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Topical Review

Molecular Regulation of Renal Phosphate Transport

M. Levi[1], S.A. Kempson[2], M. Lötscher[3], J. Biber[3], H. Murer[3]

[1]Department of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, and Department of Veterans Affairs Medical Center, Dallas, Texas

[2]Department of Physiology and Biophysics, Indiana University School of Medicine, Indianapolis, Indiana

[3]Institutes of Physiology and Anatomy, University of Zürich-Irchel, Zürich, Switzerland

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Introduction

Renal proximal tubular reabsorption of phosphate (Pi) plays a critical role in the maintenance of phosphate homeostasis. Uptake of Pi occurs at the apical cell surface of the proximal tubule via a sodium gradient-dependent process (Na/Pi-cotransport). Transport across the basolateral membrane involves multiple pathways, including anion exchange mechanism and sodium-dependent Pi-cotransport, but is different from that in the apical membrane. The rate of proximal Pi reabsorption is adjusted acutely and chronically to the homeostatic needs of the body. In vivo and in vitro studies have provided evidence that physiological regulation of proximal tubular Pi reabsorption is most likely related to alterations in the transport capacity of apical membrane Na/Pi-cotransport (Murer et al., 1991; Dennis, 1992; Berndt & Knox, 1995; Biber & Murer, 1995).

Recently type I Na/Pi-cotransporters from rabbit (Werner et al., 1991), mouse (Chong et al., 1994), and human (Miyamoto et al., 1995) kidney cortex, and type II Na/Pi-cotransporters from rat and human (Magagnin et al., 1993), rabbit (Verri et al., 1995) and mouse (Collins & Ghishan, 1994; Hartmann et al., 1995) kidney cortex, flounder kidney (Werner, Murer & Kinne, 1994), OK cells (Sorribas et al., 1994), and bovine renal NBL-1 cells (Helps, Murer & McGivan, 1995) have been identified by expression cloning, and polyclonal antibodies have been raised against the C- and N-terminals of the predicted peptide sequences. Subsequent studies have provided evidence that the type II rather than the type I Na/Pi-cotransporter represents a target for the physiological and pathophysiological regulation of proximal tubular reabsorption of Pi. This review article will, therefore, describe the basic characterization and transport characteristics of the type II Na/Pi-cotransporter and its regulation by parathyroid hormone and dietary Pi, the two most important physiological and pathophysiological regulators of renal proximal tubular Pi reabsorption.

Structural Aspects

The type II Na/Pi-cotransporter cloned from a rat kidney cortex cDNA library (NaPi-2, Magagnin et al., 1993) is a protein of 637 amino acids in length. Sequence comparisons performed with current protein sequence databases revealed no significant homologies to other polypeptide sequences.

A model of the secondary structure of the rat type II cotransporter (NaPi-2) is depicted in Fig. 1. This working model was constructed based on a combination of hydropathy analysis (Kyte & Doolittle, 1982) and the TMAP program at EMBL (Persson & Argos, 1994) and predicts eight transmembrane regions, yet (needless to say) the exact number is not known at present. In addition, the proposed model predicts a hydrophilic N- and C-terminus in which both are around 100 amino acids long and in addition, a large hydrophilic and N-glycosylated (*see below*) loop. Indirect evidence using

Correspondence to: M. Levi

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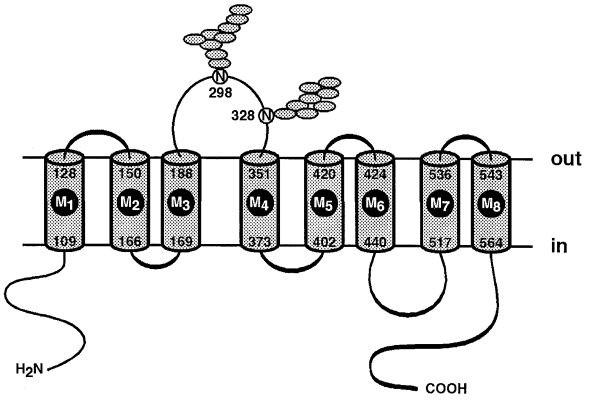

Fig. 1. Model of the secondary structure of the rat type II Na/Pi-cotransporter (NaPi-2).

antibodies directed against the N-terminus was obtained indicating that the N-terminus may be located at the cytoplasmic surface; no evidence for the localization of the C-terminus has yet been obtained.

