Differential traffic of proximal tubule Na\(^+\) transporters during hypertension or PTH: NHE3 to base of microvilli vs. NaPi2 to endosomes

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Yang, Li E., Arvid B. Maunsbach, Patrick K. K. Leong, and Alicia A. McDonough. Differential traffic of proximal tubule Na\(^+\) transporters during hypertension or PTH: NHE3 to base of microvilli vs. NaPi2 to endosomes. Am J Physiol Renal Physiol 287: F896–F906, 2004. First published July 20, 2004; doi:10.1152/ajprenal.00160.2004.—We previously reported that Na\(^+\)/H\(^+\) exchanger type 3 (NHE3) and NaPi2 are acutely retracted from the proximal tubule (PT) microvilli (MV) during acute hypertension [high blood pressure (BP)] or parathyroid hormone (PTH) treatment. By subcellular membrane fractionation, NHE3 and NaPi2 show indistinguishable redistribution patterns out of light-density into heavy-density membranes in response to either treatment consistent with a retraction from the apical MV to the intermicrovillar cleft region. This study aimed to examine the redistribution of PT NHE3 vs. NaPi2 by confocal and electron microscopy during high BP and during PTH treatment to determine whether their respective destinations overlap or are distinct. High-BP protocol: systolic BP was increased 50–60 mmHg by increasing peripheral resistance for 20 min; PTH protocol: rats were infused with 6.6 μg/kg iv of PTH followed by 0.1 μg·kg\(^{-1}\)·min\(^{-1}\) infusion for 1 h. For light microscopy, rats were infused with 25 mg of horseradish peroxidase (HRP) 10 min before kidney fixation. Kidney slices were dual labeled with either NHE3 or NaPi2 and either clathrin-coated vesicle adaptor protein AP2 or endosome marker HRP. Results demonstrate retraction of NHE3 from the MV to the base of MV during either high-BP or PTH treatment; NHE3 staining did not retract below the AP2-stained domain or to HRP-labeled endosomes in either model. In comparison, NaPi2 was retracted from MV to below the AP2-stained region in both models, a little colocalizing with HRP staining. At the electron microscopic level with immunogold labeling, during high BP NHE3 was concentrated in a distinct domain in the base of the MV while NaPi2 moved to endosomes. The results demonstrate that there are divergent routes of retraction of PT NHE3 and NaPi2 from the MV during acute hypertension or PTH treatment: NHE3 is not internalized but remains at the base of the MV while NaPi2 is internalized.

A rapid increase in blood pressure (BP) acutely decreases proximal tubule (PT) sodium reabsorption that both increases NaCl at the macula densa, a tubuloglomerular feedback (TGF) signal to normalize renal blood flow (RBF) and glomerular filtration rate (GFR), and causes a pressure-natriuresis that reduces extracellular volume, which counteracts the hypertension (8, 10, 11, 42). Na\(^+\)/H\(^+\) exchange is the major route for apical sodium entry across the PT, and the Na\(^+\)/H\(^+\) exchanger type 3 (NHE3) isoform is responsible for virtually all the Na\(^+\)/H\(^+\) exchange activity in this region (2, 7). The sodium-phosphate cotransporter NaPi2 is the key PT phosphate transporter, reabsorbing 80% of the filtered phosphate (3, 27).

This laboratory previously investigated the molecular mechanisms responsible for the decrease in PT sodium reabsorption during an experimental acute increase in BP and discovered there is a parallel retraction of NHE3 and NaPi2 from the apical microvilli (MV) to membranes of higher density enriched in intermicrovillar cleft (IMC) and endosomal markers, demonstrated by subcellular membrane fractionation (35, 41). Confocal microscopic evidence also supported that NHE3 is retracted from the apical MV in acute hypertension (20, 35). The destination of NHE3 and NaPi2 after retraction from the apical MV was, however, unclear. Like acute hypertension, in vivo parathyroid hormone (PTH) treatment inhibits both the sodium-phosphate-coupled transport and sodium/hydrogen exchange in the PT and also causes diuresis and natriuresis (1, 29). This laboratory observed a similar retraction of NHE3 and NaPi2 from the apical MV-enriched membrane fractions by density gradient centrifugation of renal cortex after in vivo treatment with PTH (43), which could contribute to the decrease in PT sodium and phosphate reabsorption. Immunocytochemical studies have already demonstrated that PTH causes NaPi2 internalization and degradation (22, 32). In contrast, the destination of NHE3 in response to PTH treatment in vivo has not been clarified.

