Redistribution of Myosin VI from Top to Base of Proximal Tubule Microvilli during Acute Hypertension

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During acute hypertension, Na⁺/H⁺ exchangers (NHE3) retract from top to base of proximal tubule microvilli (MV) and Na⁺ reabsorption decreases in proximal tubule. This study aimed to determine whether the actin-based motor myosin VI coordinates retracts with NHE3 in response to acute hypertension. BP was raised approximately 50 mmHg in rats for 20 to 30 min or sham treated, and kidneys were analyzed by subcellular fractionation or microscopy. During acute hypertension, myosin VI redistributed from low density apical MV-enriched membranes (from 23 ± 2.4 to 11.4 ± 2.2%) into higher density membranes (from 23.2 ± 0.7 to 36.9 ± 2.6%). By confocal microscopy, myosin VI was detected over the entire length of the MV in controls, then became completely focused at the base of MV during acute hypertension. For electron microscopic analysis using immunogold labeling, MV were divided into five zones from top (z1) to base (z5). In controls, myosin VI was evenly distributed through the five MV zones. In acute hypertension, myosin VI decreased in z1 (from 20.6 ± 1.9 to 10.5 ± 2.3%) and z2 (from 21.0 ± 2.0 to 13.2 ± 1.4%) and increased in z5 (from 21.1 ± 3.3 to 38.6 ± 3.0%). These results provide the first observation that acute hypertension causes myosin VI redistribution and support the idea that myosin VI may serve as the molecular motor for NHE3 retraction from top to base of MV during acute hypertension.


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Received April 6, 2005. Accepted July 15, 2005.

Published online ahead of print. Publication date available at www.jasn.org.

Materials and Methods

Acute Hypertension Protocol

As described in detail previously (8,9,16), male Sprague-Dawley rats (290 to 320 g body wt) were anesthetized intramuscularly with ketamine (Fort Dodge Laboratories, Overland Park, KS) and xylazine (1:1, vol/vol; Miles, Shawnee Mission, KS) and placed on a thermostatically controlled table (37°C). BP was recorded from carotid artery, and 4.0% BSA in 0.9% NaCl was infused (50 μl/min) to maintain euvoolemia. Mean arterial pressure was increased 50 to 60 mmHg over baseline by constricting the superior mesenteric artery, celiac artery, and abdominal aorta below the renal artery with silk ligatures.
Subcellular Fractionation and Immunoblot
As described in detail previously (5,16), 20 min after BP was elevated (or sham treated), kidneys from control and acute hypertension-challenged animal were cooled and excised; renal cortex was dissected and homogenized; and a low-speed supernatant was isolated, loaded, and resolved on a sorbitol density gradient. Twelve fractions were collected, pelleted, resuspended, and stored at −80°C pending assays.

Ten-microliter aliquots of each gradient fraction or of pooled windows (defined in Figure 1) were denatured in SDS-PAGE sample buffer (30 min, 37°C), resolved on 7.5% SDS polyacrylamide gels (17), and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Total sample protein loaded ranged from 1 μg (fraction 2) to 14 μg (fraction 7). Blots were probed with either polyclonal NHE3-C00 (8) or anti–myosin VI (T. Hasson, University of San Diego) (10) at 1:2000 dilution, then with Alexa 680–labeled goat anti-rabbit secondary antibody. Villin was detected with a mAb (Immuno-tech, Chicago, IL) at 1:1000 dilution, then with Alexa 680–labeled goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA). Signals were detected and quantified with an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

Indirect Immunofluorescence
As detailed previously (9), after BP was elevated for 10 min (or sham treated for same time), the left kidney was fixed in situ in a small Plexiglas cup in periodate-lysine-paraformaldehyde (PLP) fixative for another 20 min, during which time BP remained elevated. Kidneys were removed, postfixed in PLP, rinsed, incubated overnight in 30% sucrose/PBS, embedded, and frozen in liquid nitrogen. Cryosections (5 μm) were cut, transferred to charged glass slides, air dried, rehydrated, incubated with 1% SDS/PBS for 4 min (antigen retrieval) (18), washed, and blocked with 1% BSA/PBS. Sections were dual labeled with either polyclonal anti–NHE3-C00 or anti–myosin VI (1:100) and either monoclonal anti-villin (1:100) or anti-AP2 (clathrin adaptor; 1:50; Sigma, St. Louis, MO) in 1% BSA/PBS/PBS for 1.5 h at 25°C; then washed and incubated with a mixture of FITC-conjugated goat anti-rabbit (Cappel Research Products, Durham, NC) and Alexa 568–conjugated goat antimouse (Molecular Probes, Inc., Eugene, OR) secondaries (1:100) in 1% BSA/PBS/PBS for 1 h; and washed, mounted, and dried overnight, all as described (9). 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 (Compix Inc. Imaging Systems, Cranberry Township, PA).

