Acute hypertension provokes acute trafficking of distal tubule Na-Cl cotransporter (NCC) to subapical cytoplasmic vesicles

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Lee DH, Riquier AD, Yang LE, Leong PK, Maunsbach AB, McDonough AA. Acute hypertension provokes acute trafficking of distal tubule Na-Cl cotransporter (NCC) to subapical cytoplasmic vesicles. Am J Physiol Renal Physiol 296: F810–F818, 2009. When blood pressure (BP) is elevated above baseline, a pressure natriuresis-diuresis response ensues, critical to volume and BP homeostasis. Distal convoluted tubule Na+.Cl− cotransporter (NCC) is regulated by trafficking between the apical plasma membrane (APM) and subapical cytoplasmic vesicles (SCV). We aimed to determine whether NCC trafficking contributes to pressure diuresis by decreasing APM NCC or compensates for increased volume flow to the DCT by increasing APM NCC. BP was raised 50 mmHg (high BP) in rats by arterial constriction for 5 or 20–30 min, provoking a 10-fold diuresis at both times. Kidneys were excised, and NCC subcellular distribution was analyzed by 1) sorbitol density gradient fractionation and immunoblotting and 2) immunoelectron microscopy (immuno-EM). NCC distribution did not change after 5-min high BP. After 20–30 min of high BP, 20% of NCC redistributed from low-density, APM-enriched fractions to higher density, endosome-enriched fractions, and, by quantitative immuno-EM, pool size of APM NCC decreased 14% and SCV pool size increased. Because of the time lag of the response, we tested the hypothesis that internalization of NCC was secondary to the decrease in ANG II that accompanies high BP. Clamping ANG II at a nonpressor level by confinement of captoril (12 μg/min) and ANG II (20 ng·kg−1·min−1) during 30-min high BP reduced diuresis to eightfold and prevented redistribution of NCC from APM- to SCV-enriched fractions. We conclude that DCT NCC may participate in pressure natriuresis-diuresis by retraction out of apical plasma membranes and that the retraction is, at least in part, driven by the fall in ANG II that accompanies acute hypertension.

hypothesis; diuresis; NCC; angiotensin II; immuno-EM

THE PRESSURE-NATRIURESIS RESPONSE is central to blood pressure homeostasis. An acute increase in arterial pressure provokes a near-instantaneous increase in urinary salt and volume excretion that plays a role in returning arterial pressure to the set point level (7). Since renal blood flow (RBF) and glomerular filtration rate (GFR) are autoregulated (up to a point) during acute hypertension, the natriuresis and diuresis are attributed to a decrease in tubular salt and volume reabsorption (6). Chou and Marsh (3, 4) provided early micropuncture evidence that end proximal flow rate increased 50% during hypertension. This laboratory investigated molecular mechanisms that could account for the response and demonstrated that the increase in flow out of the proximal tubule, confirmed by lithium clearance measurements, is accompanied by a redistribution of the sodium hydrogen exchanger isoform 3 (NHE3) and the sodium phosphate transporter (NaPi2) to the base of the microvilli and to subapical endosomes, respectively, as well as inhibition of sodium pump activity (22, 24, 26, 27). Chou and Marsh also determined that downstream the fluid flow rate to the distal nephron was increased by only 11% and Cl− load by just 24% in free-flowing nephrons, significantly less than the 50% increase in flow and Cl− leaving the proximal tubule. They hypothesized that this was evidence for a flow-dependent increase in fluid reabsorption in the loop of Henle during hypertension (3). The study of Magyar et al. (13) provided molecular evidence for flow-dependent activation of loop of Henle sodium transport by demonstrating that medullary sodium pump activity increased significantly during acute hypertension and that activity also increased in response to simply increasing flow to the loop without increasing arterial pressure.

The effect of acute hypertension (high blood pressure) on regulation of sodium and volume reabsorption in the distal nephron has only recently begun to be investigated. Recently, Zhao and Navar (30) studied the role of the distal nephron in pressure natriuresis in mice infused acutely with ANG II to raise arterial pressure. The natriuretic-diuretic response (UNaV) was examined both before and after blockade of distal tubule sodium transport with bendroflumethiazide, an inhibitor of the Na+–Cl−cotransporter (NCC) in the distal convoluted tubule (DCT), and amiloride, an inhibitor of the epithelial sodium channel (ENaC) in the collecting duct. These investigators observed an inverse relationship: increased arterial pressure was associated with a decreased fractional reabsorption of distal Na+ reabsorption, supporting the hypothesis that pressure natriuresis is effected in part by inhibition of distal nephron sodium reabsorption. However, their design was complicated by the fact that hypothesis was induced by ANG II, a hormone known to stimulate distal nephron ENaC (17, 19) and known to blunt pressure natriuresis (11). DCT NCC is responsible for the reabsorption of 5–10% of filtered Na+ and Cl−. NCC regulation is critical to blood pressure control as mutations in NCC or its regulators, the WNK kinases, are known to alter baseline blood pressure. We recently provided evidence for acute regulation of surface expression of NCC in vivo by trafficking between the apical plasma membrane (APM) and subapical cytoplasmic vesicles