There is evidence for regulated endocytosis of NHE3 in cultured cell lines. In Chinese hamster ovary cells, transfected NHE3 is localized to endosomal compartments in addition to the plasma membranes and the trafficking of NHE3 depends on dynamin and cytoskeleton (12, 15, 30). In opossum kidney (OK) cells, PTH or dopamine acutely stimulates the endocytosis of NHE3 via clathrin-coated vesicles (13, 17). Also, in OK cells, NHE3-mediated endothelial acidification is implicated in the endocytosis of albumin (16). However, PT brush border is very complex morphologically including tall and densely packed MV and well-defined IMC and coated pit regions, whereas in contrast, PT-derived OK cells have very sparse MV and no analogous IMC. Considering the pronounced difference in their respective morphologies, it is not obvious that results from studies conducted in cultured cells are applicable to the PT in situ (26).

The current study aimed to determine the routes of retraction of NHE3 vs. NaPi2 in vivo during these two distinct natriuretic
stimuli, acute elevation of BP or PTH treatment, employing confocal and immunoelectron microscopy (immuno-EM) and dual labeling with markers of coated pits and endosomes. The results indicate that the retracted NHE3 and NaPi2 are routed to different membrane regions: NHE3 is redistributed to the base of the MV (not to endosomes), whereas NaPi2 is internalized to endosomes and perhaps lysosomes.

**EXPERIMENTAL PROCEDURES**

**Animal preparation.** All animals protocols were approved by The University of Southern California Institutional Animal Care and Use Committee. Experiments were performed in male Sprague-Dawley rats (290–320 g body wt) that had free access to food and water before the experiment. Rats were anesthetized intramuscularly with ketamine (Fort Dodge Laboratories) and xylazine (Miles; 1:1 vol/vol) and then placed on a thermally controlled warming table to maintain body temperature at 37°C. A polyethylene catheter was placed into the carotid artery to monitor BP. The jugular vein was cannulated using 4.0% BSA in 0.9% NaCl at 50 μL/min throughout the entire experimental period to maintain euvoeulation.

**Acute hypertension (high BP) protocol.** Mean arterial pressure was increased 50–60 mmHg by constricting the superior mesenteric artery, celiac artery, and abdominal aorta below the renal artery by tying silk ligatures around the vessels (35, 41). Endosomes were functionally labeled by horseradish peroxidase (HRP) uptake. Specifically, after 10-min acute hypertension, 25 mg of HRP in 1 ml of PBS were injected into the jugular vein, and then 10 min later kidneys were fixed in situ for 20 min while BP was recorded; thus the experimental time point was between 20 and 40 min.

**PTH protocol.** The synthetic bovine PTH fragment bPTH-(1–34) (Peninsula Lab, Belmont, CA) was dissolved in 4.0% BSA in 0.9% NaCl. PTH was infused intravenously in a bolus dose of 6.6 μg/kg followed by an infusion at 0.1 μg·kg⁻¹·min⁻¹ for 1 h. After 50 min, HRP was injected as described in the high-BP protocol, and then 10 min after HRP injection kidneys were fixed in situ for 20 min (during which PTH was continuously infused at 0.1 μg·kg⁻¹·min⁻¹); thus the PTH infusion time point is between 60 and 80 min. In one series designed to examine early effects of PTH, kidneys were fixed starting 10 min after the PTH bolus and continuous infusion (for 20 min), without HRP injection; thus the PTH infusion time point is between 10 and 30 min.

**Homogenization and subcellular fractionation.** The procedure for subcellular fractionation of renal cortex membranes has been described previously (41, 42). In brief, kidneys from control and treated animals were cooled in situ by placing the isolated kidney in a small Plexiglas cup and bathing it in PLP fixative (2% paraformaldehyde, 75 mM lysine, and 10 mM Na-periodate, pH 7.4) for 20 min. The kidneys were then removed and cut in half on a midsagittal plane and postfixed in PLP for another 3–4 h. The fixed tissue was rinsed twice with PBS, cryoprotected by incubation overnight in 30% sucrose in PBS, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA), and frozen in liquid nitrogen. Cryosections (5 μM) were cut using a Microm Heidelberg (Mikron Instruments, San Marcos, CA) cryomicrotome and transferred to Fisher Superfrost Plus-charged glass slides and air dried. For immunofluorescence labeling, the sections were rehydrated in PBS 10 min, followed by 10-min washing with 50 mM NH₄Cl in PBS, then with 1% SDS in PBS for 4 min for antigen retrieval (9). SDS was removed by two 5-min washes in PBS, and the sections were blocked with 1% BSA in PBS to reduce background. Dual labeling was performed by incubating with polyclonal antiserum NHE3-C00 at 1:100 dilution or anti-NaPi2 at 1:250 dilution and monoclonal anti-goat-anti-mouse (Molecular Probes, Eugene, OR) secondary antibodies diluted 1:100 in 1% BSA/PBS for 1.5 h at room temperature. After being washed three times for 5 min in PBS, the sections were incubated with a mixture of FITC-conjugated goat-anti-rabbit (Cappel Research Products, Durham, NC) and Alexa 568-conjugated goat-anti-mouse (Molecular Probes, Eugene, OR) secondary antibodies diluted 1:100 in 1% BSA/PBS for 1 h, washed three times with PBS, mounted in Prolong Antifade (Molecular Probes), and dried overnight at room temperature. Slides were viewed with a Nikon PCM Quantitative Measuring High-Performance Confocal System equipped with filters for both FITC and TRITC fluorescence attached to a Nikon TE300 Quantum upright microscope. Images were acquired with Simple PCI C-Imaging Hardware and Quantitative Measuring Software and processed with Adobe PhotoDeluxe (Adobe Systems, Mountain View, CA).