Immunoelectron Microscopy
BP was increased 50 to 60 mmHg over baseline as described in the Acute Hypertension Protocol section or sham treated. After 20 min of acute hypertension, two rats were perfusion-fixed with 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), and the others were fixed by superfusion of the kidney surface with the fixative. The immunolabeling pattern, described below, was the same with the two fixation methods. As detailed previously (9), tissue blocks were trimmed from the cortex, 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 on a Reichert Ultracut S cryoultramicrotome (Leica, Germany) or on tissue that was cryo-substituted in a Reichert AFS freeze-substitution apparatus (Leica, Germany) and embedded in Lowicryl HM20 as described previously (19).

The Lowicryl sections or ultrathin cryosection were blocked (PBS that contained 0.05 M glycine and either 0.1% skim milk powder or 1% BSA) and incubated with polyclonal anti–myosin VI (1:100, 1 h) in PBS/0.1% skim milk powder. Anti–myosin VI was visualized with goat anti-rabbit IgG conjugated to 10 nm of colloidal gold particles (GAR.EM10; BioCell Research Laboratories, Cardiff, UK; 1:50 in PBS/0.1% skim milk powder and 5 mg/ml polyethylene glycol). Locivirc sections were stained with uranyl acetate, and the ultrathin cryosections were stained with 0.3% uranyl acetate in 1.8% mennyehcellulose for 10 min. Immunolabeling controls were incubated with nonimmune rabbit IgG or without primary antibody. All controls showed absence of staining. Electron micrographs were taken at ×16,000 in a Morgagni FEI electron microscope (Hillsboro, OR) from brush border areas where the microvilli were sectioned longitudinally. Thus, the micrographs represent random samples of microvilli in PT segments S1 and S2.

Quantitative Analysis of Myosin VI Distribution
The distribution of myosin VI, demonstrated by immunogold labeling, was determined in cryosections from six controls and five animals with high BP over three areas: The MV, where the MV were close to parallel with the section; the intermicrovillar zone (IMZ); and the apical cytoplasmic zone (ACZ) as defined in Figure 2. The MV was divided in five zones, counted from top to base of the microvilli. Zone 1 from 0 to 20% of the length of a given microvillus, zone 2 from 20 to 40%, etc. Zone 5 thus represents the very base of the MV and does not include the membrane between the MV or the endocytic invaginations. The IMZ begins where the MV end and is arbitrarily defined to extend to a (measured) depth of 0.5 μm into the apical part of the cell. It contains the plasma membrane between the MV, the coated invaginations, small coated endocytic vacuoles, dense apical tubules, and sometimes large

Figure 1. Myosin VI and Na+/H+ exchanger isoform 3 (NHE3) redistribute from low-density membranes to high-density membranes during acute hypertension (high BP). Renal cortices from the rat kidneys were removed and subjected to subcellular fractionation on sorbitol density gradients and collected as 12 fractions. A constant volume of sample from each gradient fraction was resolved by SDS-PAGE. Typical immunoblots of myosin VI, NHE3, and villin from control and acute hypertension-challenged animal were probed with either a mixture of FITC- and TRITC-conjugated goat anti-rabbit IgG conjugated to 10 nm of colloidal gold particles (GAR.EM10; BioCell Research Laboratories, Cardiff, UK) or 1:50 in PBS/0.1% skim milk powder and 5 mg/ml polyethylene glycol). Lowicryl sections were stained with uranyl acetate, and the ultrathin cryosections were stained with 0.3% uranyl acetate in 1.8% mennyehcellulose for 10 min. Immunolabeling controls were incubated with nonimmune rabbit IgG or without primary antibody. All controls showed absence of labeling. Electron micrographs were taken at ×16,000 in a Morgagni FEI electron microscope (Hillsboro, OR) from brush border areas where the microvilli were sectioned longitudinally. Thus, the micrographs represent random samples of microvilli in PT segments S1 and S2.
endocytic vacuoles. The ACZ is arbitrarily defined as the next 0.5-μm-deep zone into the apical cytoplasm. The measured location of individual gold particles, representing labeled myosin VI, was assigned to one of the five zones along the microvillus or to IMZ or ACZ. The number of gold particles within each zone was expressed as percentage of total gold particles counted in each animal. The numbers for each zone are the accumulated numbers from several sections from each animal. Mean values for each zone in controls and in animals with high BP were compared with ANOVA.

