a low-phosphate diet decreases the phosphaturic effect of PTH (23, 29, 36). The mechanism of this response is unknown but may reflect increased trafficking of Npt2a to the apical membrane in response to phosphate restriction (3, 18). While it is not certain that the biochemical pathways are identical, it is possible that proximal tubule-like cells in culture in low-phosphate media also increases Npt2a membrane expression and phosphate uptake. In intact animals, the changes in transport and BBM expression of Npt2a could be the result of specific signals read by proximal tubule cells or the result of systemic and/or hormonal changes associated with reduced dietary in-

Address for reprint requests and other correspondence: E. J. Weinman, UMH, 22 S. Green St., Rm. N3W143, Baltimore, MD 21201 (e-mail: eweinman@medicine.umaryland.edu).

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THE SODIUM-DEPENDENT PHOSPHATE TRANSPORTER IIa (Npt2a, NaPi IIa) located in the apical membrane of the renal proximal convoluted tubule is the major determinant of urinary phosphate excretion (11, 22, 30). This transporter is regulated by physiological stimuli such that the abundance of Npt2a in the apical membrane is increased in response to dietary restriction of phosphate and decreased in response to parathyroid hormone (PTH) (1, 3, 9, 17, 18, 21, 25). The processes that determine the trafficking of Npt2a to and from the apical brush-border membrane (BBM), however, have not been completely elucidated. While the transporter has a number of modifiable sites on its COOH terminus, phosphorylation of these residues has little influence on its cellular distribution or activity. These findings resulted in a continued search for regulatory factors and the identification of two novel pathways. One regulatory pathway involves a series of proteins collectedively called phosphatonin (30). The other pathway ensured the recognition that the COOH terminus of Npt2a constituted a class 1 PDZ binding motif (8, 12, 27). The renal proximal tubule expresses at least three PDZ proteins that bind Npt2a, namely, Na$^+$/H$^+$ exchanger regulatory factor-1 (NHERF-1), NHERF-2, and PDZK1. Prior studies have indicated that disruption of the NHERF-1 interaction with ezrin resulted in loss of Npt2a from the apical membrane surface of opossum kidney (OK) cells (12). Moreover, compared with wild-type mice, NHERF-1 null mice demonstrate hypophosphatemia and an increase in the urinary excretion of phosphate associated with decreased abundance of Npt2a in the apical membrane of the cells of the proximal tubule (27).

The present experiments were designed to address two questions related to Npt2a trafficking and phosphate transport in response to PTH. In OK cells using an ezrin binding domain-deficient NHERF-1 as a dominant-negative reagent, we found that basal phosphate transport was decreased but that the response to PTH remained intact (17). On the other hand, Mahon and co-workers (21), using a subclone of OK cells that had reduced NHERF-1, found a striking decrease in the inhibitory effect of PTH on phosphate transport. OK cells, in contrast to proximal renal tubules of mice, express only NHERF-1. Accordingly, the first objective of the present experiments was to study the role of NHERF-1 in modulating the effect of PTH on phosphate transport and membrane expression of Npt2a using primary proximal tubule cell cultures from wild-type and NHERF-1$^{-/-}$ mice. The second goal of the present experiments was to determine the effect of PTH on phosphate transport in proximal tubule cells from wild-type mice adapted to growth in low-phosphate media. A number of prior studies in intact animals have suggested that adaptation to a low-phosphate diet decreases the phosphaturic effect of PTH (23, 29, 36). The mechanism of this response is unknown but may reflect increased trafficking of Npt2a to the apical membrane in response to phosphate restriction (3, 18). While it is not certain that the biochemical pathways are identical, incubation of proximal tubule-like cells in culture in low-phosphate media also increases Npt2a membrane expression and phosphate uptake. In intact animals, the changes in transport and BBM expression of Npt2a could be the result of specific signals read by proximal tubule cells or the result of systemic and/or hormonal changes associated with reduced dietary in-
take of phosphate. To address this question, we have examined the effect of PTH in cultured wild-type renal proximal tubule cells from mice that have been adapted to low-phosphate media. The results of these experiments demonstrate that the inhibitory effect of PTH is blunted in NHERF-1 null cells, indicating a role for this adaptor protein in the PTH signaling pathways that regulate Npt2a expression. In addition, we demonstrate that the inhibitory effect of PTH on phosphate transport and Npt2a expression is decreased in wild-type proximal tubule cells adapted to low-phosphate media. This suggests that the mechanism of the blunted phosphaturic effect of PTH in animals on a low-phosphate diet can be explained, at least in part, by biochemical changes in the cells of the proximal tubule.

