The renal Na^+/phosphate cotransporter NaPi-IIa is internalized via the receptor-mediated endocytic route in response to parathyroid hormone

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The major renal Na^+/phosphate cotransporter, NaPi-IIa, is regulated by a number of factors including parathyroid hormone (PTH), dopamine, and dietary phosphate intake. PTH induces the acute internalization of NaPi-IIa from the brush border membrane (BBM) and its routing to and subsequent degradation in lysosomes. Previous work indicated that megalin, part of the apical receptor-mediated endocytic apparatus, may play a role in the PTH-induced removal of NaPi-IIa. Here we examined in rats the time-dependent internalization route of NaPi-IIa after acute PTH application using immunohistochemistry and markers of several endocytic compartments. NaPi-IIa removal from the BBM was detectable as early as 5 min after PTH injection. After 10–15 min, NaPi-IIa was localized in subapical compartments positive for clathrin. Shortly thereafter, NaPi-IIa appeared in endosomes stained for EEA1 (early endosomal antigen 1). After 45–60 min, NaPi-IIa was found in late endosomes/lysosomes marked with lgp120. In contrast, no change in the subcellular localization of megalin and the Na^+/H^+ exchanger NHE3 was detected up to 60 min after PTH injection. To further characterize the internalization route, insulin, as a marker for receptor-mediated endocytosis, and horseradish peroxidase (HRP) and fluorescein isothiocyanate (FITC)-dextran (10 kDa), as markers for fluid-phase mediated endocytosis, were used. NaPi-IIa colocalized with insulin 5–30 min after PTH injection but did not overlap with HRP or FITC-dextran. These results demonstrate a distinct internalization route of NaPi-IIa in response to acute PTH application that may involve the receptor-mediated endocytic pathway including clathrin-coated vesicles and EEA1-positive early endosomes, and routes NaPi-IIa to lysosomes for degradation.

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Inorganic phosphate (P_i) homeostasis is effectively balanced between intestinal absorption via the Na^+/phosphate cotransporter NaPi-IIb and the renal excretion regulated at the level of the apical Na^+/phosphate cotransporters NaPi-IIa and NaPi-IIc.[1] Transport of P_i across the proximal tubular apical membrane is mainly performed by NaPi-IIa.[2,3] A variety of stimuli regulate the abundance of NaPi-IIa and activity of Na/Pi cotransport including dietary phosphate intake, acid–base homeostasis, and a number of hormones such as parathyroid hormone (PTH), dopamine, and steroid hormones.[1,3,4] Rapid up- or downregulation of the transporter subsequent to acute changes in phosphate intake or PTH levels is based rather on post-transcriptional events, including intracellular trafficking, than on transcriptionally regulated changes in the mRNA levels of NaPi-IIa.[1,3,5] A decrease of proximal tubular reabsorption of phosphate (P_i), which can be provoked by PTH, has been shown to correlate with a decrease in the number of type IIa Na/Pi cotransporters residing in the brush border membrane (BBM).[2] Internalization of NaPi-IIa *in vivo* most likely occurs at the invaginated membrane regions at the base of the BBM as demonstrated in PTH-treated rats.[6] Internalized NaPi-IIa proteins are subsequently directed to the lysosomes for degradation.[6–8] Similar observations were made in studies with OK cells, a cell line with some proximal tubular characteristics.[7,9]

In a first step of its rapid downregulation by PTH, NaPi-IIa accumulates transiently in the apical endocytic compartment of the proximal tubule before being degraded in a second step in the lysosomes within the course of one to several hours.[7,8,10] Similarities of this process with receptor-mediated endocytosis of ligands have been highlighted.[10–13] Previously, we have investigated NaPi-IIa internalization using immunohistochemical methods and found evidence for a distinct internalization route.[6] Moreover, we have recently demonstrated in two mouse models that loss of the endocytic receptor megalin itself or its chaperone protein RAP leads to an impairment and delay of the PTH-induced

NaPi-IIa internalization.[14,15] Taken together, these data point to a possible involvement of the receptor-mediated endocytic pathway in the internalization of NaPi-IIa in response to PTH.

However, little is known about the route of internalization and the underlying mechanisms of the rapid PTH-dependent removal of NaPi-IIa molecules and their subsequent intracellular routing to lysosomes. Therefore, the present study aimed to determine the route of retraction of NaPi-IIa *in vivo* after PTH treatment, employing confocal microscopy and dual labeling with markers of specific compartments such as clathrin-coated pits and endosomes together with markers for receptor-mediated and fluid-phase endocytosis. We used insulin as a representative marker for receptor-mediated endocytosis. Upon insulin binding, activated insulin receptors complexed with insulin are released from microvilli, segregate in clathrin-coated pits, and enter the cells. In endosomes, insulin is uncoupled from its receptor and the receptor recycles back to the cell surface, whereas insulin molecules are targeted to late endosomes and lysosomes where they are degraded.[16] Horseradish peroxidase (HRP) was used as a marker of fluid-phase endocytosis.[13]

The results indicate that the retraced NaPi-IIa molecules are following the pathway of receptor-mediated endocytosis being routed via clathrin-coated vesicles to early endosomes and lysosomes.

