Parathyroid hormone-dependent endocytosis
of renal type IIc Na-Pi cotransporter

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Segawa H, Yamanaka S, Onitsuka A, Tomoe Y, Kuwahata M, Ito M, Taketani Y, Miyamoto K-i. Parathyroid hormone-dependent endocytosis of renal type IIc Na-Pi cotransporter. Am J Physiol Renal Physiol 292: F395–F403, 2007. First published September 19, 2006; doi:10.1152/ajprenal.00100.2006.—Hereditary hypophosphatemic rickets with hypercalciuria results from mutations of the renal type IIc Na-Pi cotransporter gene, suggesting that the type IIc transporter plays a prominent role in renal phosphate handling. The goal of the present study was to investigate the regulation of the type IIc Na-Pi cotransporter by parathyroid hormone (PTH). Type IIc Na-Pi cotransporter levels were markedly increased in thyroparathyroidectomized (TPTX) rats. Four hours after administration of PTH, type IIc transporter protein levels were markedly decreased in the apical membrane fraction but recovered to baseline levels at 24 h. Immunohistochemical analyses demonstrated the presence of the type IIc transporter in the apical membrane and subapical compartments in the proximal tubular cells in TPTX animals. After administration of PTH, the intensity of immunoreactive signals in apical and subapical type IIc transporter decreased in the renal proximal tubular cells in TPTX rats. Colchicine completely blocked the internalization of the type IIc transporter. In addition, leupeptin prevented the PTH-mediated degradation of the type IIa transporter in lysosomes but had no effect on PTH-mediated degradation of the lysosomal type IIc transporter. In PTH-treated TPTX rats, the internalization of the type IIc transporter occurred after administration of PTH(1–34) (PKA and PKC activator) or PTH(3–34) (PKC activator). Thus the present study demonstrated that PTH is a major hormonal regulator of the type IIc Na-Pi cotransporter in renal proximal tubules.

Address for reprint requests and other correspondence: K. Miyamoto, Dept. of Molecular Nutrition, Institution of Health Biosciences, The Univ. of Tokushima Graduate School, Kuramoto-Cho 3, Tokushima City 770-8503, Japan (e-mail: miyamoto@nutr.med.tokushima-u.ac.jp).

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To study the internalization of type IIc Na-Pi cotransporters that are subsequently routed to the lysosomes, TPTX rats were injected intraperitoneally with 6 mg of leupeptin (in 0.5 ml of PBS) 1 h before injection of PTH. To study an involvement of microtubules in transcytosis of type IIc Na-Pi cotransporter in TPTX rats was investigated after intravenous administration of bovine PTH (amino acid residues 1–34; Sigma) at a dose of 7.5 μg/100 g body wt under anesthesia (14, 27).

To determine whether the type II Na-Pi cotransporter was regulated by PTH, Western and Northern blot analyses were performed to Western and Northern blot analyses were performed to determine cortical membrane isolation and four individual rats for the preparation of membrane fraction, lysosomal fraction, and immunoblotting.

RNA Isolation and Northern Blotting
Total RNA was extracted from the kidney of rats with ISOGEN (Nippon Gene, Tokyo, Japan), and isolated poly(A)+ RNA (3 μg/ lane) was separated on a 1% agarose gel in the presence of 2.2 M formaldehyde and blotted onto a Hybond-N+ membrane (Amersham Pharmacia Biotech) as described previously (24, 25). The specific probes for each Na-Pi cotransporter were labeled with [32P]dCTP by the Megaprime DNA labeling system (Amersham Pharmacia Biotech). Hybridization proceeded for 3 h at 65°C in Rapid-hyb buffer (Amersham Pharmacia Biotech). The blot was evaluated by autoradiography using Fujix bioimaging analyzer BAS-1500 (Fujifilm, Tokyo, Japan) (24, 25).

