Effects of ACE inhibition on proximal tubule sodium transport

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The proximal tubule (PT) is a primary target site for captopril but the molecular mechanisms for its action in PT are not well defined. The aim of this study was to determine the physiological and molecular changes in PT provoked by acute captopril treatment in the absence of changes in blood pressure or glomerular filtration rate (GFR). Captopril (infused at 12 µg/min for 20 min) did not change blood pressure or GFR but induced an immediate (<10 min) increase in PT flow measured with a nonobstructive optical method (to 117 ± 14% of baseline) along with a rapid diuresis from 2.1 ± 0.6 mg/min (baseline) to 3.7 ± 0.9 mg/min (captopril). Captopril also provoked a significant retraction of PT Na+/H+ exchanger isoform 3 (NHE3), NHE regulatory factor (NHERF)-1, myosin-VI, and Na+/P-co transporter type 2 (NaP2), but not ACE, out of apical microvillus-enriched membranes. Proteomic analysis with MALDI-TOF MS revealed an additional eight abundant membrane-associated proteins that redistribute out of the microvillus-enriched membrane during captopril treatment: megalin, myosin II-A, clathrin, aminopeptidase N, DPPIV, ezrin, moesin, and vacuolar H+-ATPase subunit β2. In summary, captopril can rapidly depress PT reabsorption in the absence of a change in GFR or BP and provokes the redistribution of a set of transporters and transporter-associated proteins that likely participate in the decrease in PT reabsorption and may also contribute to the blood pressure-lowering effect of ACE inhibitors.

sodium/hydrogen exchanger isoform 3; proximal tubular flow; blood pressure-lowering effect of ACE inhibitors.

ANGIOTENSIN-CONVERTING ENZYME (ACE) catalyzes the conversion of ANG I to ANG II and is, thus, a rate-limiting component of the renin-angiotensin system (RAS). ANG II is both a potent vasoconstrictor and a sodium-retaining hormone; degradation of renal function by ANG II has been demonstrated in many hypertensive models (36, 56). ACE inhibitors, such as captopril, which block the endogenous formation of ANG II (35), are commonly prescribed for treatment of chronic hypertension (3). There is strong evidence that ACE inhibitors exert their antihypertensive effects through actions on the kidney. In young spontaneously hypertensive rats (SHR) (19), transplantation of kidneys from control SHR into ACE inhibitor-pretreated normotensive SHR resulted in an increase in blood pressure in the recipients, indicating that the kidney is a critical site for the pressure-normalizing effects of ACE inhibition in SHR.

Within the kidney, the proximal tubule (PT) is very sensitive to changes in ANG II levels and could, therefore, be a major target site for captopril action. The PT reabsorbs over 67% of the kidney’s filtered salt and water load (26). Harris and co-workers (20) demonstrated that acute captopril administration decreased PT fluid reabsorption in both normotensive rats and two-kidney, one-clip Goldblatt hypertensive rats but the molecular mechanisms were not well defined. Previous studies from the McDonough laboratory showed that the decrease in PT Na+ reabsorption caused by acute hypertension or PTH treatment is not associated with an internalization of the major PT Na+ transporter [Na+/H+ exchanger isoform 3 (NHE3)] but, rather, with a retraction of NHE3 from the top to the base of the microvilli (58, 61) within the plane of the microvillar membrane. This finding raises the questions as to the molecular mechanisms responsible for the decrease in PT Na+ reabsorption and the mechanisms responsible for the redistribution of NHE3 within the apical microvilli (60). Interestingly, this NHE3 retraction is partially blocked when systemic [ANG II]↓ is clamped during acute hypertension, evidence for a two-stage redistribution (30).

A significant body of knowledge exists about PT transport physiology (15, 25, 34), but less is understood about how the transport is regulated, especially in vivo. To fully understand the apical transport processes and their regulation in the PT, it would help to identify the molecular components involved in signaling and protein trafficking. In the present study, we demonstrate that acute captopril treatment provokes a redistribution of NHE3 to the base of the microvilli coincident with an increase in PT fluid flow rate (i.e., decrease reabsorption). We further identified proteins that, along with NHE3, exhibited dynamic redistribution between membrane domains in response to acute captopril treatment. We used conventional immunological techniques to examine candidate proteins and a proteomic strategy to identify other abundant PT apical membrane-associated proteins. These approaches together identified a set of 11 membrane-associated proteins that redistribute out of low-density apical membranes in response to captopril treatment and may contribute to the antihypertensive action of ACE inhibitors.