**Immuno-EM.** Acute hypertension was induced for 20 min in four rats as described above. Four other rats served as controls and were treated the same way but without induction of high BP. None of the animals received HRP. In each group, two rats were perfusion-fixed with 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, and two rats were fixed by superfusion of the kidney surface with the fixative. BP was continuously monitored before and during fixation. Many surface-fixed tubules showed partial absence of lumen and some bulging of the apical cytoplasm into the tubule lumen. The immunolabeling pattern, described below, was the same with the two fixation methods. Tissue blocks were trimmed from the cortex and postfixed in the same fixative for 2 h, rinsed in buffer, infiltrated with 2.3 M sucrose, mounted on holders, and frozen in liquid nitrogen. Immunoelectron microscopy was performed either on thin (70 nm) cryosections prepared from the frozen tissue on a Reichert Ultracut S cryoultramicrotome (Leica) or on tissue that was cryosubstituted in a Reichert AFS freeze-substitution apparatus (Leica) and embedded in Lowicryl HM20 as previously described (25). Briefly, the samples were sequentially equilibrated over 3 days in methanol containing 0.5% uranyl acetate at temperatures gradually increasing from −90 to −70°C, rinsed in pure methanol, and infiltrated with Lowicryl HM20 at −45°C and, finally, UV-polymerization for 2 days at −45°C and 2 days at 0°C.

The Lowicryl sections or ultrathin cryosection were first blocked by incubation in PBS containing 0.05 M glycine and either 0.1% skim milk powder or 1% BSA. The sections were then incubated for 1 h at room temperature with polyclonal antiserum NHE3-C00 at 1:100...
fractionation) vs. the discarded low-speed pellet (P_o) by density gradient for fractionation. These apical proteins are recovered in S_o, which is loaded onto the density membrane marker characteristics of the fractions collected from the sorbitol density gradients have been reported previously (35, 42). In brief, fractions 3-5 [window I (WI)] are enriched in plasma membrane apical brush-border markers alkaline phosphatase, dipetidyl-peptidase IV (DPPIV), and NHE3 as well as the basolateral marker Na-K-ATPase, fractions 6-8 [window II (WII)] also contain apical membrane markers as well as most of the IMC marker megalin, fractions 9-11 [window III (WIII)] are enriched in the endosomal marker rab 5a and the lysosomal marker β-hexosaminidase and also contain megalin and the CCV adaptor protein AP2. In data collected from four density fractions, the NHE3, expressed as percent of total, is as follows: 24 ± 2% in WI, 63 ± 1% in WII, 13 ± 2% in WIII. NHE3 density distribution normalized to protein distribution provides an estimate of the concentration of NHE3 across the gradient, expressed as arbitrary density units/protein: 3.5 ± 1.1 in WI, 4.5 ± 0.9 in WII, 1.2 ± 0.2 in WIII. From this pattern, we conclude that WI and WII are both enriched in NHE3, and thus apical membranes, relative to WIII.