**Preparation of Membrane Fraction, Lysosomal Fraction, and Immunoblotting**

Methods for preparation of BBM vesicles (BBMVs) with the use of calcium precipitation have been previously described (3). The preparation of cortical membrane was done according to a previously described method (29). Tissue homogenates in 0.3 M sucrose containing protease inhibitor cocktail tablets (Complete Mini, EDTA-free; Roche) were centrifuged at 600 g for 10 min, and the supernatant was centrifuged for 20 min at the same speed. The cytosol was then centrifuged at 105,000 g for 45 min, and the membrane pellet was used for Western blotting.

Lysosomes from kidney cortex were isolated according to Maunsbach (16). The homogenate was centrifuged at 150 g for 10 min; from the resulting supernatant, a crude lysosomal fraction was obtained by centrifugation at 9,000 g for 3 min. Afterward, lysosomes were purified by use of a linear sucrose gradient (1.2–2.2 M in 1 mM EDTA, 1 mM Tris·HCl; pH 7.5). The distribution pattern of the lysosomes was determined by the activities of acid phosphatase and glucosaminidase (11, 16).

Protein samples were heated at 95°C for 5 min in sample buffer in the presence of 5% 2-mercaptoethanol and subjected to 8% SDS-PAGE. The separated proteins were transferred electrophoretically to polyvinylidene difluoride transfer membranes (Hybond-P; Amersham Pharmacia Biotech). The membranes were treated with dilute rabbit affinity-purified anti-type IIC (1:500) or anti-type IIa (1:4,000) Na-Pi cotransporter antibodies (24, 25), followed by treatment with horseradish peroxidase-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories) (24, 25). Signals were detected by the ECL Plus system (Amersham Pharmacia Biotech).

**Immunohistochemistry**

Rats were anesthetized with pentobarbital sodium (100 mg/kg body wt), and their kidneys were perfused via the left ventricle with PBS followed by 4% paraformaldehyde solution (pH 7.2). Kidneys were postfixed with the same solution overnight at 4°C, washed with PBS, cryoprotected with 10% and 20% sucrose at 4°C, and embedded in OCT compound (Miles, Elkhart, IN) and frozen in hexane at −80°C. Frozen sections (5 μm) were collected onto silane-coated slides and air dried. For immunofluorescence microscopy, serial sections were incubated with rabbit anti-type IIa (1:4,000) and guinea pig anti-type IIc (1:200) Na-Pi cotransporter antibodies overnight at 4°C (26). Thereafter, sections were treated with Alexa Fluor 568 anti-rabbit (Molecular Probes) or Alexa Fluor 488 anti-guinea pig (Molecular Probes) as secondary antibody for 60 min at room temperature (26).

**Data Analysis**

Data are expressed as means ± SD. Differences among multiple groups were analyzed by ANOVA. Differences between two experimental groups were determined by ANOVA followed by Student’s t-test. P values <0.05 were considered statistically significant.

**RESULTS**

Expression of the Type IIC Na-Pi Cotransporter in TPTX Rat Kidney

Western and Northern blot analyses were performed to determine whether the type II Na-Pi cotransporter was regulated in the kidney of TPTX rats (Fig. 1). Type IIa Na-Pi cotransporter levels were significantly increased in the TPTX rat kidney compared with sham-operated controls (Fig. 1, A and B, top). In addition, type IIC Na-Pi cotransporter protein (Fig. 1A, middle) and mRNA levels (Fig. 1B, middle) were significantly increased in the TPTX rat kidney.

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**Table 1. Plasma and fractional excretion results for Pi and calcium**

<table>
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<tr>
<th></th>
<th>Plasma Pi, mM</th>
<th>Plasma Calcium, mM</th>
<th>FE Pi, %</th>
<th>Calcium, mM</th>
<th>FE, Calcium, %</th>
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<tr>
<td>Sham</td>
<td>2.2±0.09</td>
<td>2.5±0.17</td>
<td>11.2±1.6</td>
<td>0.8±0.1</td>
<td></td>
</tr>
<tr>
<td>TPTX</td>
<td>3.8±0.03*</td>
<td>1.3±0.18*</td>
<td>0.6±0.1*</td>
<td>1.5±0.1</td>
<td></td>
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<tr>
<td>Control P, diet</td>
<td>2.5±0.05</td>
<td>2.6±0.15</td>
<td>11.9±1.9</td>
<td>2.6±0.6</td>
<td></td>
</tr>
<tr>
<td>Low-Pi, diet</td>
<td>1.4±0.3*</td>
<td>3.7±0.2*</td>
<td>0.7±0.02*</td>
<td>3.6±0.4*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 3–5 rats. TPTX, thyroparathyroidectomized; FE, fractional excretion. *P < 0.05 compared with Sham vs. TPTX or control P, diet vs. low-Pi, diet.
Effect of PTH(1–34) on Type IIc Protein Levels in the TPTX Rat