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(SCV). Specifically, a high-salt diet provokes a chronic shift in NCC distribution from the APM to SCV (18) and angiotensin-converting enzyme inhibitors (ACEI) acutely do the same thing, while ANG II after ACEI acutely shifts NCC back to the APM. These responses reveal important molecular mechanisms of action of high-salt diets to raise arterial pressure and of ACEI to lower pressure (18, 19).

The purpose of the present study was to determine whether and how DCT NCC distribution between the APM and SCV is affected during acute hypertension. There were two plausible outcomes: 1) NCC activity could be inhibited by redistribution from the APM to SCV to further contribute to pressure natriuresis, analogous to the inhibition of the type 3 Na+/H+ exchanger (NHE3) and type 2 Na-P, cotransporter (NaPi2) in the proximal tubule; or 2) NCC could be activated by redistribution from SCV to the APM to further compensate for the increased volume flow, analogous to the flow-dependent activation of loop of Henle sodium transport. Our results, from both subcellular fractionation and immunoelectron microscopy (immuno-EM) analysis, demonstrate that outcome 1 is correct, that acute hypertension provokes a retraction of NCC from the APM to SCV. We went on to determine that this NCC redistribution is likely secondary to the fall in ANG II that accompanies acute hypertension.

MATERIALS AND METHODS

Animals. All studies were performed on male Sprague-Dawley rats (body wt 300–350 g) that were kept under diurnal light conditions and had free access to food and water. All animal experiments were approved by the University of Southern California Keck School of Medicine Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental protocols. The effect of hypertension on distal tubular sodium chloride cotransporter trafficking was investigated using sample sets collected for previous or concurrent studies on the effect of hypertension on proximal tubule sodium transporters, as detailed below. In all protocols, density gradient samples were stored frozen at −80°C without repeated freeze-thaw until they were assayed for this study. Distribution of proximal tubule transporter was reassayed to verify that the responses observed initially were unaltered by freezing (discussed in RESULTS). Fixed tissue blocks for immuno-EM were stored in liquid nitrogen without freeze-thaw.

Series 1: 5-min vs. 20- to 30-min acute hypertension. In this first series of rats, described in detail previously and initially reported by Yang et al. (22), rats were anesthetized intramuscularly with ketamine (100 mg/kg) and sham operated, or blood pressure was elevated for 15 min as described for series 1 (n = 5 pairs). Fifteen-minute acute hypertension provoked a significant redistribution of NCC to higher density fractions analogous to that seen in Fig. 1B (not shown). Kidneys were cooled in situ by flushing with cold PBS and then excised for homogenization and fractionation on OptiPrep flotation gradients as described below.

Fig. 1. Effects of acutely raising blood pressure (↑ BP) on density distribution of NaCl cotransporter (NCC). A: density distribution of NCC in control (●) vs. 5-min ↑ BP (○). B: density distribution of NCC in control (●) vs. 30-min ↑ BP (○). Relative abundance, determined by immunoblot of a constant volume of each fraction, is shown and expressed as percentage of total signal in all 12 fractions; n = 5 in control and 30-min groups; n = 3 in 5-min group. Values are means ± SE. *P < 0.05 compared with corresponding control, unpaired Student’s t-test. C: summary of difference in the density distribution of renal cortical NHE3 between control and 30-min high BP. D: typical immunoblots of constant volume of fractions 2–12.
Series 3: ANG II clamp during acute hypertension. In the third series, as reported previously (10), rats were anesthetized and surgically cannulated as described for series 1. Urine output, GFR, and endogenous Cl− measurements made were as described (10). Three groups of rats were studied: 1) control, sham-operated and infused; 2) acute hypertension as described for series 1 (for 20 min); and 3) ANG II clamp followed by acute hypertension (20 min). The ANG II level was clamped by inhibiting de novo ANG II synthesis with the ACEI captopril, followed by infusion of ANG II at a rate that restored baseline blood pressure. Specifically, captopril was infused intravenously at 12 μg/min for 10 min before the infusion of ANG II (12 μg/min) and ANG II (20 ng·kg⁻¹·min⁻¹; both from Sigma) for another 20–30 min to verify basal blood pressure, followed by raising of blood pressure for 20 min. In this study, n = 3 in each group. One rat from each group was analyzed on the same day, and samples were processed and assayed in parallel.