Table 1. Relative abundance of proximal tubule proteins in S_o vs. P_o

<table>
<thead>
<tr>
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<th>% of Total</th>
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<tr>
<td>NHE3</td>
<td>98.8</td>
<td>1.2</td>
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<tr>
<td>NaPi2</td>
<td>87.5</td>
<td>12.5</td>
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<tr>
<td>Villin</td>
<td>90.9</td>
<td>9.1</td>
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<tr>
<td>Megalin</td>
<td>97.1</td>
<td>2.9</td>
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<td>AP2</td>
<td>92.6</td>
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Renal cortex from a control rat was dissected, homogenized, and centrifuged at 2,000 g for 10 min. Relative abundance of NHE3, NaPi2, the intermicrovillar cleft receptor protein megalin, the clathrin-coated pit adaptor protein AP2, and microvillar actin-bundling protein villin were examined by immunoblots of a constant amount of protein in the low-speed spin supernatant (S_o) vs. the discarded low-speed pellet (P_o). The conclusion is that between 87 and 98% of these apical proteins are recovered in S_o, which is loaded onto the density gradient for fractionation.

RESULTS

Subcellular fractionation of renal cortex. To assess transporter recovery during membrane fractionation of renal cortex, we examined the recovery of NHE3, NaPi2, the IMC receptor protein megalin, the clathrin-coated pit adaptor protein AP2, and microvillar actin-bundling protein villin in the low-speed fractionations 6-8 [window I (WI)] of NHE3 across the gradient, expressed as arbitrary density units/protein: 3.5 ± 1.1 in WI, 4.5 ± 0.9 in WII, 1.2 ± 0.2 in WIII. From this pattern, we conclude that WI and WII are both enriched in NHE3, and thus apical membranes, relative to WIII.

Similar redistribution of NHE3 and NaPi2 by subcellular membrane fractionation. Our previous studies established that NHE3 and NaPi2 are acutely retracted from the PT MV during acute hypertension (high BP) or PTH treatment (35, 43), which accounts for, at least in part, the decrease in PT sodium and phosphate reabsorption during acute hypertension or PTH treatment. By subcellular membrane fractionation, NHE3 and NaPi2 undergo similar redistribution patterns out of light-density (WI) into heavier-density (WII and WIII) membranes in response to high BP or PTH corresponding to a retraction from the apical MV. Figure 1 contains typical immunoblots of density gradient fractions probed with antibodies to NHE3, NaPi2, and villin from control vs. 20 min high-BP-challenged rats. Both NHE3 and NaPi2 transit from apical membrane-enriched fractions 3-5 (WI) to IMC-enriched fractions 6-8 (WII) and to IMC and coated pit and endosome-enriched fractions 9-11 (WIII). The redistribution responses of NHE3 and NaPi2 assessed by subcellular fractionation are indistinguishable. The actin-bundling protein villin broadly distributes between fractions 4-12, in a pattern unaltered by high BP or PTH treatment, indicating a translocation of the sodium transporters out of the apical MV rather than a change in the density of villin-associated membranes containing NHE3 and NaPi2.

Confocal immunofluorescence analysis of NHE3 redistribution. To determine the destination of NHE3 during acute hypertension or PTH treatment, double labeling with antibo-
ies to NHE3 vs. markers of early endosomes and clathrin-coated pits was performed on cryosections of kidneys harvested from control, 20-min high-BP and PTH-treated rats. During PTH treatment (described in EXPERIMENTAL PROCEDURES), BP dropped transiently for less than 5 min after the bolus injection and then returned to baseline for the remainder of the 1-h infusion. We previously demonstrated that PTH provokes two- to threefold increases in both lithium clearance (indicator of decreased PT sodium reabsorption) and urine output (43). Kidneys were surface rather than perfusion fixed to maintain defined renal perfusion pressure. In Fig. 2, NHE3 was labeled with polyclonal antiserum NHE3-C00 and FITC-conjugated goat-anti-rabbit secondary antibody. Early endosomes were identified by HRP internalization detected with monoclonal anti-HRP and Alexa 568-conjugated goat-anti-mouse secondary antibody. In time-paired saline-infused control rats, NHE3 labeling is enriched in the apical brush border (Fig. 2, left), shown previously to colocalize with the microvillar actin bundling protein villin (36) and shown here to be distinct from the subapical endosomal labeling with HRP. During both acute hypertension (Fig. 2, middle) and PTH treatment (Fig. 2, right), NHE3 labeling retracts from the top of the MV and concentrates at the base of the MV. In previous studies in which NHE3 is labeled with green and villin with red secondary antibodies, nearly all of the green NHE3 retracts below the red villin staining (20, 35). However, NHE3 is not internalized to colocalize with HRP-labeled subapical early endosomes in response to acute hypertension or PTH treatment.