**MATERIALS AND METHODS**

**Animals and preparation of renal proximal tubule cells.** Male NHERF-1−/− mice (B6.129-Slc9a3r1tmSsl/Ss1) bred into a C57BL/6 background for six generations and parental wild-type inbred control C57BL/6 mice age 12–16 wk were used in the current experiments (27). All animal protocols and procedures were approved by Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. Primary renal proximal tubule cell cultures were prepared as recently described from this laboratory (5). Kidney cortices were dissected, minced, and digested using 1% Worthington collagenase type II and 0.25% soybean trypsin inhibitor. The samples were then resuspended in 35 ml of 45% Percoll and centrifuged at 26,891 g for 15 min at 4°C. The 5- to 10-mL layer containing the proximal tubule cells was aspirated, centrifuged, washed to remove the remaining Percoll, and resuspended in 6–10 ml of DMEM/F-12 containing 50 U/ml of penicillin, 50 μg/ml streptomycin, 10 ng/ml epidermal growth factor, 0.5 μM hydrocortisone, 0.87 μM bovine insulin, 50 μM prostaglandin E1, 50 mM sodium selenite, 50 μg/ml human transferrin, and 5 μM 3,3′,5-triiodo-l-thyronine. The proximal tubule cells were plated on Matrigel-coated coverslips or plastic cell culture dishes coated with Matrigel and maintained in an incubator at 37°C in 5% CO2. The cultures were left undisturbed for 36 h, after which the media was replaced every 2 days until the cells achieved confluence.

**Transport assays.** Phosphate transport was measured by determination of the sodium-dependent uptake of radiolabeled phosphate (5, 17). Except in the phosphate adaptation studies, the cells were grown in DMEM/F-12 containing ~0.9 mM phosphate. In the adaptation experiments, cells were grown in DMEM/F-12 containing 0.3 mM phosphate for 24 h. One-half of the wells of a given culture were incubated with DMSO for 2 h, whereas the other half were treated with PTH (10−7 M) for 2 h (Table 1). Sodium-independent phosphate transport averaged 0.47 ± 0.10 and 0.44 ± 0.07 nmol·mg protein−1·10 min−1 in the absence or presence of PTH, respectively, indicating that PTH inhibited the sodium-dependent component of phosphate uptake. The decrease in PTH-associated sodium-dependent phosphate transport was associated with a 32.7 ± 5.2% decrease in membrane expression of Npt2a (Fig. 1, Table 2). Sodium-dependent phosphate transport in proximal tubule cells from NHERF-1−/− mice grown in DMEM/F-12 was significantly lower than in wild-type cells grown in the same media and averaged 2.9 ± 0.4 nmol·mg protein−1·10 min−1 (P < 0.05; Table 1). Treatment of the NHERF-1−/− cells with PTH for 2 h resulted in a small but significant decrease in sodium-dependent phosphate uptake to 2.7 ± 0.4 nmol·mg protein−1·10 min−1 (P < 0.05; Table 1). There were no signific-

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<th>Table 1. Effect of PTH (10−7 M) on sodium-dependent phosphate uptake in wild-type and NHERF-1−/− proximal tubule cells</th>
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<td>n</td>
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<tr>
<td>Wild-type, normal-phosphate media</td>
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<tr>
<td>NHERF-1−/−, normal-phosphate media</td>
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<tr>
<td>Wild-type, low-phosphate media</td>
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Values are means ± SE expressed as nmol·mg protein−1·10 min−1. n, No. of preparations studied: NHERF, exchange regulatory factor; PTH, parathyroid hormone. The effect of PTH on sodium-dependent phosphate uptake in wild-type and NHERF-1−/− cultured proximal tubule cells incubated in normal-phosphate media (0.9 mM) and in wild-type cells incubated in low-phosphate media (0.3 mM) for 24 h before the study is shown. *P < 0.05 compared with wild-type cells grown in normal-phosphate media. †P < 0.05 compared with control.
In parallel studies, the signaling pathways of the parathyroid 1R receptor (PTH1R) were examined. PTH (10^{-7} M) stimulated cAMP generation 43.3 ± 6.8-fold and PKC activity by 36.6 ± 0.8% in wild-type proximal tubule cells grown in DMEM/F-12 (Table 3). The response to PTH was not significantly different in NHERF-1 null proximal tubule cells grown in the same media in which PTH stimulated cAMP generation 42.7 ± 6.2-fold and PKC activity by 32.3 ± 3.3%. In wild-type cells grown in the low-phosphate media, PTH stimulated cAMP generation by 45.8 ± 6.5-fold and PKC activity by 33.2 ± 2.2%. These values were not significantly different from the results obtained in wild-type cells grown in DMEM/F-12 (Table 3). Prior studies in OK cells have indicated that PTH significantly increases activation of ERK1/2 (2, 4, 14, 16).