METHODS

Animal preparation and experimental design. Experiments were performed using male Sprague-Dawley rats (250–350 g body wt) that
were kept under diurnal light conditions and had free access to food and water. Rats were anesthetized intramuscularly with Inactin (Sigma; at 125 mg/kg) and body temperature was maintained thermostatically at 37°C. Polyethylene catheters (PE-50) were placed into the carotid artery to monitor blood pressure and into the jugular vein to infuse 4.0% BSA in 0.9% saline (at 50 µl/min) to maintain euolemia. The left ureter was cannulated with a Surflwo I.V. catheter (Terumo) for urine collection. At the completion of all surgical procedures, the rats were allowed to recover for >60 min before infusion of drugs and collection of in vivo data. Urine was collected at 10-min intervals and volume was determined gravimetrically. Glomerular filtration rate (GFR), calculated for one kidney (as urine was collected from just one ureter), was measured by infusion of FITC-inulin (5 mg/ml) as previously described by Lorenz and Grunestein (32), who found that dialysis of FITC-inulin did not reveal any unbound FITC. Urine samples were protected from direct ambient light, diluted with PBS (pH 7.4), and the fluorescence was measured (excitation = 480 nm and emission = 530 nm) in 1-cm cuvettes with a PerkinElmer LS5 spectrofluorometer. The rats that were used for measurement of urine output, GFR, protein subcellular distribution, proteomic analysis, and confocal microscopy analysis were infused with captopril intravenously via the jugular vein at 12 µg/min for 20 min. Control rats were sham-operated and infused with 4.0% BSA in 0.9% saline. At the end of the in vivo experiment, the kidneys were removed for immediate analysis. A different set of rats was used for measurement of PT flow rate: tubular flow was recorded continuously during baseline conditions (0.9% saline infusion for 10–15 min), followed subsequently by captopril infusion (12 µg/min for 20 min), then coinfusion of captopril (12 µg/min) and ANG II (20 ng·kg⁻¹·min⁻¹) for >20 min. All animal experiments were approved by the University of Southern California Keck School of Medicine and conducted in accord with the Guide for the Care and Use of Laboratory Animals (Washington, DC: National Academy Press, 1996).

Measurement of proximal tubular flow. PT flow was measured by a method developed by Chou and Marsh (11, 53) as described previously. These animals were infused with 20 µl/min saline to maintain euolemia. In brief, a bolus of lissamine green dye was injected intravenously to identify early segments of PTs. PTs were selected for observation only if they had a long segment (>1 mm) that ran on the surface. In brief, a micropipette (2- to 3-μm outer diameter) filled with synthetic proximal tubular fluid (127 mM NaCl, 25 mM NaHCO₃, 3 mM KCl, 1 mM MgSO₄·7H₂O, 1 mM K₂HPO₄, 5 mM urea, and 1.8 mM CaCl₂) containing 1% solution of rhodamine-isothiocyanate 20S-labeled dextran (MW 17,200, Sigma) was infused into the proximal convoluted tubule. Injection was driven by a pneumatic picopump (PV830, WPI). Injection frequency and volume were kept under diurnal light conditions and had free access to food and water. Rats were anesthetized intramuscularly with Inactin (Sigma; at 125 mg/kg) and body temperature was maintained thermostatically at 37°C. Polyethylene catheters (PE-50) were placed into the carotid artery to monitor blood pressure and into the jugular vein to infuse 4.0% BSA in 0.9% saline (at 50 µl/min) to maintain euolemia. The left ureter was cannulated with a Surflwo I.V. catheter (Terumo) for urine collection. At the completion of all surgical procedures, the rats were allowed to recover for >60 min before infusion of drugs and collection of in vivo data. Urine was collected at 10-min intervals and volume was determined gravimetrically. Glomerular filtration rate (GFR), calculated for one kidney (as urine was collected from just one ureter), was measured by infusion of FITC-inulin (5 mg/ml) as previously described by Lorenz and Grunestein (32), who found that dialysis of FITC-inulin did not reveal any unbound FITC. Urine samples were protected from direct ambient light, diluted with PBS (pH 7.4), and the fluorescence was measured (excitation = 480 nm and emission = 530 nm) in 1-cm cuvettes with a PerkinElmer LS5 spectrofluorometer. The rats that were used for measurement of urine output, GFR, protein subcellular distribution, proteomic analysis, and confocal microscopy analysis were infused with captopril intravenously via the jugular vein at 12 µg/min for 20 min. Control rats were sham-operated and infused with 4.0% BSA in 0.9% saline. At the end of the in vivo experiment, the kidneys were removed for immediate analysis. A different set of rats was used for measurement of PT flow rate: tubular flow was recorded continuously during baseline conditions (0.9% saline infusion for 10–15 min), followed subsequently by captopril infusion (12 µg/min for 20 min), then coinfusion of captopril (12 µg/min) and ANG II (20 ng·kg⁻¹·min⁻¹) for >20 min. All animal experiments were approved by the University of Southern California Keck School of Medicine and conducted in accord with the Guide for the Care and Use of Laboratory Animals (Washington, DC: National Academy Press, 1996).