To determine whether NHE3 is retracted into clathrin-coated pits, dual labeling of NHE3 and the clathrin adaptor protein AP2 was performed on cryosections from both acute hypertension and PTH treatment. During neither acute hypertension (Fig. 3, middle) nor PTH treatment (Fig. 3, right) does the retracted NHE3 colocalize with AP2 in the clathrin-coated pits. In addition, there is no NHE3 below AP2 staining in either model. These results suggest that NHE3 is retracted to a domain at the base of the MV above the clathrin-coated vesicles in response to acute hypertension or PTH treatment. The findings in Figs. 2 and 3 were confirmed with a different antibody to NHE3 (AB3085, from Chemicon, Temecula, CA), not shown.

Confocal immunofluorescence analysis of NaPi2 redistribution. NaPi2 redistribution was analyzed in tandem, analogous to the NHE3 redistribution studies. NaPi2 was detected with polyclonal anti-rat NaPi2 antibody, and then FITC-conjugated goat-anti-rabbit secondary antibody. In time-paired saline-infused control rats, the staining of NaPi2 is mainly in the brush border, although there is also a slight amount of punctate labeling below the apical membrane overlapping with HRP labeling (Fig. 4, top). During acute hypertension, some NaPi2 remains in the brush border, but there is pronounced internalization of NaPi2 to early endosomes colocalizing with HRP (Fig. 4, middle), indicating this transporter is internalized via the route used by HRP. Additionally, NaPi2 staining is detected around the nuclei. During PTH treatment, there is even more pronounced internalization of NaPi2 to intracellular compartments and less NaPi2 staining remaining in the brush border (Fig. 4, bottom). Surprisingly, the NaPi2 is not colocalized with HRP after PTH, suggesting that either the internalized NaPi2 has already passed through the early endosomes en route to lysosomes by this time point or that the route of NaPi2 internalization during PTH treatment is via an endocytic pathway distinct from that used by HRP.

To determine whether NaPi2 is internalized through clathrin-coated pits, double labeling of NaPi2 and AP2 was examined. During acute hypertension, NaPi2 is retracted from the MV and appears as the punctate staining either overlapping with AP2 or below AP2 staining (Fig. 5B). After a shorter treatment with PTH of only 10 min, NaPi2 is already retracted from the MV to the region below AP2 staining with occasional overlapping with AP2 staining (Fig. 5C). After infusion of PTH for 1 h, the retracted NaPi2 is in the cytoplasm with some NaPi2 staining around nuclei but does not overlap with AP2 staining (Fig. 5D), indicating that NaPi2 might be internalized into late endosomes or lysosomes by this time point.

Fig. 2. Redistribution of NHE3 during either 20-min high-BP or PTH treatment compared with endocytic compartment labeled with horseradish peroxidase (HRP). HRP was injected intravenously into rats, after 10 min the kidneys were fixed in situ with PLP for 20 min, followed by in vitro fixing for another 3–4 h. Kidney surface sections from control (left), 20-min high-BP (middle), and PTH treatment (right) at a dose of 6.6 μg/kg bolus followed by 1-h infusion at 0.1 μg·kg⁻¹·min⁻¹ were double labeled with polyclonal NHE3-C00 and then FITC-conjugated goat-anti-rabbit secondary antibody (green), and with monoclonal anti-HRP antibody, and then Alexa 568-conjugated goat-anti-mouse secondary antibody (red). NHE3 is retracted from the microvilli (MV) to the base of MV during both 20-min high-BP and PTH treatment, with no evidence that NHE3 moves into endocytic tracer HRP-labeled compartments. Bar = 7 μm.
FIG. 3. Redistribution of NHE3 during either 20-min high-BP or PTH treatment compared with the CCV adaptor protein AP2-stained domain. Kidneys were prepared as in Fig. 2. Surface sections from control (left), 20-min high-BP (middle), and PTH treatment as in Fig. 2 (right) were double labeled with polyclonal NHE3-C00 antibody and then FITC-conjugated goat-anti-rabbit secondary antibody (green), and with monoclonal anti-AP2 antibody, and then Alexa 568-conjugated goat-anti-mouse secondary antibody (red). NHE3 is retracted from the MV to the base of MV during both 20-min high-BP and PTH treatment. NHE3 staining does not retract to below the CCV adaptor protein AP2-stained domain in either model, thus there is no evidence that NHE3 is internalized. Bar = 7 μm.

**DISCUSSION**

This study demonstrates the distinct trafficking patterns of NHE3 vs. NaPi2 during either acute BP elevation or PTH treatment. Both confocal and electron microscopic images provide clear evidence for retraction of NHE3 from the tops of the MV to the IMC regions, that is, not internalized (Figs. 2, 3, and 6), and for the internalization of NaPi2 from the MV into endocytic vacuoles and/or lysosomes (Figs. 4, 5, and 7) stimulated by either acute hypertension or PTH. Although not shown in this study, this sodium transporter retraction coincides with increased urine output and decreased PT sodium reabsorption between 5 and 30 min of hypertension (35, 41).