Homogenization and subcellular fractionation on sorbitol gradients. The procedure for subcellular fractionation of the renal cortex membranes has been described in detail previously (22) and was identical in all three series. In brief, renal cortices were rapidly dissected and homogenized in isolation buffer (5% sorbitol, 0.5 mM aprotinin, and 5 mM histidine-imidazole buffer, pH 7.5) and centrifuged at 2,000 × g for 10 min. The pellet was homogenized and centrifuged again, and the low-speed supernatants (S₁₀₀) were pooled, loaded at the interface between two hyperbolic sorbitol gradients (ranging between 35 and 70% sorbitol), and centrifuged in a swinging bucket rotor (100,000 × g for 5 h). Twelve fractions were collected from the top, diluted with isolation buffer, pelleted by centrifugation (250,000 × g for 1.5 h), resuspended in 1 ml of isolation buffer, and stored in aliquots at −80°C, pending assays.

Isolation of lipid raft fractions by flotation gradient. S₁₀₀ membranes were isolated as described above and then centrifuged at 200,000 × g for 75 min and the pellets resuspended in TNE buffer [50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and Complete Protease Inhibitor Cocktail (Roche Diagnostics)] through a 25-G needle on ice. Lipid raft fractions were isolated using a modification of methods previously reported (8, 12). Total membranes were incubated in TNE buffer containing 1% Triton X-100 on ice for 30 min with occasional vortexing. Proteins in ordered lipid domains are resistant to detergent extraction and are more buoyant than proteins in nonordered domains that are extracted by the detergent. These populations can be separated on density gradients. The extract was adjusted to 50% OptiPrep (Sigma). A volume of 600 μl was loaded at the bottom of an ultracentrifuge tube and overlayed with 1 ml each of 40, 30, and 20% and 400 μl of 10% OptiPrep in TNE. Following centrifugation at 170,000 × g at 4°C for 4 h, a continuous density gradient was formed. Six 250-μl fractions were collected from the top followed by collection of five 500-μl fractions.

Immunoblot analysis and antibodies. To assess the density distribution and flotation gradient patterns of NCC, a constant volume of sample from each fraction was assayed. Samples were denatured in SDS-PAGE sample buffer for 20 min at 60°C, resolved on 7.5% SDS-polyacrylamide gels according to Laemmli (9), and transferred to polyvinylidene difluoride membranes (Millipore Immobilon-P). One-half of the volume of a couple of the samples was assayed in parallel to verify linearity of the detection system. Blots were probed with polyclonal antiserum against NCC (TSβcα, D. Ellison, Oregon Health & Science University, Portland, OR) at a 1:1,000 dilution, incubated with Alexa 680-labeled goat anti-rabbit serum (Molecular Probes), and detected and quantitated with the Odyssey Infrared Imaging System (Li-COR, Lincoln, NB) and accompanying Li-COR software.

Density gradient data analysis. As described previously (18, 19) in the analysis of density gradient distributions, two-way ANOVA was applied to determine whether there was a significant effect of treatment on the overall density distribution pattern. After significance was established, the location of the difference in the pattern was assessed by an unpaired two-tailed Student’s t-test assuming equal variance, with Bonferroni adjustments for multiple comparisons. Density gradient results are expressed as means ± SE. Differences were regarded significant at P < 0.05.