Subcellular fractionation. The procedure for collection, homogenization, and subcellular fractionation of total cortical membranes has been described in detail previously (61, 62). In brief, cortical tissue homogenate was subjected to centrifugation at 100,000 g for 5 h in a hyperbolic sorbitol gradient, and the cortical membranes were collected as 12 fractions for the density gradient distribution analysis of various proteins. We previously reported the membrane marker assignment for these fractions (57): fractions 3-5 are enriched in both apical membrane markers alkaline phosphatase and dipeptidyl-peptidase IV (DPPIV) as well as basolateral membrane marker Na-K-ATPase α₁; fractions 6-8 contain a mixed pool of apical membrane markers such as DPPIV and the intermicrovillar cleft marker megalin; and fractions 9-11 are enriched in endosomal (rab5a) and lysosomal (β-hexosaminidase) membrane markers as well as the intermicrovillar cleft marker megalin. With the use of the current fractionation protocol, this “membrane marker map” has been found to be very consistent and reproducible between gradients.

Immunoblot analysis. To determine the density distribution of proteins, a constant volume of each membrane fraction was assayed and expressed as the percentage of the total in all 12 fractions for each gradient. Samples were denatured in SDS-PAGE sample buffer for 30 min at 37°C, resolved on 5 or 7.5% SDS polyacrylamide gels according to Laemmli (29), and transferred to polyvinylidene difluoride membranes (Millipore Immobilon-P). Membrane blots were probed with the following antibodies: NHE3-C00 against NHE3, Me-Na⁺-P, co-transporter type 2 (NaPi2) against NaPi2 (both 1:2,000; McDonough laboratory, Univ. Southern California), 464.6 against Na-K-ATPase α₁-subunit (1:200; M. Kashgarian, Yale Univ.), sc-12187 against angiotensin-converting enzyme (ACE; 1:200; Santa Cruz Biotechnology), anti-myosin VI against myosin VI (1:2,000; T. Hasson, Univ. of California San Diego), R-1046 against NHE regulatory factor (NHERF)-1 (1:3,000; E. Weinman, Univ. of Maryland), anti-gp105 against DPPIV (1:1,000; M. Farquhar, Univ. of California San Diego), 459 against megalin (1:5,000; M. Farquhar, Univ. of California San Diego), BT561 against myosin II-A (1:2,000; Biomedical Technologies), MAS 401 against the heavy chain of clathrin (1:200; Harlan Sera-Lab), sc-15360 against aminopeptidase N (1:100; Santa Cruz Biotechnology), sc-6409 against ezrin and moesin (1:1,000; Santa Cruz Biotechnology), and A1565eF1 against the β₂-subunit of the vacuolar H⁺-ATPase (1:100; D. Brown, Harvard University). Polyclonal primary antibodies (against ACE, aminopeptidase N, DPPIV, NHE3, NHE-RF, NaPi2, megalin, and myosin II-A) were detected with Alexa 680-labeled goat anti-rabbit or Alexa 680-labeled donkey anti-goat secondary antibody (Molecular Probes), and polyclonal chicken anti-β₂ primary antibody was probed with monoclonal mouse anti-chicken secondary antibody (Sigma) followed by detection with Alexa 680-labeled goat anti-mouse secondary antibody (Molecular Probes). The density of the protein bands was quantitated using the Odyssey Infra-red Imaging System and quantitation software (LI-COR, Lincoln, NE).