Much of the recent physiological data describing the regulation of NHE3 in the PT has been derived from studies of stable epithelial cell lines such as OK and LLC-PK1. These studies built on our initial in vivo observation that NHE3 is regulated by trafficking between apical MV and an unidentified pool that was either at the base or below the MV (42). Some of the most convincing data supporting a role of membrane trafficking in the regulation of NHE3 comes from the Moe laboratory (13, 17, 18, 34). These studies in cultured cells used membrane-impermeant, cleavable biotinylation reagents to distinguish between plasma membrane and intracellular pools of NHE3 and demonstrated that inhibition of NHE3 activity by PTH or dopamine is accompanied by trafficking of NHE3 between the plasma membrane and intracellular pools of NHE3 and demonstrated that inhibition of NHE3 activity by PTH or dopamine is accompanied by trafficking of NHE3 between the plasma membrane and an intracellular compartment. As predicted, these effects are dependent on the cells having an intact cytoskeleton, recently reviewed by Szaszi and co-workers (30, 31). Furthermore, also in OK cells, active NHE3 has been shown to facilitate the initial step of endocytosis of complexes of ligands bound to the scavenger receptor megalin (16), suggesting that a NHE3-megalin complex might be also important in receptor-mediated endocytosis.

There are striking differences in the phenotypes of renal cell lines contrasted with renal PT cells (26). The brush border of the PT is very dense and consists of two distinct microdomains: the MV and the intermicrovillar domain, which can be subdivided into IMC and the intermicrovillar coated pits (ICP). Cultured PT cells have sparse MV and the ICP microdomain of the PT is all but lacking in cell lines. Although there is evidence for substantial intracellular pools of NHE3 in cultured...
cells, reviewed above, evidence for a significant pool of intracellular NHE3 in vivo at baseline, analogous to the pool of water channels seen in collecting duct cells at baseline, is all but lacking. Our previous studies demonstrate the appearance of a putative endosomal pool by subcellular fractionation after acute hypertension or PTH treatment: NHE3 shifts to high-density membranes enriched in endosomal markers and, by confocal microscopy, from MV (where NHE3 overlaps with villin staining) to a region just below villin staining (20, 35). However, the results of this current study contradict our previous interpretation that NHE3 was actually endocytosed. Confocal and electron microscopy results from the present study showed that NHE3 is redistributed between a pool in the MV to a pool in the intermicrovillar region, that is, redistribution within the apical membrane without endocytosis.

The presence of a recruitable pool of NHE3 at the base of the MV is physiologically relevant. This laboratory previously established that redistribution of NHE3 in response to acute hypertension is a reversible response: when BP is restored, NHE3 returns to its original density distribution pattern and sodium transport in the PT is restored (41). We recently reported that in hypertension induced by renal injury/sympathetic nervous system activation, NHE3 and NaPi2 abundance in MV-enriched low-density membranes increases, whereas transporter abundance in the high-density membranes enriched in IMC, ICP, and endosomes decreases (36, 37), a response

Fig. 4. Redistribution of NaPi2 during either 20-min high-BP or PTH treatment compared with endocytic compartment labeled with HRP. HRP was injected intravenously into rats and then kidneys were fixed as in Fig. 2. Kidney surface sections from control (top), 20-min high-BP (middle), and PTH treatment as in Fig. 2 (bottom) were double labeled with polyclonal anti-rat NaPi2 antibody, and then FITC-conjugated goat-anti-rabbit secondary antibody (green), and with monoclonal anti-HRP antibody, and then Alexa 568-conjugated goat-anti-mouse secondary antibody (red). Overlapping of NaPi2 and HRP appears yellow. NaPi2 is retracted from the MV to endocytic tracer HRP-stained compartments with some below the HRP staining after 20-min high BP. NaPi2 is retracted below HRP staining after PTH treatment. Bar = 7 μm.