The rat Na/Pi cotransporter contains four N-glycosylation sites (NXS/T). Recent studies using site-directed mutagenesis demonstrated that only the asparagine residues 298 and 328 are glycosylated (Hayes et al., 1994). In transport studies (tracer flux and electrophysiological methods) performed with oocytes of *Xenopus laevis* injected with mutated cRNA, evidence was obtained showing that the lack of N-glycosylation at positions 298 and 328 does not impair the kinetic parameters (apparent affinities for Pi and Na) nor the pH-dependence of the cotransporter (Hayes et al., 1994). However, immunofluorescence studies provided evidence that showed that in oocytes injected with mutated NaPi-2 cRNA the delivery of mutagenized, unglycosylated NaPi-2 cotransporter to the oolemma was impaired, leading to a reduced rate of Na/Pi-cotransport compared with oocytes injected with normal NaPi-2 cRNA. Therefore, glycosylation may be important for the targeting of the NaPi-2 cotransporters to the plasma membrane.

Furthermore, the type II Na/Pi-cotransporter contains several consensus sites for phosphorylation by protein kinase C or casein kinase II. To address the question whether predicted consensus sites for protein kinase C phosphorylation may be involved in protein kinase C-mediated inhibition of NaPi-2 related Na/Pi-cotransport in oocytes, the sites predicted to be located at the cytoplasmic surface (S5, S91, S462, T508 and S625) were mutated (Hayes et al., 1995). After injection of such mutated NaPi-2 cRNA into oocytes, no difference to normal cRNA was observed with respect to the inhibition provoked by the addition of phorbol ester. These data suggest either an involvement of unpredictable phosphorylation sites in the phorbol-ester-mediated inhibition or an indirect phosphorylation reaction. It remains to be elucidated whether or not a direct phosphorylation of the cotransporter may play a role such as in parathyroid-hormone-mediated inhibition of proximal tubular Pi-reabsorption.

Interestingly, two overlapping leucine zipper motifs are present within the predicted transmembrane spanning domain M4 (Fig. 1). As leucine zippers have been associated with the dimerization of a variety of proteins, it could be speculated that these leucine zipper motifs may participate in dimerization of these transporters. The only evidence that the functional unit of the type II Na/Pi-cotransporter could consist of a homomultimeric complex was obtained by target-size analysis of Na/Pi-cotransport in isolated proximal tubular brush-border membranes (Delisle et al., 1992). These experiments demonstrated a functional molecular mass of 230 kDa which would be consistent with two molecules of the type II cotransporter.

Transport Characteristics

The Na/Pi-cotransporter has been cloned based on its function, Na-dependent Pi-transport. Results obtained with tracer flux studies using oocytes injected with NaPi-2 cRNA revealed that the kinetic parameters of the cloned cotransporter are similar or identical to those obtained in transport experiments using isolated proximal tubular brush-border membranes (Murer et al., 1991; Magagnin et al., 1993). Apparent affinities for Pi (at 100 mM NaCl) and sodium are around 0.1–0.2 mM and 50 mM respectively.

Recent electrophysiological studies demonstrated that superfusion of oocytes injected with NaPi-2 cRNA with phosphate in a sodium-containing medium induces cell depolarization and provokes an inwardly directed current (*I*p) under voltage clamp conditions (Fig. 2) suggesting the movement of a positive charge during the transport process (Busch et al., 1994). At a constant concentration of Pi, *I*p changed as a function of the extracellular concentration of sodium, and the calculated Hill coefficient suggested a 3:1 coupling ratio of Na *vs.* Pi. Furthermore, it could be demonstrated that the Km for Pi is a function of the Na-concentration. As the external Na concentration was changed from 100 to 50 mM the Km for Pi ranged from 0.1 to 0.5 mM (Busch et al., 1994). This finding is in agreement with an ordered interaction of Na which is bound first and Pi which is bound second.

Similarly as described in transport studies using brush-border membranes, in oocytes injected with NaPi-2 cRNA Na/Pi-cotransport increased by increasing the external pH (Murer et al., 1991; Magagnin et al., 1993; Busch et al., 1994). Recent studies demonstrated that this pH dependence is not primarily a consequence of preferential transport of divalent Pi at high pH since the carrier likely accepts both mono- and divalent Pi.