Next, administration of PTH on expression of the type IIc Na-Pi cotransporter was examined via Western blot analysis in TPTX rats (Fig. 2). Two hours after administration of PTH, type IIa Na-Pi cotransporter protein levels were significantly decreased in the apical membrane. Type IIa Na-Pi cotransporter protein levels remained low until 8 h after administration of PTH and gradually increased from 12 h after administration of PTH (Fig. 2). By contrast, type IIc Na-Pi cotransporter protein levels remained unchanged at 2 h after administration of PTH (Fig. 2). Four hours after administration of PTH, type IIc transporter levels decreased to 85% of the initial values in the BBMV fractions. Thereafter, the amounts of the type IIc transporter protein gradually increased in the BBMV fractions and recovered to baseline levels at 24 h. Of note, the effect of PTH on type IIc transporter was slower than that on the type IIa transporter.

Effects of PTH on the Endocytosis of Type IIc Na-Pi Cotransporter in TPTX Rats

Cryosections of rat kidneys were immunohistochemically stained with a specific antibody directed against type IIa and type IIc Na-Pi cotransporters. In TPTX rat kidneys, most of the immunoreactive signals of the type IIc transporter colocalized with those of the type IIa transporter in the apical membrane region, and the type IIc transporter also localized to the base of the proximal tubule microvilli as described previously (Fig. 3A, a–c) (26). Immunoreactive signals for the type IIa transporter were markedly decreased in the apical membrane regions at 15 min and 2 h after PTH treatment and were detected as small dots in intracellular compartment, probably in lysosomal fraction (Fig. 3A, d, f, g, and i). In contrast, the intensity of the type IIc immunoreactive signals remained unchanged in the apical membrane region, although it seemed to increase in the base of the microvilli at 15 min and 2 h after PTH treatment (Fig. 3A, d, f, g, and i).

Fig. 1. Expression of the type IIc Na-Pi cotransporter in the kidney of thyroparathyroidectomized (TPTX) rats. A: immunoblotting of n = 3 rats. Brush-border membrane vesicles (BBMVs, 20 μg/lane) isolated from kidney of sham-operated or TPTX male rats were loaded into each lane. Actin was used as internal control. B: Northern blotting of n = 3 rats. Poly(A)⁺ RNA was extracted from kidneys of sham-operated and TPTX male rats. Each lane was loaded with 3 μg of RNA. GAPDH was used as internal control.