Differential Trafficking of NHE3 and NaPi2
Fig. 5. Redistribution of NaPi2 during either 20-min high-BP or PTH treatment compared with the CCV adaptor protein AP2-stained domain. The kidneys were fixed as in Fig. 2. Surface sections from control (A), 20-min high-BP (B), PTH treatment at a dose of 6.6 μg/kg bolus followed by 10-min infusion at 0.1 μg·kg⁻¹·min⁻¹ (C), and PTH treatment at a dose of 6.6 μg/kg bolus followed by 60-min infusion at 0.1 μg·kg⁻¹·min⁻¹ (D) were double labeled with polyclonal anti-rat NaPi2 antibody, and then FITC-conjugated goat-anti-rabbit secondary antibody (green), and with monoclonal anti-AP2 antibody, and then Alexa 568-conjugated goat-anti-mouse secondary antibody (red); overlapping of NaPi2 and AP2 appears in yellow. NaPi2 is retracted from the MV to below the AP2-stained region in both 20-min high-BP and PTH treatment, some colocalizing with AP2 staining in both 20-min high-BP and 10-min PTH infusion. Bar = 7 μm.
that may contribute to the generation and maintenance of hypertension. Besse-Eschmann et al. (4) demonstrated a similar mechanism of NHE3 regulation in puromycin aminonucleoside-induced nephrotic syndrome; that is, NHE3 is shifted from an inactive pool to an active pool in the apical brush border, which would contribute to the sodium retention observed in nephritic syndrome.

The factors that constrain the reserve compartment of NHE3 to the base of MV remain to be defined. The current results indicate there is little if any NHE3 colocalization with AP2 or HRP during acute hypertension or PTH treatment. Immuno-EM results showed that NHE3 did not (or only to a very small extent) move into the components of the endocytic apparatus including clathrin-coated endocytic invaginations/pits, dense apical tubules, small and large endocytic vacuoles, or lysosomes. The EM results are consistent with the confocal results that localized NHE3 above AP2 during PTH or high-BP treatment (Fig. 3). In contrast, the results of Yip et al. (38) studying the one-clip Goldblatt hypertensive model, also using confocal microscopy, suggested that NHE3 colocalizes with AP2. This difference may be due to differences in the animal models, kidney fixation protocols, or the resolution by light microscopy.

Can the retraction of NHE3 observed during acute BP elevation cause the decrease in sodium entry into the PT? Yip et al. (39) measured Na+/H+ exchanger activity in vivo after loading cells with the intracellular pH indicator BCECF and determined that 20-min acute hypertension caused a 50% reduction in Na+/H+ exchanger activity. Studies from this laboratory, using the acridine orange quench method to assess Na+/H+ exchanger transport activity in membrane fractions from control vs. acute hypertension renal cortex, concluded that there was no change in Na+/H+ exchanger activity/ transporter when NHE3 retracted to the intermicrovillar domain. In a related analysis, Biemesderfer et al. (5) fractionated unstimulated rabbit renal brush-border membranes, assayed Na+/H+ exchange activity using the same acridine orange quench method, and concluded that NHE3 exists in two oligo-

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Fig. 6. Immunoelectron microscopic (immuno-EM) analysis of the NHE3 redistribution during acute hypertension. The tissue was fixed with paraformaldehyde and embedded in Lowicryl HM20. The Lowicryl sections were immunolabeled with polyclonal anti-NHE3 antibodies, which were detected with goat-anti-rabbit IgG conjugated to 10-nm colloidal gold. There is very little or no labeling of NHE3 in the apical cytoplasm in either control (A) or 20-min acute hypertension (B). A: NHE3 is present along the whole length of the MV in control rats (arrowheads). B: NHE3 is retracted to the base of the MV after 20-min high BP. C: after 20-min high BP, NHE3 is present in the intermicrovillar membrane, i.e., the membrane between the base of the MV (*) and the beginning of the coated endocytic invaginations (arrows). There is essentially no labeling of the membrane of the coated pits (arrowheads). A and B: bars = 0.5 μm; C: bar = 0.2 μm.
meric states: an active 9.6S form present in brush border MV and an inactive 21S megalin-associated NHE3 in dense vesicles containing markers of the IMCs. The authors postulated that the 21S form could serve as a reservoir of NHE3 for its rapid regulation. There are clear differences between the membrane preparation methods used in the two studies, and it remains to be determined whether key regulatory elements were lost in our fractionation procedure. Taken together, these studies are consistent with the hypothesis that during hypertension, NHE3 is rapidly retracted from the MV to the IMC where transporters form complexes with regulatory proteins that reversibly inactivate activity and decrease PT Na\(^+\) reabsorption, without NHE3 internalization.

How does NHE3 move within the plane of the MV? It is well established that brush-border MV are filled with bundled actin filaments, and evidence suggests that NHE3 can be tethered to the actin via the PDZ domain protein NHE RF and ezrin (40). Recently, the unconventional myosin VI, which moves toward the pointed ends of actin filaments, was, in fact, localized in the PT, mainly to the base of the MV (6). Together, these findings suggest myosin VI is a good candidate for moving cargo proteins such as NHE3 and/or NaPi2 along the MV down to the IMC/ICP region. Evidence from subcellular fractionation in our laboratory showed that myosin VI redistributes with NHE3 during acute BP elevation (in preparation).