Results

Coincident Redistribution of Myosin VI and NHE3 by Subcellular Membrane Fractionation

Membrane marker characteristics of the fractions collected from the sorbitol density gradients have been reported previously (5,8). In brief, fractions 3 to 5 (window I [WI]) contain plasma membrane markers, including apical markers alkaline phosphatase, dipeptidyl-peptidase IV, NHE3, and basolateral Na,K-ATPase; fractions 6 to 8 (window II [WII]) also contain apical membrane markers as well as most of the intermicrovillar cleft (IMC) marker megalin and the clathrin-coated pit adaptor protein AP2; fractions 9 to 11 (window III [WIII]) are enriched in the endosomal marker rab 5a and the lysosomal marker β-hexosaminidase as well as some apical membrane and intermicrovillar cleft markers. We established that NHE3 is acutely retracted from the top to the base of the proximal tubule MV during acute hypertension (high BP) (9). Figure 1 demonstrates typical density gradient patterns in renal cortex from control versus 20-min high BP challenged rats probed with antibodies to myosin VI, NHE3, and villin. Myosin VI and NHE3 transit in concert out of light density (WI) into higher density (WII and WIII) membranes in response to high BP. The broad distribution pattern of the actin-bundling protein villin (between fractions 4 and 12) is unchanged by high BP, demonstrating that there is not an overall change in density of the apical membranes. For quantification of the density shift, the density gradient fractions were pooled into the three windows as defined in Figure 1, and percentage of total in each window was calculated. Twenty-minute high BP provoked coincident redistribution of myosin VI (Figure 3A) and NHE3 (Figure 3B) out of low-density WI membranes: Myosin VI decreased from 23 ± 4.8% (control) to 11.4 ± 4.4% (high BP), and NHE3 decreased from 23.7 ± 4.8% (control) to 8.1 ± 3.9% (high BP). Both proteins redistributed to higher density WII membranes: Myosin VI increased from 23.2 ± 1.4% (control) to 36.9 ± 5.2% (high BP), and NHE3 increased from 13.5 ± 3.4% (control) to 22.1 ± 4.8% (high BP). Villin distribution was not changed (Figure 3C) and total protein distribution changed only mini-

Figure 2. Schematic drawing and subdivision of apical surface of proximal tubule (PT) cell for immunogold analysis of myosin VI distribution. The locations of gold particles were determined over three areas of PT cells: Microvilli (MV), intermicrovillar zone (IMZ), and apical cytoplasmic zone (ACZ). The IMZ is arbitrarily defined to extend 0.5 μm into the apical part of the cell. It contains the plasma membrane between the MV, the coated invaginations, coated or uncoated endocytic vacuoles, and dense apical tubules (striated). The ACZ is defined as an adjacent 0.5-μm-wide zone deeper in the apical cytoplasm. The location of individual gold particles, representing labeled myosin VI, was measured from the tip of each microvillus and related to the measured length of the same microvillus. Each gold particle then was referred to one of five equal zones of the MV: Zone 1 from 0 (tip of MV) to 20% of the length of a given microvillus, zone 2 from 20 to 40%, etc. Zone 5 thus contains the very base of the MV. The percentage of colloidal gold particles in each zone was determined for each animal.