In the current studies, PTH stimulated ERK1/2 phosphorylation in OK cells by 1.6 ± 0.3 fold. By contrast, PTH did not stimulate ERK1/2 phosphorylation at 15–30 min in wild-type mouse proximal tubule cells grown in normal phosphate media but actually inhibited ERK1/2 by 27.5 ± 4.2%. Similar results were observed in NHERF-1^{-/-} cells grown in normal phosphate media where ERK1/2 phosphorylation was decreased by 26.9 ± 7.7%. In wild-type cells grown in low-phosphate media PTH inhibited ERK1/2 activation by 20.5 ± 3.7% (Table 3).

**DISCUSSION**

The factors that regulate BBM Npt2a expression and, as a consequence, renal phosphate excretion are under intense study. Recently, a role for the PDZ domain adaptor protein NHERF-1 in the regulation of Npt2a expression has been advanced (8, 12, 27). Initially, NHERF-1 was identified as an interacting protein in a yeast two-hybrid screen using the Npt2a gene as bait (8). Consistent with these findings, NHERF-1^{-/-} mice demonstrated hypophosphatemia, an increase in the urinary excretion of phosphate, and decreased Npt2a expression in BBM of the renal proximal tubule (27). In intact animals, we have recently demonstrated that the adaptive response to a low-phosphate diet is impaired in NHERF-1^{-/-} mice (35). Moreover, we have reported that NHERF-1 was required for the adaptive increase in sodium-dependent phosphate transport in primary cell cultures of renal proximal tubule cells of mice grown in low-phosphate media (5). In the present experiments, we address two aspects of the regulation of phosphate transport by PTH in the renal proximal tubule, namely, the role of NHERF-1 and the effect of adaptation to low-phosphate media.

**Table 2. Effect of PTH (10^{-7} M) on plasma membrane abundance of Npt2a in wild-type and NHERF-1^{-/-} proximal tubule cells**

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>%Decrease from Control</th>
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<tbody>
<tr>
<td>Wild-type, normal-phosphate media</td>
<td>6</td>
<td>32.7 ± 5.2</td>
</tr>
<tr>
<td>NHERF-1^{-/-}, normal-phosphate media</td>
<td>6</td>
<td>12.8 ± 6.3</td>
</tr>
<tr>
<td>Wild-type, low-phosphate media</td>
<td>5</td>
<td>12.5 ± 5.0</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as % decrease from control cells treated with DMSO. n, No. of cultures studied; Npt2a, sodium-dependent phosphate transporter IIa. The effect of PTH on plasma membrane abundance of Npt2a in wild-type and NHERF-1^{-/-} proximal tubule cells incubated in normal-phosphate media and in wild-type cells incubated in low-phosphate media for 24 h before the study is shown. *P < 0.05 compared with wild-type cells grown in normal-phosphate media.
Table 3. Effect of PTH (10⁻⁷ M) on cAMP generation, PKC activity, and ERK1/2 activation in cultured proximal tubule cells from wild-type and NHERF-1⁻/⁻ cells