The difference in the retraction patterns between NHE3 and NaPi2 was clearly demonstrated by their tandem analysis using both confocal and immuno-EM. During acute hypertension, there is an apparent internalization of NaPi2 to early endosomes evident by colocalizing with HRP (Fig. 4, middle) consistent with the subcellular fractionation results previously published by this laboratory (43). Fifteen minutes after injecting 100 \(\mu\)g of PTH in rats, Traebert et al. (32) detected significant colocalization of PTH with HRP. After 2-h high-phosphate-diet feeding, NaPi2 was colocalized with Golgi and lysosomal markers (21). During 1-h PTH treatment, we ob-

Fig. 7. Immuno-EM analysis of the NaPi2 redistribution during acute hypertension. The tissue was fixed as in Fig. 6 and thin cryosections were immunolabeled with rabbit polyclonal anti-NaPi2 antibodies, which were detected with goat-anti-rabbit IgG conjugated to 10-nm colloidal gold. A: in control rats, most of the labeling is present over the MV, but occasionally there is also labeling associated with the dense apical tubules (AT) or occasional coated or uncoated endocytic vacuoles (E; arrowheads). B: after 20-min high BP, there is a decrease in NaPi2 in the MV and an increase in the apical cytoplasm, in particular in coated or uncoated E and dense AT (arrowheads). Bar = 0.5 \(\mu\)m.
served a pronounced internalization of NaPi2 to intracellular vesicles including perinuclear compartments (Fig. 4, bottom) and no NaPi2 colocalization with HRP, suggesting that by 1 h NaPi2 has already passed through the early endosomes en route to lysosomes. During acute hypertension or 10 min after PTH infusion, NaPi2 appears as the punctate staining either colocalizing with AP2 or right below AP2 staining region (Fig. 5, B and C), consistent with the findings from Traebert et al. (32) and indicating that clathrin-coated pits may contribute to the endocytosis of NaPi2. The beaded appearance of NaPi2 staining right below the clathrin staining domain was also observed in a NHERF1 knockout mouse that has a problem inserting NaPi2 to the brush border (33).

The immuno-EM results from the current study indicate that during acute hypertension, NaPi2 enters the normal endocytic pathway, which transports proteins from the tubule lumen into the cell (23). Thus NaPi2 is present in the small coated endocytic vacuoles, as well as in the uncoated large endocytic vacuoles and the dense apical tubules, which are located around the vacuoles. The apical tubules have been shown to connect directly to the vacuoles and may return membranes from the endocytic vacuoles to the apical cell membrane in a recycling mechanism (24). When the dense apical tubules move away from the endocytic vacuoles, their membranes evidently include NaPi2 but not the clathrin coats and most of the endocytosed protein is left behind. Therefore, in double immunofluorescence, there is overlap between NaPi2 and AP2 in many coated endocytic vacuoles, but the surrounding cytoplasmic regions contain many apical tubules labeled only with NaPi2. This is probably the case in Fig. 5, B and C, and explains why some NaPi2 staining is present below the AP2 staining zone.

In conclusion, the parallel study of NHE3 and NaPi2 redistribution provides direct in vivo evidence that NHE3 and NaPi2 are regulated via distinct trafficking pathways. Although both are retracted from the top of the MV and the mechanism for the initial retraction may be shared, after that point the retraction pathways appear distinct. There is no evidence for a significant pool of intracellular NHE3 in vivo at either baseline or the presence of natriuretic stimuli such as increased BP or PTH. The well-defined intermicrorivillar domain in the PT, not observed in cultured cells, appears to serve as a storage pool for NHE3. Thus caution must be applied in studies using cultured renal cell lines to define molecular mechanisms of sodium transport regulation. The NHE3 pool at the base of the MV and IMC is likely important for the rapid retraction and insertion of NHE3, necessary to change PT Na+ transport to generate the autoregulatory signal relayed to the macula densa during changes in BP or GFR. In contrast, NaPi2 is not retracted to a pool at the base of the MV in the apical surface but internalized, likely through clathrin-coated pits, and destined to endosomes/lysosomes. It remains to be determined whether internalized NaPi2 is degraded, as is evident after PTH treatment (22), and/or recycled back to the cell surface via the dense apical tubules like internalized insulin receptors (28).

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