Figure 3. Summary of myosin VI, NHE3, villin, and protein distribution in three windows, expressed as the percentage of the total signal in all three windows. On the basis of previous marker analyses (8), membrane fractions were pooled into three windows and subjected to Western blot analysis: Fractions 3 to 5 were pooled into WI, fractions 6 to 8 were pooled into WII, and fractions 9 to 11 were pooled into WIII. A constant volume of sample from each window was resolved by SDS-PAGE. Values are means ± SD; n = 4 in each group. *P < 0.05 versus control, assessed by ANOVA and followed by paired t test.
nally (Figure 3D) by high BP, indicating that the redistribution of myosin VI and NHE3 are specific.

**Immunofluorescence Evidence for Myosin VI Redistribution**

Double labeling with antibodies to myosin VI *versus* markers of brush border MV or clathrin coated-pits was performed on kidneys that were harvested from control and acute high BP challenged rats. Kidneys were surface rather than perfusion fixed in this series. Villin was stained to indicate position of MV; its distribution is not altered by acute hypertension (7). At baseline pressure (Figure 4A, left), there is a significant amount of myosin VI both in the proximal tubule MV (arrow) and at the base of the MV (arrowhead) as previously reported (11). During high BP (Figure 4A, right), there is a shift in myosin VI distribution to the base of the MV (green, arrowhead) leaving the red villin staining in the MV.

To define further the destination of the retracted myosin VI during high BP, we performed dual labeling of myosin VI and the clathrin adaptor protein AP-2. In controls (Figure 4B, left), myosin VI is partially co-localized with AP-2 at the base of the MV (arrowhead), with a significant amount of myosin VI located in the MV above AP-2 staining (arrow). After high BP (Figure 4B, right), myosin VI is concentrated at the base of the MV evident either right above or overlapping with AP-2 staining (yellow, arrowhead). However, there is no obvious myosin VI internal to AP-2 staining. These results indicate that myosin VI is retracted from the top to the base of the MV during high BP rather than to pools below the MV.

**Immunoelectron Microscopic Analysis of Myosin VI Redistribution**

In Figure 5, myosin VI labeling is observed along the length of the MV in both controls and animals with high BP. However, in controls, labeling is evenly distributed along the MV (Figure 5A), whereas after acute hypertension, gold particles appear more frequent over the inner half of the MV (Figure 5B). This difference in labeling of myosin VI between controls and animals with high BP was documented in a quantitative analysis of myosin VI distribution as revealed by the colloidal gold particles, which eliminates cell-to-cell variability (Figure 6A): Myosin VI–associated particle density decreased significantly in the top of the MV (zones 1 and 2) and doubled at the base of the MV (zone 5). In both controls and animals with high BP, myosin VI was also present in the IMZ and in the ACZ farther into the cytoplasm. The quantitative analysis showed that myosin VI distribution, as percentage of total in the slice, was not altered by high BP in MV (approximately 50% of total myosin VI), IMC (approximately 30%), and ACZ (approximately 20%; Figure 6B). That is, there was significant redistribution *within* the MV during high BP but not between MV, IMC, and ACZ zones as defined.

Comparison of villin labeling of the MV in controls and animals with high BP revealed no difference in villin distribution. Specifically, there was no evidence of decreased labeling in the top of the MV (Figure 5C).

**Discussion**

This laboratory previously established that in response to either acute hypertension or parathyroid hormone treatment, NHE3 is retracted from the top to the base of the proximal tubule MV associated with decreased PT Na\(^+\) reabsorption; that is, NHE3 is retracted *within* the apical membrane plane without endocytosis (7,9). This study addresses how NHE3 moves within the plane of the MV membrane. The trafficking of
the membrane proteins in epithelial cells often utilizes actin as a track or relies on the interaction of the membrane proteins with actin and its molecular motors. Recently, Wagner et al. (20) showed that expression of myosin I (Myo1c), an actin-based unconventional myosin in M1 collecting duct cells, participates in the regulation of the Na⁺/H⁺ channel after ADH stimulation. It is well established that the brush border MV are dense and filled with bundled actin filaments. The unconventional myosin VI is highly enriched in the PT brush border region (10,11).