More likely it also includes an altered affinity of the transporter for Na-ions at different pH values (Hartmann et al., 1995).

The electrogenic nature of Na/Pi-cotransport by the NaPi-2 cotransporter also revealed new insights with respect to its specificity. Thus far the only substrates to be transported are phosphate and Na-ions and in addition also arsenate but with a Km about tenfold higher than Pi (Hartmann et al., 1995). No evidence for transport of the oxyanion sulfate has been obtained thus far (Markovich et al., 1993). Furthermore, the competitive inhibitor phosphonoformic acid (Kempson, 1988) is not transported (Busch et al., 1996).

Regulation of Renal Na/Pi-cotransporter by Parathyroid Hormone

The action of parathyroid hormone (PTH) on the kidney has been studied extensively (Berndt & Knox, 1995; Murer & Biber, 1992). The PTH-induced increase in urinary excretion of Pi is due primarily to inhibition of Pi reabsorption in the convoluted and straight segments of the proximal tubule. The inhibition in the straight segment is more marked and may be more important for final regulation of Pi excretion by PTH, especially in the rabbit (Berndt & Knox, 1995). The Na/Pi cotransporter in the apical brush border membrane (BBM) appears to be a major target of the signaling pathways activated by PTH. A decrease in the V_{max} of Na/Pi cotransport across the BBM (Hammerman, Karl & Hruska, 1980) probably accounts for the decrease in tubular Pi reabsorption induced by PTH. Receptors for PTH are located on the basolateral membrane (Murer & Biber, 1992) and are G protein-linked (Juppner et al., 1991; Dunlay & Hruska, 1990). Binding of PTH activates both the adenylate cyclase/protein kinase A system and the phospholipase C/protein kinase C system. Direct activation of either kinase in renal cells is accompanied by inhibition of Na/Pi cotransport (Dunlay & Hruska, 1990). There is considerable evidence that both protein kinase A- and protein kinase C-dependent phosphorylation are important for PTH-induced inhibition of Na/Pi cotransport. Furthermore, these two pathways may act in a cooperative manner since the full inhibitory effect of PTH requires that both pathways are intact (Segal & Pollock, 1990). It is not known if different subpopulations of PTH receptors are coupled to the two intracellular pathways. Phosphorylation of renal BBM proteins in vitro results in inhibition of Na/Pi cotransport (Hammerman & Hruska, 1982) and PTH action on OK cell monolayers changed the abundance and phosphorylation state of specific proteins present in an apical membrane fraction (Reshkin et al., 1990). However, it has not been determined if the Na/Pi cotransporter itself is phosphorylated when the intracellular signaling pathways are activated by PTH. Detailed discussions of these issues have been published previously (Murer, 1992; Dunlay & Hruska, 1990) and they will not be discussed further in this review.

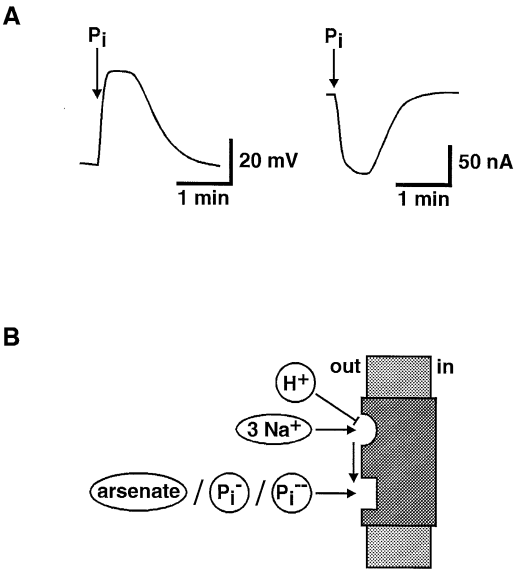

Fig. 2. Properties of the type II Na/Pi-cotransporter. (*A*) Superfusion of oocytes injected with NaPi-2 cRNA induces membrane depolarization and inward current. (*B*) A scheme summarizing the interactions of Na, Pi, arsenate and protons with the Na/Pi-cotransporter at the extracellular surface.