RESULTS

A low-P_i diet increases the expression of NaPi-IIa cotransporters predominantly in the brush border of superficial nephrons, the sites that under normal dietary conditions display a more moderate expression of the cotransporter.[14,17] Therefore, all animal experiments were performed on rats fed a low-P_i diet for 3 days, in order to obtain maximal NaPi-IIa expression in all nephron generations as the starting point.

Effect of PTH treatment on NaPi-IIa distribution

The method of injecting PTH into the vena cava in anesthetized rats allowed us to perform the hormone treatment for periods as short as 5 min and, without noticeable side effects from anesthesia, as long as 60 min. The initial immunohistochemical evaluation of the PTH effect at various time points (i.e. 5, 15, 30, and 60 min after injection) showed that the onset of NaPi-IIa internalization from the BBM was already detectable after 5 min and was very pronounced after 15 min. With prolonged treatment (30 and 60 min), the expression of NaPi-IIa cotransporters decreased in superficial as well as in juxtamedullary nephrons (Figure 1). NaPi-IIa in control animals showed almost exclusively BBM localization (the BBM was stained against actin in red, NaPi-IIa in green, overlay of NaPi-IIa and actin appeared in bright yellow). Higher magnifications of cross-sections of proximal tubules in PTH-injected rats illustrate that the intensity of the NaPi-IIa cotransporter staining (green label) was decreased in the BBM with prolonged PTH treatment (Figure 2). In PTH-injected animals, NaPi-IIa-

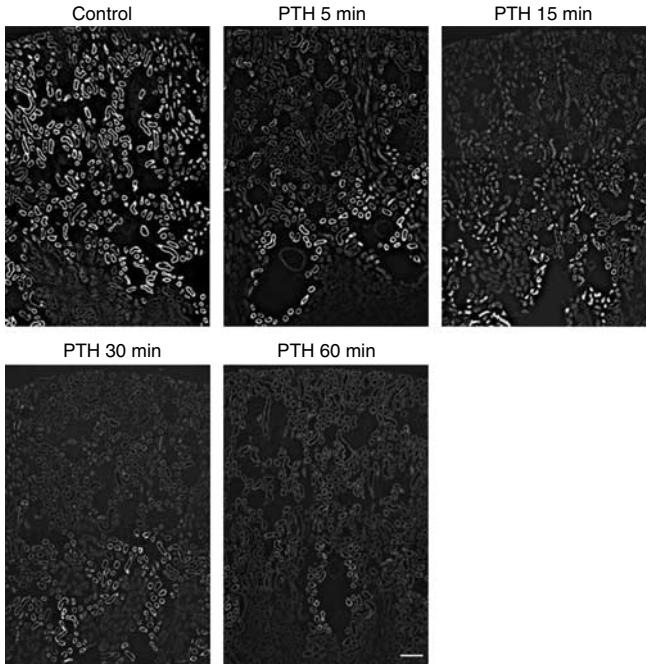

Figure 1 | Type IIa NaPi cotransporter (NaPi-IIa) protein in the kidney cortex of control rats and of rats 5, 15, 30, or 60 min after injection with 1-34 PTH; cryostat sections, immunofluorescence. NaPi-IIa labeling was strongly reduced in kidneys immediately 5 min after PTH application. After prolonged periods after PTH injection (15 and 30 min), NaPi-IIa staining remained strongest in proximal convoluted tubules of juxtamedullary nephrons, which almost completely disappeared 60 min after PTH administration. Bar = ~200 μm.

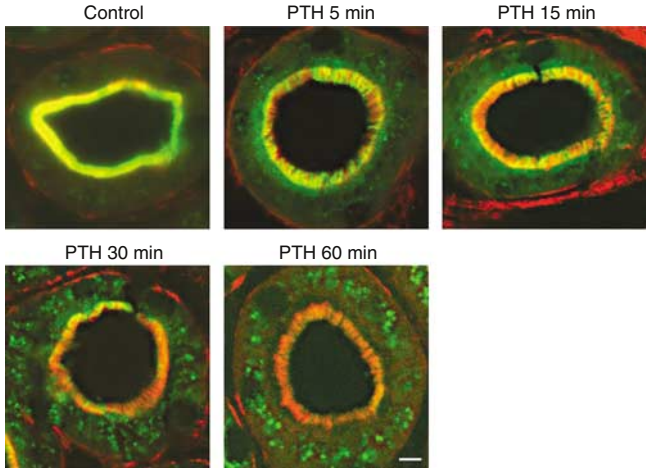

Figure 2 | S1 segments of superficial nephrons stained with an antibody against NaPi-IIa (green label) and with rhodamine-phalloidin against β-actin filaments (red); cryostat sections, immunofluorescence. Under control conditions, a high degree of overlap (yellow appearance) between NaPi-IIa and actin is seen, demonstrating the predominant localization of NaPi-IIa in the BBM. At 5–15 min after 1-34 PTH injection, the intensity of NaPi-IIa-related staining was decreased in the BBM (orange appearance) and a large green fluorescent rim appears below the BBM. Thirty minutes after PTH injection, the intensity of the intracellular NaPi-IIa staining (green) below the subapical region is increased. After 60 min, intracellular NaPi-IIa staining has disappeared and BBM was colored in red. Bar = ~10 μm.