Effect of PTH(1–34) on Type IIC Protein Levels in the TPTX Rat

Next, administration of PTH on expression of the type IIc Na-Pi cotransporter was examined via Western blot analysis in TPTX rats (Fig. 2). Two hours after administration of PTH, type IIa Na-Pi cotransporter protein levels were significantly decreased in the apical membrane. Type IIa Na-Pi cotransporter protein levels remained low until 8 h after administration of PTH and gradually increased from 12 h after administration of PTH (Fig. 2). By contrast, type IIc Na-Pi cotransporter protein levels remained unchanged at 2 h after administration of PTH (Fig. 2). Four hours after administration of PTH, type IIc transporter levels decreased to 85% of the initial values in the BBMV fractions. Thereafter, the amounts of the type IIc transporter protein gradually increased in the BBMV fractions and recovered to baseline levels at 24 h. Of note, the effect of PTH on type IIc transporter was slower than that on the type IIa transporter.
Fig. 3. Immunofluorescence microscopy of cryostat sections from TPTX rat kidneys treated with PTH(1–34). A: untreated TPTX rat kidney sections (a–c) and sections 15 min (d–f) and 2 h (g–i) after administration of PTH. B: TPTX rat kidney sections 4 h (a–c) and 8 h (d–f) after administration of PTH. C: TPTX rat kidney sections 12 h (a–c) and 24 h (d–f) after administration of PTH. Sections were double labeled for type IIa Na-Pi transporter protein (a, d, and g; red) and for type IIc transporter protein (b, e, and h; green). Merged images are shown in c, f, and i. Bar = 10 μm. Arrowheads, immunoreactive signals of the type IIc Na-Pi cotransporter in the apical membrane. *Immunoreactive signals of the type IIc Na-Pi cotransporter in the base of the microvilli.
Four hours after administration of PTH to TPTX rats, the intensity of type IIa and type IIc transporter immunoreactive signals was weaker in the apical membrane than in untreated TPTX rats (Fig. 3B, a–c). Similar findings were observed in the proximal tubule cells 8 h after administration of PTH (Fig. 3B, d–f). Twelve hours after administration of PTH, the levels of type IIa Na-Pi cotransporter protein recovered to baseline levels (Fig. 3C, a and c). In contrast, the immunoreactive signals of the type IIc transporter were detected only in the base of the microvilli and not in the apical membrane regions (Fig. 3C, b and c). Twenty-four hours after administration of PTH, immunoreactive signals for the type IIc transporter returned to baseline levels and were localized exclusively in the apical membrane (Fig. 3C, e and f).

**PTH-Mediated Degradation of the Type IIc Transporter**

Next, we investigated whether the reduction of the type IIc transporter in the BBMV was due to translocation to the intracellular compartment or due to degradation in the intracellular fraction. As shown in Fig. 4, a high-Pi diet stimulated translocation of the type IIc transporter from the apical membrane to the intracellular compartments. However, administration of a high-Pi diet did not reduce the amounts of the type IIc protein in the cortical membrane fraction but did markedly suppress the levels of the transporter in the BBMV fraction (Fig. 4).

By contrast, 8 h after PTH treatment, type IIc transporter levels were significantly decreased in the cortical membrane fraction, whereas type IIa transporter levels gradually increased and returned to baseline levels (Fig. 4). These observations suggest that PTH induced degradation of the type IIc transporter in the cellular fraction.

**Microtubule-Mediated Regulation of the Type IIc Na-Pi Cotransporter**

Next, we studied whether internalization of the type IIc Na-Pi cotransporter from apical membrane to the intracellular compartments was dependent on the microtubule network (Fig. 5). Colchicine did not prevent PTH-induced downregulation of the type IIa Na-Pi cotransporter at 8 h after administration of PTH but did prevent the internalization of the type IIc Na-Pi cotransporter.

At 24 h after administration of PTH, the immunoreactive signals for the type IIa or type IIc Na-Pi cotransporter returned to baseline (Figs. 2 and 3). As shown in Fig. 5, pretreatment of TPTX rats with colchicine completely inhibited PTH-induced increases in type IIa and IIc Na-Pi cotransporters levels at 24 h after PTH injection. These data suggest that the microtubule network is an important pathway for membrane insertion of the type IIc and type IIa transporters in the proximal tubular cells.
Effect of a Lysosomal Inhibitor on the Degradation of the Type IIc Na-Pi Transporter

To investigate whether internalized type IIc Na-Pi cotransporters were routed to the lysosomes, TPTX rats were injected with the lysosomal inhibitor leupeptin before treatment with PTH (Fig. 6). Eight hours after PTH administration, BBMVs and lysosomal fractions were isolated from the kidney cortex tissue of TPTX rats. As shown in Fig. 6, in the BBMV fraction, type IIa and type IIc Na-Pi cotransporter protein levels were significantly lower in PTH-treated TPTX rats than in untreated TPTX rats. Treatment with leupeptin did not affect type IIa and type IIc transporter protein levels in the BBMVs. In the

Fig. 5. Involvement of microtubules in down- and upregulation of the type IIc Na-Pi transporter. TPTX rats were treated with colchicines (1.0 mg/kg body wt, dissolved in saline and administered intraperitoneally) 3 h before injection of PTH or 8 h after injection of PTH. A: Western blots of type IIa Na-Pi transporter (top), type IIc Na-Pi transporter (middle), and actin as internal control (bottom). B: relative intensities of type IIa (a) or type IIc (b) Na-Pi, transporter proteins. The immunoreactive band intensity for TPTX rat control (−PTH, −colchicine) was 1.0. Values are means ± SD; n = 3–5 rats. *P < 0.05 compared with TPTX rat control (−PTH, −colchicine).