Recent collaborative efforts between several laboratories have focused on determining if the net result of PTH action, regardless of the signaling pathway, is a decrease in the abundance of the Na/Pi cotransporter in the BBM. The possibility that PTH might promote endocytic removal of NaPi-2 was first raised by the observation 20 years ago that colchicine, a microtubule poison, blocked the phosphaturic action of PTH in rats (Dousa, Duarte & Knox, 1976). More direct evidence that PTH action on Na/Pi cotransport may be mediated in part by endocytosis was obtained in OK cells. PTH action on Na/Pi cotransport was significantly impaired when endocytosis was inhibited (Kempson et al., 1990). Subsequent data from PTH-treated rats provided further support for this idea. The Na-dependent Pi-protectable binding of phosphonoformic acid, a specific inhibitor of Na/Pi cotransport, was decreased in BBM vesicles from PTH-treated rats compared to controls. This was due to a decrease in the binding capacity rather than the affinity (Hoppe et al., 1991) and is consistent with the V_{max} change in Na/Pi cotransport (Hammerman et al., 1980). Additional work in OK cells has shown that the onset of PTH action on Na/Pi cotransport does not require de novo protein synthesis but the recovery step, after PTH removal, is protein synthesis-dependent (Malmstrom &

Murer, 1987). This leads to the conclusion that the Na/Pi cotransporter, once internalized, is probably degraded and not immediately available for recycling by exocytosis to the plasma membrane. Recovery from PTH action will require synthesis of new transporters and trafficking to the plasma membrane. Incubation of OK cells with PTH, under conditions that inhibited plasma membrane Na/Pi cotransport (V_{max} effect), did not change total endocytic activity (Paraiso, McAteer & Kempson, 1995). It was proposed that PTH must stimulate selective removal of Na/Pi cotransporters without changing the overall rate of constitutive endocytosis. One possible role of the protein phosphorylation pathways may be to phosphorylate the Na/Pi cotransporter so that the attached Pi serves to label and/or inactivate it for shuttling into areas where endocytic vesicles are being formed. Several potential phosphorylation sites are present in the cytoplasmic domains of the deduced amino acid sequence of NaPi-2 (Biber & Murer, 1995).

A polyclonal antibody to the predicted peptide sequence of NaPi-2 proved to be invaluable for further studies. One approach was to examine isolated ''endosomal'' vesicle fractions for the presence of NaPi-2. Both Na/Pi cotransport function and NaPi-2 protein was detected in heavy endosomes from rat kidney cortex (Abraham, Burckhardt & Kempson 1992; Loghman-Adham et al., 1995) but subsequent work suggests that these fractions may not represent an intracellular compartment (Hammond et al., 1994). Subsequent work focused on a fraction of light endosomes that likely represents true intracellular endosomal vesicles (Hammond & Verroust, 1994). Since this fraction is much less abundant than the heavy endosomes, binding of NaPi-2 antibody was studied with flow cytometry. This technique detected specific binding of the antibody to nondenatured light endosomes (Kempson et al., 1995). There was no specific binding to nondenatured BBM, presumably because antigenic sites are on the inside surface of BBM vesicles and are only exposed by denaturation. Thus the binding to light endosomes indicates the presence of a true intracellular pool of NaPi-2 that cannot be attributed to BBM contamination of the endosomal preparation.

The NaPi-2 antibody has been used also in studies designed to monitor shifts in the distribution of NaPi-2 protein between the apical BBM and the cytoplasm of cells of the proximal tubule. Western blots of isolated denatured BBM have shown unequivocally that the NaPi-2 abundance in BBM of parathyroidectomized (PTX) rats was decreased by a 2-hr treatment with PTH at a dose that inhibited Na/Pi cotransport (Kempson et al., 1995). The loss of NaPi-2 from the BBM was observed in proximal tubules throughout the cortex, both superficial and juxtamedullary, but there was no change in immunofluorescence specific for gamma-glutamyltranspeptidase2, a BBM marker enzyme. Only a limited amount of intracellular NaPi-2 was detected by the immunohistochemical technique, lending support to the notion that internalized NaPi-2 may be rapidly degraded. Preliminary data obtained in PTX rats after PTH treatment for shorter time intervals (35–60 min) have revealed high abundance of NaPi-2 protein in an intracellular subapical location in parallel with decreased NaPi-2 protein in the BBM (Loetscher et al., 1995; Loetscher et al., 1996). In contrast, the Na/sulfate cotransporter was not internalized, which illustrates that the change in distribution of NaPi-2 proteins was selective. In other words, the mechanism is unlikely to involve wholescale nonspecific internalization of fragments of the BBM. This is consistent with the finding that PTH did not change the constitutive endocytic rate in OK cells (Paraiso et al., 1995). Taken together, these findings strongly suggest that one mechanism by which PTH inhibits renal Na/Pi cotransport at the cellular level involves selective endocytic removal of Na/Pi cotransporters from the BBM of the renal proximal tubule. Initially, the endocytosed cotransporters are redistributed to the cytoplasm but they appear to be targeted for rapid lysosomal degradation rather than recycling to the BBM if the PTH treatment is prolonged.