<table>
<thead>
<tr>
<th></th>
<th>cAMP Generation</th>
<th>PKC Activity</th>
<th>ERK1/2 Activation</th>
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<tbody>
<tr>
<td>Wild-type, normal-phosphate media</td>
<td>43.3±6.8*(n=4)</td>
<td>36.6±0.8*(n=3)</td>
<td>−26.9±7.7*(n=5)</td>
</tr>
<tr>
<td>NHERF-1⁻/⁻, normal-phosphate</td>
<td>42.7±6.2*(n=3)</td>
<td>32.3±3.3*(n=3)</td>
<td>−27.5±4.2(n=5)</td>
</tr>
<tr>
<td>Wild-type, Low-phosphate media</td>
<td>45.8±6.5*(n=4)</td>
<td>33.2±2.2*(n=3)</td>
<td>−20.5±3.7*(n=3)</td>
</tr>
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Values are means ± SE. n, No. of preparations studied. cAMP results are expressed as fold-stimulation above background, PKC was assayed as activity and expressed as percent stimulation above background, and ERK1/2 results are expressed as % inhibition. The effect of PTH on the generation of cAMP and the activation of PKC and ERK1/2 are shown. PTH significantly stimulated cAMP accumulation and PKC activity and inhibited ERK1/2 activation compared with control cells treated with DMSO (*P < 0.05). There were no significant differences in PTH stimulation of cAMP, PKC, or inhibition of ERK1/2 among the groups.

Two prior studies have addressed the potential role of NHERF-1 on the inhibitory effect of PTH on Npt2a expression and sodium-dependent phosphate transport (17, 21). In OK cells, expression of an ezrin binding domain-deficient NHERF-1 as a dominant-negative reagent resulted in decreased basal phosphate transport but no change in the relative magnitude of the inhibitory effect of PTH (17). By contrast, more recent studies using a subclone of OK cells that expresses low levels of NHERF-1 demonstrated a blunted effect of PTH (21). OK cells, while a favored proximal tubule cell model for study of PTH signaling and phosphate transport, may not mirror events in all proximal tubule cells. Like the proximal tubule of the rat, OK cells express NHERF-1 but not NHERF-2 (32). Human and mouse proximal tubules cells, on the other hand, express both NHERF-1 and NHERF-2, and the potential interaction between these proteins may affect the response to physiological stimuli (33, 34). In wild-type proximal tubule cells, PTH inhibited membrane Npt2a expression and sodium-dependent phosphate transport by ~30%. By contrast, the inhibitory effect of PTH on Npt2a expression and phosphate transport was significantly less in NHERF-1⁻/⁻ cells. When considered with our prior studies, the present results indicate involvement of NHERF-1 in the two major regulatory controllers of phosphate excretion: limitation of the dietary intake of phosphate or incubation of cultured cells in low-phosphate media, which increases phosphate transport, and the response to PTH, which inhibits phosphate transport (5). The precise interaction between NHERF-1 and Npt2a is not known, but we have previously speculated that NHERF-1 serves as a retention signal for Npt2a in BBM. We favor this speculation rather than postulating that NHERF-1 is required for the likely independent processes that recruit Npt2a to the apical membrane in response to restriction of dietary phosphate intake as well as the processes that retrieve Npt2a from the BBM in response to PTH. We would also propose that the NHERF-1/Npt2a pool of Npt2a is the PTH-responsive pool. We recognize that these hypotheses await experimental verification.

The adaptation to dietary restriction of phosphate intake is rapid and associated with recruitment of Npt2a to the BBM and, as a consequence, decreased urinary excretion of phosphate (3, 18, 25, 35). Several prior studies in intact animals have indicated that the phosphaturic effect of PTH is blunted in animals adapted to a low-phosphate diet (23, 29, 36). The mechanism of this relative resistance to the phosphaturic effect of PTH is unknown but may be the result of specific biochemical changes in renal proximal tubule cells or changes in systemic factors that alter PTH signaling. Proximal tubule cells from wild-type mice demonstrate increased phosphate transport when incubated in low- compared with high-phosphate media (5). This model system, then, permits examination of the effect of PTH on phosphate transport and Npt2a expression in the absence of changes in systemic factors. Our findings indicate that while basal phosphate transport is higher in cells grown in the low- compared with normal-phosphate media, there is a decrease in the inhibitory effect of PTH on Npt2a expression and phosphate transport in cells adapted to low-phosphate media. It is acknowledged that the adaptive response to incubation of cells in low-phosphate media may not be identical to restriction of the dietary intake of phosphate. To the degree that both types of responses utilize similar mechanisms, however, the present studies indicate that the biochemical changes reside, at least in part, in the cells of the proximal tubule and are not the consequence of systemic factors. Our prior studies have indicated that the abundance of NHERF-1 and NHERF-2 was not different between mice proximal tubule cells grown in low- vs. high-phosphate media, making it unlikely that the NHERF proteins are involved in the blunted response to PTH in adapted cells (5). We did observe a modest increase in PDZK1 expression in cells grown in low-phosphate media (5). The role of PDZK1 in phosphate transport is not fully elucidated. PDZK1 mRNA is increased in rats fed a low-phosphate diet, and initially it was believed that this protein might be involved in the recruitment of Npt2a to the BBM (6). On the other hand, changes in PDZK1 protein have not been consistently observed, and PDZK1⁻/⁻ mice on a normal-phosphate diet have no apparent abnormalities in Npt2a targeting in the kidney, or in blood or urine phosphate concentrations (15). Nonetheless, a role for PDZK1 cannot be excluded. In our view, it remains possible that PDZK1 recruited to the BBM in mice proximal cells grown in low-phosphate media may interfere with the mechanisms subserving PTH-mediated reduction of Npt2a abundance in the BBM.