SDS-PAGE analysis, in-gel trypsin digestion, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, and peptide mass fingerprinting. The protein concentration in the apical membrane-enriched fraction 4 of the density gradient was measured with the BCA assay kit (Pierce Technology) and 35 µg of protein were loaded onto each lane of a self-cast 7.5%, 140 × 140-mm, SDS-PAGE gel. The gel was run at 60 V for 18 h at 4°C after which the gel was incubated in 100 ml of Coomassie blue stain (Bio-Safe Coomassie Blue G250, BioRad) at room temperature for 48 h. Protein bands that showed a marked difference in density between control and captopril-treated samples were excised for proteomic analysis.

Excised protein spots were subjected to in-gel trypsin digestion as previously described by this laboratory (50). Trypsin-generated peptides were applied by a thin film-spotting procedure for matrix-
assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis using α-cyanohydroxycinnamic acid as the matrix on stainless steel targets, as previously described (44). Mass spectral data were obtained using a ToF-Spec 2E (Micromass) and a 337-nm N₂ laser at 20–35% power in the reflector mode. Spectral data were obtained by averaging 10 spectra, each of which was the composite of 10 laser firings. Mass axis calibrations were accomplished using peaks from tryptic autohydrolysis. Peptide masses obtained by MALDI-MS were analyzed using the Mascot search engine (www.matrixscience.com) by comparison to the NCBI protein database. A probability-based MOWSE (MOlecular Weight SEarch) score greater than 74 indicated a significant match that was not a random event.

**Indirect immunofluorescence.** As detailed previously (58), the left kidneys of a subset of the control and captopril-treated rats were fixed in situ in a small Plexiglas cup in PLP fixative for 20 min in preparation for indirect immunofluorescence analysis. After in situ fixation, the kidneys were removed, postfixed in PLP, rinsed, incubated 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) (9), washed, and blocked with 1% BSA/PBS. Sections were dual labeled with monoclonal anti-villin at 1:100 (Immunotech) and one of the following antibodies: anti-NHE3 at 1:100 (NHE3-C00; polyclonal), anti-myosin VI at 1:100 (MUD-19; Sigma; monoclonal), and anti-NaPi2 at 1:250 (J. Biber, Univ. of Zürich; polyclonal) in 1% BSA/PBS (1.5 h, 25°C), then incubated with a mixture of FITC-conjugated goat-anti-rabbit (Cappel Research Products) and Alexa 568-conjugated goat-anti-mouse (Molecular Probes) secondaries at 1:100 in 1% BSA/PBS for 1 h, washed, mounted, and dried overnight, all as previously described (58). 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.

**Statistical analysis.** All data, unless otherwise stated, are expressed as means ± SD. Two-tailed Student’s t-test was applied for pairwise comparisons with Bonferroni’s adjustments. A P value <0.05 was considered significant.

**RESULTS**

**Effect of captopril on mean arterial pressure and renal function.** Captopril infusion at 12 μg/min had no significant effect on mean arterial pressure (MAP; baseline: 124.5 ± 12.5 mmHg; with captopril: 125.3 ± 11.3 mmHg; Fig. 1A) but did induce a significant diuretic response: urine output increased from 2.1 ± 0.6 mg/min at baseline to 3.7 ± 0.9 mg/min after 20 min of captopril infusion (Fig. 1B). This increase in urine output occurred in the absence of a change in GFR (baseline: 0.59 ± 0.13 ml/min; with captopril: 0.51 ± 0.08 ml/min; Fig. 1C). Consistent with the captopril-induced diuretic response, mean normalized PT flow increased to 117 ± 14% after 10 min of captopril infusion (Fig. 2). Subsequent infusion of ANG II at 20 ng·kg⁻¹·min⁻¹ immediately (<3 min) reversed tubular flow to basal level indicating that the captopril-mediated depression in reabsorption was due to a decrease in ANG II effect in the PT.