Immuno-EM. To analyze NCC subcellular distribution at the ultrastructural level, kidneys from five animals each from control and 20-min acute hypertension regimes were analyzed by immuno-EM. As described previously (18, 19, 24), kidneys were either perfusion-fixed or fixed in situ by superfusion with 4% paraformaldehyde in 0.1 M Na cacodylate buffer, pH 7.2. The immunolabeling pattern was the same with either fixation method. Tissues were postfixed for 2 h, infiltrated with 2.3 M sucrose, and frozen in liquid nitrogen. Ultrathin (70-nm) cryosection or Lowicryl sections were cut on a Reichert Ultratrac S cryoultramicrotome (Leica), blocked (in PBS, 0.05 M glycine and 0.1% skim milk), incubated overnight at 4°C with polyclonal antibody to NCC (TSCα, D. Ellison), diluted at 1:1,600 dilution. As described and demonstrated previously (18, 19), immunolabeling controls were performed with nonimmune rabbit IgG or without primary antibody. NCC were visualized using goat anti-rabbit IgG conjugated to 10-nm colloidal gold particles (GAR EM10, BioCell Research Laboratories, Cardiff, UK) diluted 1:50 in PBS with 0.1% skim milk powder and polyethylene glycol (5 mg/ml), and the cryosections were stained for 10 min in 0.3% uranyl acetate in 1.8% methyl-cellulose. Cryosections were examined with a FEI Morgagni electron microscope and recorded for quantitation at ×16,000 on Kodak 163 film and printed at ×50,000.

Immuno-EM data analysis. Quantitation of immunoglobulin labeling was performed on electron micrographs of DCT cells from five animals in each group as previously described (18, 19). The following a priori criteria were applied to the selection of cells for quantitation. Cryosections were viewed at low magnification first, and then micrographs were recorded at high magnification in the EM of all DCT cells in the section that, at low magnification, appeared to have correct orientation. All recorded micrographs were then analyzed provided they 1) were devoid of sectioning defects (folds, scratches, compression); and 2) were judged to be oriented approximately at right angle to the apical cell surface. From each rat, four to nine DCT cells were analyzed that satisfied these criteria. Gold particles were referred to the apical cell membrane when located either on its outer surface, directly above, or within 30 nm from its inner surface. Gold particles over the apical cytoplasm were quantitated below the analyzed stretch of apical cell membrane down to a depth of 2 μm as described previously (19); zone 1 extended from 30 nm to 1 μm below the plasma membrane, and zone 2 from 1 to 2 μm into the cytoplasm. The percentage of particles associated with the cell membrane and in the two cytoplasmic zones was determined as fractions of all counted particles in that cell. The mean percentage was then calculated for each animal with a specific treatment, and the means for control and acute hypertension groups were compared by a two-tailed Student’s t-test for unpaired samples. Counts were corrected for background labeling as described previously (19).

RESULTS

Effect of Acute Hypertension on Subcellular Distribution of Renal NCC

As previously reported (22), raising blood pressure by 50–60 mmHg for as little as 5 min provoked a rapid and sustained diuretic response to acute hypertension. Specifically, hypertension increased Cl−, a measure of flow out of the proximal tubule, sixfold and increased urine output ninefold. These responses were sustained for at least 30 min (Table 1).

We previously reported, using the series 1 sample set and others, that during acute hypertension proximal tubule NHE3 and NaPi2 rapidly redistribute from the body to the base of the
microvilli or subapical endosomes, respectively. The responses have been demonstrated by both direct immuno-EM (24) as well as redistribution of the transporters on sorbitol density gradients. Redistribution of villin, associated with apical membrane, is not affected by acute hypertension. The immuno-EM determination that APM NCC decreases by ~20% of the total NCC redistributes to higher density membranes. Specifically, at normal blood pressure, 54.3 ± 2.1% of NCC is localized to fractions 1–5 and 45.7 ± 2.1% to fractions 6–12, and when arterial pressure is raised for 30 min the percentage in fractions 1–5 falls to 33.9 ± 4.3% (P < 0.005) and the percentage in fractions 6–12 increases to 66.1 ± 4.3% (P < 0.005). Figure 1D provides typical immunoblots from a set of three membrane fractionations prepared from animals studied on the same day and immunoblotted on the same day. Figure 2A illustrates that proximal tubule NHE3 redistributes to higher density membranes at both 5 and 30 min (in a sample set included in the analysis and assayed at the same time as NCC), and Fig. 2B illustrates that the apical microvillar bundling protein villin does not redistribute during hypertension at either time point. We conclude that ~20% of NCC redistributes out of low-density putative apical-enriched membranes during hypertension after a lag time of longer than 5 min, which is subsequent to the onset of the pressure diuresis and redistribution of NHE3 which are evident at 5 min.