Regulation of Renal Na/Pi-cotransporter by Dietary Pi

A major regulator of Na/Pi cotransport is changes in dietary Pi content. Dietary Pi restriction is associated with an adaptive increase of the overall proximal tubular capacity to reabsorb Pi (Stoll, Kinne & Murer, 1979; Kempson & Dousa, 1979; Barrett, Gertner & Rasmussen, 1980; Cheng, Liang & Sacktor, 1983; Levi, Baird & Wilson, 1990). Part of this phenomenon is independent of extrarenal factors such as parathyroid hormone, 1α, 25-dihydroxyvitamin D_3, plasma calcium, or growth hormone (Murer et al., 1991). The adaptive phenomenon also occurs in renal tubular cells grown in culture upon lowering of the culture media Pi content (Caverzasio et al., 1985; Biber, Forgo & Murer, 1988). In response to the in vivo or cell culture Pi restriction, the increase in apical brush border membrane (BBM) Na/Pi cotransport is mediated by an increase in the V_{max}, while there are no changes in the affinities of the transporter for Pi or Na ions.

Previous in vivo and cell culture studies indicated that adaptation to chronic Pi restriction can be abolished by inhibitors of transcription and protein synthesis (Caverzasio et al., 1985; Biber et al., 1988; Shah et al., 1979). However, it could not be determined whether the proteins involved in the adaptive response represent newly synthesized Na/Pi cotransporters or if other regulatory proteins are involved.

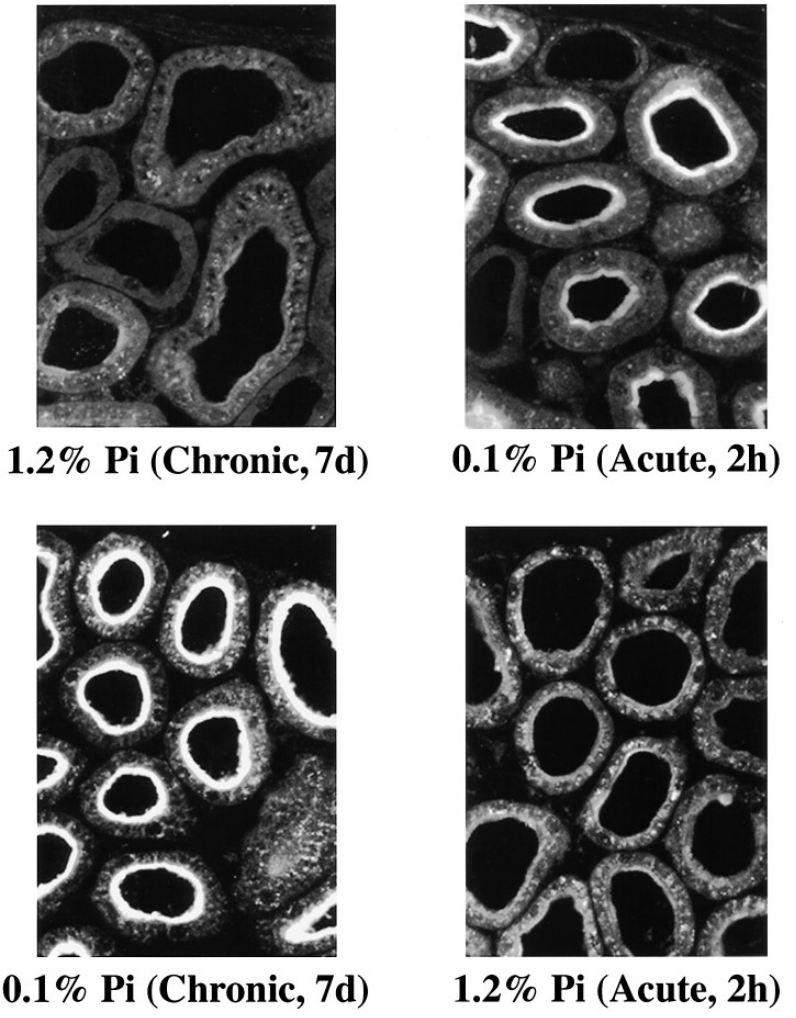

Fig. 3. Immunostaining of NaPi-2, with a rabbit antiserum and or FITC-labeled goat anti-rabbit antibody in superficial cortex of rat kidney:cryostat sections.