**Effect of captopril on the subcellular distribution of PT Na⁺ transporters and candidate regulators in the renal cortex.** Because NHE3 is the primary Na⁺/H⁺ exchanger isoform responsible for PT apical NaCl and NaHCO₃ reabsorption (37, 41, 42), we tested the hypothesis that the captopril-induced increase in PT flow (i.e., decrease in PT reabsorption) (Fig. 2), in the absence of GFR change (Fig. 1C), was associated with a redistribution of NHE3 out of low-density apical enriched membranes (fractions 3–5). Indeed, captopril induced a significant decrease in NHE3 abundance in the enriched microvillar
membranes (fractions 4 and 5) and a parallel increase in intermicrovillar membrane-enriched fraction 7 (Fig. 3A). Captopril also provoked an accompanying redistribution of NHERF-1 [Na\(^+/\)H\(^+\) exchanger regulatory factor 1 which interacts with NHE3 (17, 48)] (Fig. 3B), myosin VI [an abundant unconventional myosin located at the base of the PT microvilli (5)] (Fig. 3C), and NaPi2 [type IIa Na-phosphate cotransporter (38)] (Fig. 3D). On the other hand, captopril did not alter the subcellular density distribution of ACE (Fig. 3E), a potential target site for captopril, nor did it alter the distribution of the basolaterally located Na-K-ATPase \(\alpha_1\) catalytic subunit (Fig. 3F). Acute captopril treatment did not change total cortical protein abundance (data not shown).

Indirect immunocytochemistry of NHE3, myosin VI, and NaPi2 in the PT in response to acute captopril treatment. Confocal immunofluorescence analysis was conducted as an independent strategy to evaluate the predictions of the membrane fractionation analysis (Fig. 3). The actin bundling protein villin labels the brush-border microvilli as well as the microvillar core rootlets in the terminal web (46). In controls, NHE3 (green) was present primarily in the brush border where it colocalized with villin (red) as evidenced by the yellow labeling denoting red and green overlay (Fig. 4A, left). In response to captopril, NHE3 moved out of the top of the brush-border microvilli (revealing a red-labeled villin layer) and focused at the base of the microvilli, overlaying villin (yellow) (Fig. 4A, right). These observations are consistent with the conclusion of the membrane fractionation analysis that captopril provokes a redistribution of NHE3 from low- to higher-density membrane fractions (Fig. 3A). Myosin VI displayed a similar redistribution response to captopril as NHE3. At baseline, myosin VI was located along the length of the microvilli (Fig. 4B, left). In response to captopril, there was a shift in myosin VI distribution to the base of the microvilli (revealing a compact green-labeled myosin VI layer; Fig. 4B, right) analogous to our recent finding of coincident redistribution of NHE3 and myosin VI to the base of the microvilli during acute hypertension (59). NaPi2 has a similar baseline distribution as NHE3 where it colocalized with villin in the PT brush border (yellow; Fig. 4C, left). However, captopril failed to induce a discernible shift of
NaPi2 within the microvilli (Fig. 4, right). This suggests that only a very small fraction of NaPi2 redistributes to higher-density membranes leaving a significant fraction overlaying villin staining, unlike what we previously observed in response to hypertension or PTH treatment (58).

Proteomic identification of additional proteins that redistribute out of low-density apical-enriched membranes after captopril treatment. Our candidate protein approach revealed that captopril provokes redistribution of NHE3, NHERF-1, myosin VI, and NaPi2 out of the apical membranes in the PT
(Fig. 3). We also aimed at identifying additional membrane-associated proteins that redistribute to higher-density membranes along with NHE3 in response to acute captopril treatment that could potentially contribute to the regulation of PT reabsorption. We focused on microvillus-enriched membrane fraction 4, where a large difference in NHE3 distribution after captopril treatment was evident (Fig. 3). A constant amount of protein from fraction 4 from both control and captopril-treated rats was resolved by SDS-PAGE (7.5%). In-gel staining with Coomassie blue revealed eight discernible bands that redistributed out of fraction 4 during acute captopril treatment (Fig. 5). The identity of the proteins, analyzed by MALDI-TOF MS, is summarized in Table 1: megalin (345 kDa); myosin II-A (227 kDa); clathrin (193 kDa); aminopeptidase N (110 kDa); DPPIV (89 kDa); ezrin (69 kDa); moesin (68 kDa); vacuolar H+-ATPase subunit β2 (57 kDa).