Effect of 20-Minute Acute Hypertension on NCC Distribution in DCT Cells

Immuo-EM was used to determine the subcellular localization of NCC in DCT cells of control rats and those subjected to 20-min acute hypertension. As reported previously, 1) NCC-containing subapical vesicles were 0.01–0.05 μm in diameter, and the frequency of labeled vesicles decreased with increasing distance from the apical plasma membrane; 2) there was no specific label observed in the basal cytoplasm and adjacent cells, including those of the proximal tubule, cortical collecting tubules, and intercalated cells; and 3) an overall labeling pattern was observed in both DCT I and DCT II (18, 19). In this study, the overall fine structure of the APM and basolateral plasma membrane as well as the microvilli was the same in control and 20-min hypertension groups. In both groups, NCC was associated with the APM as well as a population of vesicles located in the apical regions of the cytoplasm, but the relative subcellular distributions were characteristic for each group. In both groups, NCC labeling was observed both over the small apical microvilli and between the microvilli (Fig. 3, A and B). Quantitation of the relative abundance of NCC at the apical membrane, in the apical zone down to 1 μm below the membrane and the zone between 1 and 2 μm below the membrane (Z2) is summarized in Fig. 4. In controls, 61.8 ± 7.7% of the NCC gold labeling was associated with the APM. Acute hypertension reduced NCC in APM to 47.3 ± 4.6% (P < 0.01) and caused a coordinate increase in labeling of cytoplasmic vesicles, which was significant in Z2 (P = 0.014). The immuno-EM determination that APM NCC decreases by 25% when blood pressure is raised for 20 min agrees with the subcellular fractionation finding that about 20% of the total NCC re-distributes from low density apical membrane en-

Table 1. Physiological measurements

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<th>5-min Basal</th>
<th>5-min High BP</th>
<th>30-min Basal</th>
<th>30-min High BP</th>
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<td><strong>Mean arterial</strong></td>
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<td></td>
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<td>164 ± 11*</td>
<td>98 ± 13</td>
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<td>72 ± 60*</td>
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Values are means ± SD. BP, blood pressure. Data are summarized from Ref. 22. *P < 0.05 high BP vs. basal value for same time period.
riched fractions to higher density endosomal enriched fractions.

Membrane Domain Properties of NCC During Acute Hypertension

Because there was such a marked change in density gradient distribution of NCC with acute hypertension, as well as a significant redistribution from APM to SCV by immuno-EM, we tested the hypothesis that NCC redistributed to membranes with distinct domain properties. To determine whether NCC is localized to ordered lipid domains such as rafts, to nonraft domains, or both to determine whether there is a shift between domains as NCC redistributes out of the apical membrane during hypertension, renal cortex homogenates from animals at baseline blood pressure and those with acutely elevated blood pressure were subjected to cold Triton X-100 extraction followed by fractionation on OptiPrep gradients. Proteins that remain in ordered lipid domains after detergent extraction are more buoyant than those that are extracted by the detergent. For improved resolution of the detergent-resistant lipid raft domains at the top of the 4-ml gradients, six 250-μl fractions were collected followed by five 500-μl fractions. As illustrated in Fig. 5A, the classic lipid raft marker flotillin was distributed in the top 1.5 ml and the distribution was unaffected by raising the percentage decreases significantly to 47% and increases correspondingly in the subapical cytoplasmic vesicles in Z1 and Z2.

Fig. 4. Effect of 20-min hypertension on subcellular distribution of NCC in DCT cells analyzed by immunoelectron microscopy. Colloidal gold particles, representing NCC antibodies, were counted along the apical cell membrane, in the apical zone down to 1 μm below the membrane (Z1), and the zone between 1 and 2 μm below the membrane (Z2). Counts were corrected for background labeling, which was very low (~1 particle/μm²). Results were collected from 5 rats/group. Within each rat, 4–9 cells were analyzed and means were calculated. Results are expressed as means ± SD. The total grains in apical membrane + Z1 + Z2 is defined as 100%. *P < 0.05 compared with corresponding control, unpaired Student’s t-test. In controls, 61% of the NCC is associated with the apical membranes, while after 20-min hypertension the percentage decreases significantly to 47% and increases correspondingly in the subapical cytoplasmic vesicles in Z1 and Z2.