Fig. 6. Effect of leupeptin on the degradation of type IIa and type IIc transporters in the lysosomal fraction. TPTX rats were treated with leupeptin (6 mg leupeptin/0.5 ml PBS) 1 h before injection of PTH(1–34). Eight hours after administration of PTH, we prepared the lysosomal fractions and BBMVs used for the experiment. Immunoblotting was performed by using BBMVs (20 μg/lane) (A) and lysosomes (100 μg/lane) (B); blots show type IIa Na-Pi, transporter (top), type IIc Na-Pi, transporter (middle), and actin (A) or lamp2 (B) (internal control; bottom). For A and B, n = 3–5 rats.
lyosomal fractions of kidneys isolated from TPTX rats treated with PTH, the amounts of the type IIa and type IIc transporter were not different from amounts in untreated TPTX rats. However, type IIa transporter protein levels were significantly higher in TPTX rats treated with PTH and leupeptin than in TPTX rats treated with PTH alone (Fig. 6). In contrast, type IIc Na-Pi cotransporter protein was not detected in the lysosomal fraction of PTH-treated TPTX rats (Fig. 6). Furthermore, type IIc transporters were absent in the lysosomal fractions isolated from leupeptin-treated animals at 12 and 22 h after administration of PTH (data not shown).

**Effect of PTH(1–34) and PTH(3–34) on Expression of Type IIc Na-Pi Cotransporter**

Next, we investigated whether PTH analogs, signaling either through PKA and PKC [PTH(1–34)] or only through PKC [PTH(3–34)], regulate the type IIc Na-Pi cotransporter in TPTX rat kidneys. As shown in Fig. 7A, administration of PTH(1–34) or PTH(3–34) (8 h) stimulated endocytosis in the TPTX rat.

Animals fed a low-Pi diet have a blunted phosphaturic response to PTH (20). Therefore, we investigated whether PTH-induced downregulation of the type IIc transporter by PTH was blunted in rats fed a low-Pi diet (Fig. 7B). Type IIa and type IIc Na-Pi protein levels were significantly higher in the kidney BBMVs of animal fed a 0.02% low-Pi diet than in those fed a 0.6% control P diet, (Fig. 7B) (24). Administration of PTH(1–34) and PTH(3–34) did not significantly downregulate type IIa Na-Pi cotransporter levels in the kidney BBMVs of rats fed a low-Pi diet (Fig. 7B). By contrast, administration of PTH(1–34) and PTH(3–34) markedly reduced type IIc Na-Pi transporter levels in the kidney BBMVs of rats fed a low-Pi diet (Fig. 7B). Thus, the response to PTH on type IIc transporter in P-restricted rats was distinct from that on the type IIa transporter.

**DISCUSSION**

The present study demonstrated that PTH regulates the type IIa and type IIc transporters. Our group (26) previously showed that acute feeding of a high-Pi diet decreased the amounts of the type IIc transporter protein in the apical membrane of the proximal tubular cell. Compared with the time course of the internalization of the type IIa transporter, internalization of the type IIc transporter was slightly delayed (26). Western blot and immunohistochemical analyses also demonstrated that the type IIc transporter is mainly localized to the subapical (or the base of the proximal tubule microvilli) regions after acute administration of a high-Pi diet. Indeed, the amounts of the type IIc transporter protein were not changed in cortical membrane fractions after acute feeding of the high-Pi diet, whereas type IIa Na-Pi, cotransporter levels were significantly decreased (26). Furthermore, type IIc transporter levels were slightly increased in the subapical regions with a simultaneous decrease in immunoreactive intensity at the apical membrane (26). Thus, with acute feeding of a high-Pi diet, type IIa Na-Pi cotransporter is translocated from the entire brush border to the intracellular compartments (26).