Immunoblot confirmation of captopril-responsive proteins identified by proteomics. To confirm the MALDI-TOF MS findings, immunoblot analyses of the subcellular density distribution of the eight identified candidate proteins in renal cortex were conducted in sets from control and captopril-treated rats (Fig. 6). Consistent with the proteomic analysis of fraction 4 (Table 1), seven of the eight proteins demonstrated a significant captopril-mediated redistribution out of low-density apical membrane-enriched fractions (fractions 4 and 5; Fig. 6A, C-H). There was no significant decrease in myosin II-A in fraction 4, although there was a tendency for a decrease in myosin II-A abundance in fractions 5 and 6 during captopril treatment (Fig. 6B) that is consistent with the proteomic analysis (Fig. 5).

DISCUSSION

In this study, we investigated the acute effect of captopril on reabsorption in the PT and the corresponding molecular mechanisms involved. The protocol was designed to minimize changes in MAP and GFR during captopril treatment (Fig. 1) to ensure that we addressed the primary effects of captopril on PT transport. We found that systemic captopril infusion at 12 μg/min provokes an acute decrease in PT reabsorption and the retraction of a specific array of PT apical membrane-associated proteins from the top of the microvilli independent of change in MAP or GFR. Harris and co-workers (20) previously reported that acute captopril administration decreases PT fluid reabsorption in rats but did not define the signaling and molecular mechanisms. Although most of the beneficial effects of ACE inhibitors have generally been attributed to the blockade of the ACE-dependent formation of ANG II (35), ACE inhibitors also induce the accumulation of bradykinin (10, 33) and activate the ACE signaling cascade (16) that together would exert a complex array of effects on the kidney. The issue of whether the captopril-mediated effects were mediated by accumulation of bradykinins or lack of ANG II was indirectly addressed in the tubular flow assay: the captopril-mediated decrease in PT reabsorption (increase in tubular flow) was rapidly reversed by acute infusion of ANG II, suggesting that the effects stemmed from the blockade of endogenous ANG II formation rather than bradykinin accumulation (Fig. 2). Furthermore, work in progress indicates that the apical protein retraction induced by captopril can be reversed by acute ANG II infusion (Leong PKK, unpublished observations). The literature also suggests that the acute effects of captopril on PT

![Image](http://ajprenal.physiology.org/)

**Fig. 5.** One-dimensional gel electrophoresis and direct in-gel Coomassie staining of membrane fraction 4 generated by subcellular fractionation of total cortical membranes as in Fig. 3. Two representative fraction 4 samples from control saline-treated (Con 1 and 2) and captopril-treated (Cap 1 and 2) samples are shown. Arrows indicate the protein spots that were excised and subjected to in-gel tryptic digestion followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The identity of each protein spot identified by MALDI-TOF MS is shown next to the corresponding arrow.

Table 1. Protein set identified by MALDI-TOF MS that redistributed out of low-density membranes during captopril treatment

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight, kDa</th>
<th>NCBI Identifier (GI#)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Megalin; glycoprotein 330</td>
<td>345</td>
<td>34864634</td>
</tr>
<tr>
<td>Myosin II-A; MYH9 (gene)</td>
<td>227</td>
<td>20137006</td>
</tr>
<tr>
<td>Clathrin, heavy polypeptide (Hc)</td>
<td>193</td>
<td>9506497</td>
</tr>
<tr>
<td>Kidney aminopeptidase N(M)</td>
<td>110</td>
<td>13591914</td>
</tr>
<tr>
<td>Dipeptidylpeptidase (DPPIV)</td>
<td>89</td>
<td>6978773</td>
</tr>
<tr>
<td>Ezrin</td>
<td>69</td>
<td>32363497</td>
</tr>
<tr>
<td>Moesin</td>
<td>68</td>
<td>13540689</td>
</tr>
<tr>
<td>ATPase H+-transporting, vacuolar proton pump, subunit β2</td>
<td>57</td>
<td>17105370</td>
</tr>
</tbody>
</table>
reabsorption are likely due to inhibition of systemic rather than intrarenal ACE-mediated ANG II formation: 1) 1-wk systemic administration of captopril has no significant effect on the percentage of renal excretion of ANG II, an index of the kidney’s ability to metabolize ANG II (22), 2) there is no difference in PT reabsorption between wild-type mice and those that lack brush-border-associated ACE (21), indicating that intrarenal PT ACE activity does not significantly affect PT reabsorption, and 3) acute ACE inhibition has no significant effects on intraluminal concentration of ANG II in the PT (8).