Fig. 3. Immunoelectron microscope localization of NCC in distal convoluted tubule (DCT) cells. NCC was detected with polyclonal anti-NCC antibody followed by goat anti-rabbit IgG conjugated to 10-nm colloidal gold particles. A: typical control DCT cell. B: typical DCT cell from a rat subjected to acute hypertension for 20 min. In both conditions, NCC is associated with the apical membrane (arrows) as well as subapical cytoplasmic vesicles (arrowheads). However, the relative proportions are different, as quantified in Fig. 4. M, mitochondria.
the blood pressure. The transferrin receptor (TfR), a classic nonraft marker, distributed primarily in the bottom 2 ml of the gradient (Fig. 5B), although there was a lesser peak at 1.5 ml that coincided with the major protein peak, indicating that there may be some nonraft membranes in that region of the gradient. More than 85% of the NCC distributed in the top 1.5 ml of the gradient overlapping with the lipid raft marker. There is scant NCC evident above 1.5 ml, where most of the TfR was fractionated. Most of NCC is localized to a peak at 1.25–1.5 ml that contains both flotillin and some TfR. We cannot conclude that all the NCC localized to ordered lipid domains, but since ~40% of the NCC is located in cytoplasmic vesicles (Fig. 4), we can infer that NCC is localized to buoyant membranes with similar domain properties in both the apical membranes and SCV at basal blood pressure. Thus it is not surprising that NCC distribution on the flotation gradient was unaltered by acute hypertension, confirming that the lipid environment of NCC is similar in both APM and SCV.

Effect of Clamping ANG II Levels on Redistribution of NCC During Acute Hypertension

The time course of NCC redistribution during hypertension suggested the hypothesis that trafficking from apical to sub-apical membranes was secondary to the inhibition of the renin-angiotensin system (RAS) that is believed to occur during acute hypertension (16, 20). This hypothesis is also consistent with our previous studies demonstrating that 1) preventing the drop in ANG II during acute hypertension (systemic ANG II clamp) blunts the pressure diuretic response (11); 2) ANG II clamp blunts the redistribution of proximal tubule NHE3 to higher density membranes during acute hypertension (10); 4) acute RAS inhibition leads to a retraction of NCC to cytoplasmic vesicles (19); and 4) recovery from pressure natriuresis takes about 20 min (27), suggesting the response involves more than physical factors per se. To address the hypothesis, we examined a sample set in which ANG II was clamped by coinfusing captopril (12–20 ng·kg⁻¹·min⁻¹) for 20–30 min to verify basal blood pressure followed by raising blood pressure for 20 min (10). One of these animals was studied on the same day with an animal subjected to acute hypertension without ANG II clamp and a third sham-operated animal. As summarized in Table 2, the ANG II clamp did not alter basal blood pressure, GFR, Clᵢ, or urine output but did blunt the increase in Clᵢ and urine output in response to raising blood pressure. To test the hypothesis that ANG II clamp would blunt the redistribution of NCC observed in response to 30-min acute hypertension, samples from each of the three rats (control, high blood pressure, ANG II clamp + high blood pressure) were fractionated and assayed on the same gel and immunoblotted. Since we were limited to 26-well gels, we assayed fractions 3–10 from each rat which contained about 95% of the total NCC, well suited to test the hypothesis. Figure 6A, comparing NCC distribution in the

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Values are means ± SD. Data are summarized from Ref. 10. *P < 0.05 high BP, vs. basal or ANG II clamp value.
and that chronic decreases in APM NCC are provoked by a high-salt diet or treatment of spontaneously hypertensive rats (SHR) with the ACEI enalapril (18, 19, 23, 25). We postulated that the DCT NCC would respond to acute arterial hypertension, which is accompanied by an increased volume flow to the DCT, by either 1) redistribution out of the plasma membrane (APM) into the cell (SCV) to further contribute to pressure natriuresis, analogous to the inhibition of NHE3 and NaPi2 in the proximal tubule during hypertension (24); or 2) redistribution from stores in the cell (SCV) to the plasma membrane (APM) to further compensate for the increased volume flow, analogous to the flow dependent activation of loop of Henle sodium transport during hypertension (13). Our results indicate that 20–25% of NCC eventually redistributes out of the plasma membrane into the cell when blood pressure is raised, decreasing the pool of APM NCC, consistent with the hypothesis that NaCl reabsorption by the DCT is depressed during acute hypertension. However, we found that the NCC redistribution lags behind the rapid natriuresis-diuresis, behind the increase volume flow out of the proximal tubule measured by $C_{Li}^+$, and behind the rapid trafficking of proximal NHE3 measured in the same samples. This finding leads us to conclude that the redistribution is not critical for the initial rapid natriuresis-diuresis but may be important to sustaining the response. The redistribution of NCC out of the APM did not occur when ANG II levels were clamped during acute hypertension, leading us to conclude that the redistribution is secondary to the fall in ANG II that is predicted to occur when blood pressure is raised (11).