The recent cloning and the generation of highly specific antibodies to the Na/Pi-cotransporter protein made it possible to determine whether modulation of Na/Pi-cotransporter specific mRNA and/or protein abundance, *vs.* other regulatory proteins, play a role in the renal tubular adaptation to alterations in dietary Pi content.

Cellular Mechanisms of Chronic Adaptation to Low-phosphate Diet

In rats chronically (7 days) fed a low Pi diet the adaptive increase in BBM Na/Pi cotransport activity was associated with a similar increase BBM NaPi-2 protein abundance, as determined by Western blotting and also by immunohistochemistry (Fig. 3). The increases in BBM Na/Pi cotransport activity and BBM NaPi-2 protein abundance were paralleled by a similar parallel increase in NaPi-2 mRNA abundance (Levi et al., 1994).

Similarly, in rabbits chronically fed a low Pi diet, the adaptive increase in renal cortical brush border membrane Na/Pi-cotransport was paralleled by increases in the abundance of the type II Na/Pi-cotransporter (NaPi-6) mRNA and protein (Verri et al., 1995). Interestingly and importantly there were no changes in the type I Na/Pi-cotransporter (NaPi-1) mRNA or protein abundance.

The chronic adaptation to a low-Pi diet in the rat and in the rabbit is therefore characterized by parallel increases in BBM Na/Pi cotransport activity, BBM type II Na/Pi-cotransporter protein abundance, and renal cortical type II Na/Pi-cotransporter mRNA level. These results suggest that transcriptional and translational mechanisms play a role in the chronic adaptation to low-Pi diet. These findings are in agreement with previous studies which demonstrated that inhibition of transcription (actinomycin D) or de novo protein synthesis (cycloheximide) prevented the chronic adaptive increase in Na-Pi cotransport activity in response to a low-Pi diet (Caverzasio et al., 1985; Biber & Murer, 1985; Shah et al., 1979).

Studies in the opossum kidney (OK) cell line also indicated that in cells chronically (4 hr or greater) exposed to a low Pi medium (0.1 mM Pi), the adaptive

increase in Na/Pi cotransport was associated with an increase in Na/Pi cotransporter specific mRNA (NaPi-4) abundance. In OK cells the increase in Na/Pi cotransporter mRNA abundance was, however, mediated by an increase in mRNA stability rather than increased Na/Pi cotransporter gene transcriptional rate (Markovich et al., 1995), and actinomycin D did not prevent the adaptive increase in Na/Pi cotransport activity.

Cellular Mechanisms of Acute Adaptation to Low-phosphate Diet

In rats chronically (7 days) adapted on a high Pi diet, the acute (2 hr) administration of a low Pi diet resulted in rapid upregulation of BBM Na/Pi cotransport activity and BBM NaPi-2 protein abundance (Levi et al., 1994). Immunohistochemistry similarly demonstrated rapid upregulation of NaPi-2 protein expression in the apical membrane of the proximal tubule (Fig. 3). The acute upregulation of BBM Na/Pi cotransport activity has been shown to be independent of endogenous parathyroid hormone activity (Levine et al., 1986; Loetscher et al., 1996). In contrast to their effect to prevent the chronic adaptive response, inhibition of transcription with actinomycin D or de novo protein synthesis with cycloheximide did not prevent the acute adaptive response to a low-Pi diet (Loetscher et al., 1996). Furthermore, Northern blot analysis also demonstrated that there was no change in renal cortical NaPi-2 mRNA abundance (Levi et al., 1994).

Similar results have been obtained with OK cells where the acute (2 hr) adaptive increase in Na/Pi cotransport in response to a low Pi medium occurred in spite of no difference in Na/Pi cotransporter specific mRNA (NaPi-4) abundance (Markovich et al., 1995).