Our findings shed light on the mechanisms of captopril-mediated inhibition of PT reabsorption. The captopril-mediated changes in the PT Na⁺ transporters appear to be fairly specific for NHE3: there is remarkable redistribution of nearly all of the NHE3 to the base of the microvilli (Figs. 3A and 4A), analogous to what we observed during acute hypertension (58),
yet little if any retraction of NaPi2 (Fig. 4C), another PT apical Na+ transporter that is conspicuously internalized to endosomes during acute hypertension or PTH treatment (31, 58). Furthermore, there was no apparent redistribution of the basolaterally located α3-subunit of the Na+-K+-ATPase (Fig. 3E).

Related to mechanisms of transport inhibition, our subcellular fractionation results demonstrate that captopril provokes the coordinate redistribution of proteins that are known or postulated to regulate NHE3 activity through protein-protein interactions (Figs. 3 and 6) including: NHERF-1 (28, 51), ezrin (28, 51), megalin (4, 6), DPPIV (17, 18), and clathrin (12). Interestingly, four of these candidate proteins were independently identified by our proteomic analysis of abundant proteins that moved out of low-density apical membranes (fraction 4) during captopril (Fig. 5 and Table 1). NHERF-1 is one of several related PDZ-containing proteins expressed in the PT brush-border membrane (BBM) that appear to play an essential role in cAMP-mediated inhibition of NHE3 activity (51, 55). NHERF-1, in turn, links NHE3 in the PT to the actin cytoskeleton through ezrin (51). Megalin has also been shown to interact with a population of NHE3 in rabbit renal BBM primarily in the coated-pit microdomain of the brush border where evidence suggests NHE3 is inactive (4). In contrast to megalin, DPPIV, a major PT BBM proteins and serine protease (23), is distributed mostly in the microvillar domain where NHE3 is active (17). Recent studies in cultured kidney cells (OK cells) provide evidence that the protease activity of DPPIV can actually activate NHE3 transport activity (18), that is, that DPPIV interaction can potentially impact NHE3 activity in the microvillar domain. Although we observed captopril-mediated redistribution of a small fraction of DPPIV to high-density membranes along with NHE3, there was no discernible change in the subcellular distribution of DPPIV by confocal microscopy (data not shown), indicating that NHE3 moved out of the DPPIV-enriched microvillar domain. In summary, these results are consistent with a model in which captopril activates a chain of events in which NHE3 leaves the DPPIV-enriched domain and moves to the megalin-enriched domain and associates with NHERF-1, changes consistent with a decrease in NHE3 transporter activity. The actual redistribution in the plane of the microvilli may be effected by a tethering to the cytoskeleton mediated by NHERF-1 and ezrin.

ACE, the target binding site for captopril, is also expressed in the PT microvilli (16), yet, ACE displayed no significant change in subcellular distribution in response to acute captopril treatment. This suggests that local ACE trafficking is not requisite for captopril to exert its effects on PT protein redistribution. ACE inhibitors have recently been shown to induce phosphorylation of ACE and activate a distinct ACE signaling cascade in isolated human endothelial cells (27). Whether this occurs in the PT remains to be determined.

Association between myosin VI and NHE3 is likely relevant to the active translocation of NHE3 to the base of the microvilli. This unconventional myosin moves toward the minus ends of actin filaments that are located at the base of the microvilli in the PT (5). In the present study, our confocal data also demonstrated that both NHE3 (Fig. 4A) and myosin VI (Fig. 4B) moved away from the top of the microvilli in response to captopril treatment. Furthermore, we recently demonstrated coincident retraction of membrane-associated myosin VI and NHE3 from the top to the base of the microvilli in response to acute hypertension, thus, providing evidence that myosin VI may participate in driving the rapid trafficking of Na+/H+ exchangers within the apical microvillar domain of the PT (59).