Subcellular fractionation is a useful strategy for analyzing renal apical sodium transporter trafficking because these transporters have a very well-defined and limited distribution along the nephron and because specific antibodies can be used to define their density distributions within the mix of all the other membranes in the kidney. In this study, we observed that acute hypertension provoked a significant change in the physical property of the environment of NCC from lower to higher density membranes. We have previously examined the distribution of many classic subcellular membrane markers in the same sorbitol gradients and found that, generally, renal plasma membrane markers were found at low densities and renal endocytic and lysosomal markers at higher densities, albeit with significant overlap between marker distributions (14, 27, 28).

Immuno-EM has been used before (18, 19) and in this study to establish the source and destination relevant to the NCC redistribution response. Hypertension for 20–30 min reduced the APM pool of NCC ~25%, assessed by immuno-EM (Figs. 3 and 4), and provoked a redistribution of ~20% of the total NCC out of low-density APM-enriched membranes to higher density SCV-enriched membranes, assessed by density gradient centrifugation (Figs. 1 and 6). The agreement between the magnitude of redistribution assessed by immuno-EM vs. density gradient fractionation confirms once again that membrane fractionation can be used to analyze NCC redistribution and that APM containing NCC have a lower density than SCV containing NCC (18, 19). The magnitude of the redistribution is very similar to that observed when rats are infused for 20 min with the ACEI captopril, a response that is reversed when rats are infused with ANG II (19).
Given the evidence for discrete populations of NCC in APM and in SCV with distinct densities, and the evidence for redistribution between the two populations when blood pressure was increased, we aimed to determine the membrane domain properties of NCC at baseline blood pressure and in acute hypertension-treated kidneys by detergent extraction of total renal cortical membranes with Triton X-100 followed by fractionation on Optiprep flotation gradients, which separates the proteins in buoyant detergent-resistant membranes from those extracted into the soluble phase (2, 8, 15). The results indicated that NCC is localized to ordered lipid domains that are resistant to Triton extraction and thus float in the lighter fractions of the Optiprep gradient, overlapping with the classic lipid raft marker flotillin. The flotation gradient profile of membranes from rats subjected to acute hypertension was essentially identical to that of total membranes from rats at baseline blood pressure, indicating that there is not likely to be a redistribution from ordered to nonordered lipid domains when the NCC redistributed out of the APM to the SCV inside the cell during acute hypertension. We can only speculate that expression of NCC in ordered lipid domains may be important for reversible redistribution between the plasma membrane and intracellular pools. The results suggest that isolation of ordered lipid domains after Triton extraction and density gradient analysis would be a logical strategy to coenrich for proteins that associate with NCC, since they would presumably be localized to the same domain. We have recently examined the domain distribution properties of proximal tubule apical Na+ transporters NHE3 and NaPi2 (17a) using the same strategy. NHE3 distribution overlaps with flotillin, like NCC, while the distribution of NaPi2 overlaps with that of the nonraft marker TFR. These results indicate that not all the renal apical sodium transporters are expressed in ordered lipid domains.

Chou and Marsh (3) were the first to rigorously assess volume and Cl− reabsorption in the proximal portion of the nephron during acute hypertension. Their study demonstrated that reabsorption of both volume and Cl− were inhibited 40% in the proximal tubule, yet volume flow to the early distal tubule was increased by only 10%, suggesting increased reabsorption in the loop of Henle en route to the distal tubule. In this laboratory, we provided molecular evidence for increased reabsorption in the loop of Henle: medullary Na-K-ATPase activity increased 40% during acute hypertension and also during an analogous increase in volume flow from the proximal tubule provoked by a carbonic anhydrase inhibitor (13), implicating the volume flow rather than increased arterial pressure per se. The Cl− delivery to the early distal nephron increased 25% during acute hypertension in free-flow collections in the Chou and Marsh study (3). Our assays do not include a measure of specific changes in NaCl transport in the DCT during hypertension, but we postulate that if the pool size of apical NCC decreases in the face of increased flow, there will be less fractional NaCl reabsorption mediated by NCC.