By contrast, in PTH-induced endocytosis, the amounts of type IIc transporter protein in the apical membrane and intracellular pool were significantly decreased. Immunoblotting and immunohistochemical analyses suggested that internalized type IIa Na-Pi cotransporters are directed to the lysosomes for degradation as described previously (5, 11, 19, 20, 31). By contrast, the type IIc Na-Pi cotransporter was internalized to the intracellular pool but not degraded in the lysosomes. Further studies are needed to clarify the routing and/or degradation pathway of the type IIc transporter in vitro.

The signaling pathways utilized by PTH to inhibit phosphate transport and apical membrane type IIa transporter expression include activation of PKA and PKC (1, 23, 32). The present study suggests that the PKA and PKC pathways are involved in PTH-induced endocytosis of the type IIc transporter. By contrast, administration of PTH(1–34) and PTH(3–34) to rats fed a low-Pi diet induced endocytosis of the type IIc transporter but not the type IIa transporter (32). In P-restricted animals, PTH-dependent signaling appears to be distinct when considering the type IIa transporter vs. the type IIc transporter.

Several studies in intact animals have indicated that the phosphaturic effect of PTH is blunted in animals adapted to a low-Pi diet (15). 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 affect PTH signaling (15). It is interesting to note that the lack of phosphaturia during the administration of PTH to Pi-depleted animals is observed despite a normal increment in urinary cAMP, its generation by renal cortical slices, and normal activation of PKA. These data suggest that the cAMP-independent pathway or the PTH-signaling pathway may be blunted in P-restricted animals. Inhibition of cellular metabolic steps beyond the PKA system is responsible for the lack of effect of PTH on Pi reabsorption. In these contexts, we suggest that systemic factors distal to PTH signaling may be different when considering type IIa vs. type IIc Na-Pi cotransporters.

PTH receptors are located on the apical and basolateral side of the renal proximal tubule cells (10, 18). The basolateral receptor signals through adenylyl cyclase and PKC, whereas the apical receptor is believed to signal exclusively through PKC (10, 18). Because PTH is in part cleared from the circulation by glomerular filtration, the functional importance of apical PTH receptors may be dependent on the capacity of PTH-degradation mechanisms at the luminal surface (32). Clearly, at early tubular sites (S1 segment), PTH fragments capable of activating PTH receptors may be present in the tubular fluid and may therefore contribute to transport regulation, such as Na-Pi cotransport (32). Indeed, the localization of the type IIa transporter in each nephron is S1, S2, and S3 segments but that of the type IIc is restricted in S1 segment. Apical PTH receptors may preferentially be coupled to a cAMP-independent signaling pathway and may regulate the type IIc transporter in the proximal tubule cells.

Finally, type IIc Na-Pi, cotransporter levels are increased in TPTX animals, and administration of PTH suppresses the levels of the transporter. The mechanisms of the internalization are not well known. The present study indicates that PTH is a major hormonal regulator of the type IIc Na-Pi, cotransporter in renal proximal tubules.

**GRANTS**

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Fig. 7. Effect of PTH(1–34) and PTH(3–34) on the expression of type IIc Na-Pi cotransporter. BBMVs (20 μg/lane) were isolated from TPTX rats (A) and rats fed a low-Pi diet (B) without or with PTH(1,34) or PTH(3–34) (8 h). Blots show type IIa Na-Pi transporter (top), type IIc Na-Pi transporter (middle), and actin as internal control (bottom). LPD, fed a low-Pi (0.02% Pi) diet; CPD, fed a control Pi (0.6% Pi) diet. The intensity of the immunoreactive band for TPTX rats or rats fed a low-Pi diet was 1.0. Values are means ± SD; n = 3–5 rats. *P < 0.05 compared with TPTX rats or rats fed a low-Pi diet. †P < 0.05 compared with rats fed a control Pi diet.
REFERENCES