The physiological importance of ANG II and its complex signaling pathways in the kidney (39) has generated significant interest to study the genes and proteins that are regulated by ANG II. DNA microarray studies in human PT cells (7) demonstrate that ANG II elicits a significant induction of many transcription factors, hormones, and antioxidant genes as well as a 2.5-fold increase in NHE3, underscoring the importance of NHE3 as a target of ANG II in the PT. In contrast, the present study was designed to investigate the acute nongenomic effects of depression of systemic ANG II levels on the subcellular distribution of NHE3 and other related apical proteins. One of our goals was to identify key apical proteins in the PT that, in response to captopril treatment, display subcellular redistribution that may contribute to the decrease in PT reabsorption. We implemented a targeted proteomic strategy that combined subcellular fractionation and one-dimensional (1-D) SDS-PAGE to identify abundant apical membrane-associated proteins that demonstrate dynamic captopril-mediated movement between different membrane domains. The absence of NHE3 in our proteomic analysis likely indicates that NHE3 is not an “abundant” protein in the cortex. In addition, the analysis by 1-D SDS-PAGE provided efficient solubilization of membrane proteins that is not always seen in 2-D gel electrophoresis. Recently, Cutillas and co-workers (13) characterized the membrane proteome of BBM vesicles isolated from the rat renal cortex and, among the 251 BBM proteins identified were megalin, DPPIV, aminopeptidases, and V-ATPase subunits, analogous to the protein profile identified in our scheme. Our current identification of proteins that were removed from low-density membranes after acute captopril treatment identified eight abundant proteins (Table 1). All of these have previously been shown to be expressed in the PT [megalin (24), myosin II-A (2), clathrin (45), aminopeptidase N (49), DPPIV (23), ezrin and moesin (48), vacuolar H+-ATPase β2 (40)] and a subset (megalin, DPPIV, and ezrin) has been linked to the regulation of NHE3 activity (4, 18, 51). The redistribution of clathrin out of low-density membranes may indicate a general increase in clathrin-mediated endocytosis in response to captopril treatment; however, there was no discernable NHE3 or NaPi2 endocytosis. Vacular H+-ATPases, which mediate vectorial transport of protons across membranes (52), can also mediate PT bicarbonate reabsorption (54); thus, the redistribution of a pool of the β2 subunit of the H+-ATPase out of the apical membrane may contribute to the decrease in PT fluid reabsorption during acute captopril treatment. Aminopeptidase N (APN), a metallohydrolase expressed in the brush border of PT cells (49), can metabolize ANG III, which has similar physiological effects as ANG II to ANG IV (1). It is logical to postulate that the redistribution of APN out of the apical membranes, or its inhibition, may reflect a compensatory response to reduce ANG III degradation rate in response to reduced ANG II formation during captopril treatment. Related to this point, angiotensin receptor blocker valsartan reduces membrane APN activity in the renal cortex (43). Myosin II-A is a conventional class II nonmuscle myosin heavy chain encoded by the Myh9 gene (47). MYH9-related diseases are characterized by deafness, cataract, and nephropathy (14). In
adult human kidney, myosin II-A is expressed primarily in the podocytes where it is a component of the actin-myosin contractile apparatus (2) but is also expressed in the PT brush border (2), where its function is undefined. We postulate that the redistribution of cytoskeletal-associated myosin II-A during captopril may facilitate transporter trafficking along the microvilli. In summary, our current proteomic analysis identified proteins that redistributed with NHE3 during captopril treatment that were heretofore not postulated to play a role in the diuretic action of this ACE inhibitor including clathrin, moesin, vacular H^+–ATPase, and APN. Future analyses are required to understand how these candidates interact to regulate PT reabsorption and contribute to normalization of blood pressure. In the future analyses of direct protein–protein interactions, these proteins will be examined as candidates to establish whether there are functional interactions with the transporter machinery.

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