Very recently, Zhao and Navar (30) also addressed the role of the distal nephron in the pressure-natriuresis response using a different approach: arterial pressure was acutely increased in mice with a pressor dose of ANG II which provoked a twofold increase in urinary volume and sodium excretion. The fractional reabsorption of distal sodium delivery (FRDSD) was estimated by comparing the natriuresis seen during ANG II hypertension in the presence and absence of the simultaneous blockade of both NCC with a thiazide diuretic and ENaC with amiloride. A significant inverse relationship between FRDSD and arterial pressure was calculated, implicating a role of the distal nephron in ANG II pressure natriuresis (30). Since both NCC and ENaC were inhibited, it is not possible to dissect out whether one or both of the transporters were specifically inhibited. An important issue with raising the arterial pressure by ANG II infusion, as opposed to arterial constriction, is that ANG II will stimulate sodium transporters, such as NHE3 and NCC, which will likely counteract and/or blunt the natriuresis and diuresis seen with a similar elevation in pressure without ANG II. Specifically, Leong et al. (11) measured the diuretic responses to elevating arterial pressure (50−60 mmHg) with and without infusing a nonpressor dose of ANG II and found that the diuresis was reduced 50%, from fourfold to two fold, in the ANG II-infused group, to the same level as measured in the Zhao and Navar study (30) using ANG II to raise arterial pressure.

Completing a picture of the signals that connect an increase in arterial pressure to a decrease in sodium transport has been challenging, but parts of the picture are becoming clear. An attractive overall hypothesis is that acute hypertension stimulates nonautoregulated preglomerular vasculature to release a mediator(s) that depresses sodium transport in their local vicinity (6). Candidate mediators may include cytochrome P-450 metabolites, nitric oxide, and suppression of the RAS (1, 5, 6, 21, 29).

Leong and colleagues (10, 11) explored the role of suppression of the RAS during pressure natriuresis by clamping ANG II at a subpressor level before raising blood pressure. The ANG II clamp protocol blunted the natriuretic and diuretic responses to hypertension −50%, as evident in Table 2 (taken from one of these studies), yet there was still a significant, albeit blunted, retraction of proximal tubule NHE3 from the microvilli (10). The timing of the redistribution of NCC after raising blood pressure (Fig. 1) and the inhibition of the response when ANG II levels were clamped (Fig. 6) implicate the fall in ANG II, rather than a factor released from the vasculature, as stimulating the redistribution of NCC during arterial hypertension. For systemic ANG II to be an important signaling component of the pressure diuretic response, it is necessary that plasma concentration of ANG II be rapidly responsive. We previously observed (11) that captopril infusion induced an immediate (<3 min) and significant drop in blood pressure, leading us to two conclusions: endogenous ANG II has a short half-life, and baseline physiological levels of ANG II contribute significantly to baseline blood pressure. Our results predict, as discussed above, that the pressure-natriuresis response resulting from infusing pressor doses of ANG II (30) would prevent the redistribution of NCC from the APM to SCV and blunt the natriuretic response to a given increase in pressure. Whether clamping ANG II has an analogous effect on the response of ENaC to arterial hypertension remains to be determined. To answer these questions, it will be necessary to compare the fractional reabsorption of distal sodium delivery, the FRDSD, in the arterial vasoconstriction vs. ANG II models of acute hypertension.

Putting together these recent studies on the pressure-natriuresis response, we propose that the initial and rapid natriuretic response is driven by rapid generation and action of 20-HETE in the proximal tubule and that the repression of the RAS and
fall in ANG II, which lags behind the initial response, are important for sustaining the response by reducing sodium transport in ANG II-sensitive regions along the nephron, including the DCT.

Regarding the clinical significance of these findings, this study demonstrates that the DCT NCC, which is the thiazide diuretic receptor, retracts from the apical membrane during an acute rise in arterial pressure. From this finding, we postulate that the DCT NCC participates in the pressure-natriuresis response, which is critical to overall blood pressure regulation. This study also demonstrates that the redistribution of NCC during hypertension does not occur if plasma ANG II levels are clamped, implicating a fall in ANG II levels in the response. We have recently reported that ACE inhibitors provoke a very similar redistribution of NCC from the apical to subapical membranes (19), revealing a previously unreported mechanism of action of ACE inhibitors. We subsequently demonstrated that when SHR are treated chronically with ACE inhibitors, there is a chronic redistribution of NCC out of apical-enriched membranes, demonstrating a previously unreported therapeutic action of chronic ACEI (23). Overall, this study demonstrates that arterial pressure and the RAS regulate distal tubule action of chronic ACEI (23). Overall, this study demonstrates membranes, demonstrating a previously unreported therapeutic response, which is critical to overall blood pressure regulation. We have recently reported that ACE inhibitors provoke a very important for sustaining the response by reducing sodium pump activity along the nephron during acute hypertension. Am J Physiol Renal Physiol 283: F1197–F1208, 2007.


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REFERENCES