Myosin VI moves toward the pointed end of actin filaments (21), which are located at the base of the MV in the setting of the PT, suggesting that it could move cargo proteins such as NHE3 along the MV down to the base of the MV. The findings from subcellular fractionation (Figures 1 and 3) and confocal (Figure 4) and electron microscopy (Figures 5 and 6) provide evidence for movement of myosin VI from the top to the base of the MV in response to acute hypertension, coincident with redistribution of NHE3 within the plane of membrane along the microvillus. Myosin VI staining did not retract to below AP-2–stained domain (Figure 4B), suggesting that there is no detectable myosin VI–involved endocytosis in acute high BP. At the electron microscopic level, quantitative analysis of the gold particles that label myosin VI indicates that at baseline pressure, approximately half of the myosin VI protein is located in the MV, the other half split between the IMZ and the ACZ. High BP did not cause redistribution of myosin VI among these three defined regions; rather, it provoked significant retraction of myosin VI from the tops to the bases of the MV. These results, taken
together, support the hypothesis that myosin VI may play a role in the regulated decrease in proximal tubule sodium transport that occurs during acute hypertension (4,6,22), specifically by driving the retraction of NHE3 within the MV. Considering the results from another angle, they provide the first evidence for regulation of renal myosin VI distribution, establishing this motor as a candidate that could regulate a number of PT functions. We have evidence (not shown) that the redistribution of myosin VI is not isolated to high BP treatment as there is also a coordinate redistribution of myosin VI and NHE3 from low to higher density membranes in rats infused with the angiotensin-converting enzyme inhibitor captopril, which occurs in the absence of a change in BP (manuscript submitted).

This study also highlights the notion of rapid regulation of membrane transporters and membrane-associated proteins in vivo between subdomains of the plasma membrane (without internalization) associated with a change in Na+ transport. NHE3 apparently moves to a reservoir pool from which it could be rapidly recruited when a need for increased Na+ reabsorption occurs. Recently, Inoue et al. (23) demonstrated that in potassium deficiency, NaPi IIa is increasingly partitioned in the low-density microdomains of the apical membranes enriched in cholesteryl, sphingomyelin, and glycosphingolipid, characterized as “lipid raft” fractions on a detergent-free density gradient. By using fluorescence correlation spectroscopy, NaPi IIa lateral diffusion was decreased and NaPi IIa aggregation/ clustering increased, associated with decreased Na/Pi co-transport activity in K+ deficiency. Cha et al. (24) recently expressed chimera NHE3–enhanced green fluorescent protein (EGFP) in renal epithelial opossum kidney (OK) cells and demonstrated that the lateral mobility of NHE3 on the apical membrane of OK cells is dependent on an intact actin cytoskeleton determined by fluorescence recovery after photobleaching and confocal microscopy.

We know that during acute hypertension, NHE3 is retracted to the base of the MV, and we know that PT Na+ reabsorption is inhibited by the high BP, but we do not know whether retraction causes or is simply associated with inhibition of NHE3 transport activity. Recently, Kocinsky et al. (25) demonstrated that there is a distinct pattern of NHE3 phosphorylation in the MV: NHE3 phosphorylated at serine 552 localized to the coated pit region of the brush border membrane, whereas total NHE3 was found throughout the brush border membrane. Whether NHE3 phosphorylation at serine 552 is increased during high BP and whether it is critical for PT Na+ transport inhibition remain to be determined.

The factors that constrain the Na+ transporter and myosin VI at the base of the MV during hypertension remain to be defined. Evidence suggests that NHE3 can be tethered to the actin cytoskeleton via the PDZ domain protein NHERF and ezrin (26). Biemesderfer et al. (27) provided evidence that NHE3 in the PT exists in two oligomeric states: An active 9.6S form (26). Biemesderfer et al. (53) demonstrated that there is a distinct pattern of NHE3 phosphorylation within the MV. Considering the evidence, the hypothesis that myosin VI may play a role in the regulation of renal myosin VI distribution, establishing this motor as a candidate that could regulate a number of PT functions. We have evidence (not shown) that the redistribution of myosin VI is not isolated to high BP treatment as there is also a coordinate redistribution of myosin VI and NHE3 from low to higher density membranes in rats infused with the angiotensin-converting enzyme inhibitor captopril, which occurs in the absence of a change in BP (manuscript submitted).

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