The acute adaptive increases in Na/Pi cotransport activity and Na/Pi protein abundance, independent of a change in Na/Pi mRNA abundance, and independent of actinomycin D or cycloheximide, therefore suggest that the acute upregulation of Na/Pi cotransport and Na/Pi protein are most likely mediated by post-translational mechanisms (i.e., protein trafficking).

Microtubules have been shown to play a critical role in the organization and dynamics of membrane organelles, and in the trafficking of proteins and lipids from and to the plasma membrane (Brown, Sabolic & Gluck, 1991; Gilbert et al., 1991; Lafont, Burkhardt & Simons, 1994; Mays, Beck & Nelson, 1994; Mills, Schwiebert & Stanton, 1994; Cole & Lippincott-Schwartz, 1995). Further studies were therefore conducted to assess the role of microtubules in the rapid upregulation of proximal tubular apical membrane NaPi-2 protein abundance in response to low-Pi diet. In rats pretreated with the microtubule-disrupting agent colchicine, it was shown that colchicine completely abolished the acute upregulation of BBM Na/Pi cotransport activity and NaPi-2 protein abundance (Loetscher et al., 1996). Immunohistochemistry showed that colchicine treatment resulted in total disruption of the microtubular network in proximal tubular cells. Furthermore, translocation of NaPi-2 protein from intracellular compartments to the apical membrane, which occurs during acute adaptive upregulation, was blocked by colchicine. These results are in agreement with a recent study which also showed that the adaptive response of Na/Pi cotransport in OK cells to a low-Pi medium was significantly impaired in the presence of the microtubule disrupting agents nocodazole and colchicine (Hansch et al., 1993).

The evidence so far indicates that the acute upregulation of Na/Pi cotransport activity and NaPi-2 protein in the apical membranes of proximal tubules following acute dietary Pi restriction is mediated by a rapid, microtubule-dependent translocation of NaPi-2 protein from intracellular compartments to the apical membrane.

Cellular Mechanisms of Acute Adaptation to High-phosphate Diet

In rats chronically (7 days) adapted on a low-Pi diet, the acute (2 hr) administration of a high-Pi diet resulted in rapid downregulation of BBM Na/Pi cotransport activity and BBM NaPi-2 protein abundance (Levi et al., 1994). Immunohistochemistry similarly demonstrated rapid downregulation of NaPi-2 protein expression in the apical membrane of the proximal tubule (Fig. 3). The acute downregulation of BBM Na/Pi cotransport activity has been shown to be independent of endogenous parathyroid hormone activity (Loetscher et al., 1996). In contrast to its effect to prevent the acute upregulation of the Na/Pi cotransporter in response to acute administration of a low-Pi diet, pretreatment with colchicine did not prevent the acute downregulation of the Na/Pi cotransporter in response to acute administration of a high-Pi diet (Loetscher et al., 1996).

In summary, the evidence indicates that the rapid downregulation of BBM Na/Pi cotransport activity in response to acute administration of a high-Pi diet is most likely mediated by rapid endocytosis of Na/Pi cotransporters from the apical membrane of the proximal tubule by cellular mechanisms that are independent of endogenous parathyroid hormone activity and the microtubular network.

Summary

This review has focused on the regulation of renal inorganic phosphate (Pi) transport in response to changes in dietary Pi and parathyroid hormone (PTH). Both dietary Pi and PTH produce rapid and marked changes in proxi-

mal tubular Pi transport which is paralleled by marked alterations in the abundance of NaPi-2 protein in the apical brush border membrane (BBM) of the proximal tubule. It appears that rapid insertion and retrieval of Na/Pi cotransporters into and out of the BBM plays a major role in the acute regulation of renal Pi transport.

The acute regulation of the renal Na/Pi cotransporter thus far seems remarkably similar to the regulation of the glucose transporter (GLUT 4) by insulin (Verhey, Yeh & Birnbau, 1995; Yang et al., 1996; Zorzano et al., 1996) and the water channels (Aquaporin 1 and Aquaporin 2) by vasopressin (Elkjaer et al., 1995; Nielsen et al., 1995; Katsura et al., 1995).

In the case of the Na/Pi cotransporter, future work will be directed in defining the cellular trafficking mechanisms, the regulation of endocytosis and exocytosis by physiological stimuli, and their relationship to intracellular signaling pathways.

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