The relationship among NHERF-1, PTH signaling, and control of Npt2a activity is complex. PTH (PTH1R) receptors are located on both the apical as well as the basolateral side of renal proximal tubule cells (7, 26, 31). The basolateral receptor signals through adenylyl cyclase and PKC, whereas the apical receptor is believed to signal exclusively through PKC (31). Recent studies have indicated that NHERF-1 acts as a molecular switch by binding to and facilitating PTH receptor signaling through the PKC rather than the PKA pathway (20). In addition, NHERF-1 also regulates PTH receptor kinetics in response to PTH fragments (28). Because NHERF-1 is localized predominantly at the apical membrane of the renal proximal tubule, it is believed that it interacts with apical PTH1R (32, 33). In our prior studies in mice proximal tubule cells in...
culture, however, we were unable to demonstrate differences in cAMP generation or PKC activation in response to PTH in wild-type cells compared with NHERF-1−/− cells, results which are confirmed herein (5). At the present time, it is not known whether the normal cAMP and PKC responses represent PTH interaction exclusively with the basolateral receptor or the equivalent NHERF-2 interaction with the apical PTHR receptor (20). The signaling pathways utilized by PTH to inhibit phosphate transport and apical membrane Npt2a expression include activation of PKA, PKC, and, as recently reported, ERK1/2 (2, 4, 14, 16). In OK cells, PTH causes a vigorous two- to fivefold increase in ERK1/2 activation when assayed as activity or by phosphorylation of serine 204 (4, 14, 16). Lederer and co-workers (16) studied the effect of a specific inhibitor of ERK1/2 in OK cells. Curiously, these studies showed significant attenuation of PTH inhibition of phosphate uptake but not PTH-associated downregulation of the abundance of NaPi-4, the OK cell sodium-dependent phosphate transporter (16). Other experiments in kidney slices of mice demonstrated that PTH treatment decreased apical membrane expression of Npt2a and that this effect was blocked by an inhibitor of ERK1/2 (2). Assays of stimulation of ERK1/2 in response to PTH, however, indicated only modest (30–50%) stimulation at early time points rather than the several-fold stimulation reported in OK cells. In the present experiments, we confirm the activation of ERK1/2 in response to PTH in OK cells. In contrast, in cultured proximal tubules from mice, PTH did not stimulate ERK1/2 and, in fact, decreased ERK1/2 phosphorylation below baseline. Interestingly, there is precedence for an inhibitory effect of PTH on ERK1/2 activation in osteoblastic and bone marrow-derived cells (13, 24). Although the mechanism and biological significance remain to be elucidated, it would appear that the effect of PTH on ERK1/2 in OK cells is clearly different in mouse proximal tubule cells compared with OK cells. The fact that PTH inhibited phosphate transport and decreased plasma membrane expression of Npt2a in cultured wild-type mouse proximal tubule cells grown in normal-phosphate media suggests that activation of ERK1/2 is not an absolute requirement for mediating the inhibitory effect of PTH. Moreover, the inhibitory effect of PTH on ERK1/2 was the same in wild-type cells grown in normal- or low-phosphate media, and in NHERF-1 null cells, indicating that the differential responses to PTH are not the result of differences in ERK1/2 kinetics.

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