Increased renal renin content in mice lacking the Na\(^{+}\)/H\(^{+}\) exchanger NHE2

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Hanner F, Chambrey R, Bourgeois S, Meer E, Mucsi I, Rosivall L, Shull GE, Lorenz JN, Eladari D, Peti-Peterdi J. Increased renal renin content in mice lacking the Na\(^{+}\)/H\(^{+}\) exchanger NHE2. Am J Physiol Renal Physiol 294: F937–F944, 2008. First published February 20, 2008; doi:10.1152/ajprenal.00591.2007.—Macula densa (MD) cells express the Na\(^{+}\)/H\(^{+}\) exchanger (NHE) isoform NHE2 at the apical membrane, which may play an important role in tubular salt sensing through the regulation of cell volume and intracellular pH. These studies aimed to determine whether NHE2 participates in the MD control of renin synthesis. Renal renin content and activity and elements of the MD signaling pathway were analyzed using wild-type (NHE2\(^{+/+}\)) and NHE2 knockout (NHE2\(^{-/-}\)) mice. Immunofluorescence studies indicated that NHE2\(^{-/-}\) mice lack NHE3 at the MD apical membrane, so the other apical NHE isoform has not compensated for the lack of NHE2. Importantly, the number of renin-expressing cells in the afferent arteriole in NHE2\(^{-/-}\) mice was increased 

Increased renal cortical renin content in mice lacking the Na\(^{+}\)/H\(^{+}\) exchanger NHE2. Am J Physiol Renal Physiol 294: F937–F944, 2008. First published February 20, 2008; doi:10.1152/ajprenal.00591.2007.—The Juxtaglomerular apparatus (JGA) represents a major structural component of the renin-angiotensin system and is one of the most important regulatory sites of renal salt and water conservation and blood pressure maintenance (36, 37). The JGA consists of a tubular component (the macula densa, MD), the extraglomerular mesangium, and a vascular component that includes the terminal part of the afferent arteriole containing the renin-producing juxtaglomerular (JG) cells (2). Two major regulatory functions are performed by the JGA: the high distal tubular NaCl concentration ([NaCl])-induced afferent arteriolar vasoconstriction (tubuloglomerular feedback, TGF) and the low tubular [NaCl]-induced renin release (3, 5, 22, 23, 36–38). MD-mediated renin release signaling include, at least, the low tubular salt-induced activation of p38 and ERK1/2 MAP kinases, cyclooxygenase-2 (COX-2), and microsomal prostaglandin E synthase (mPGES) expression, indicating MD-specific mechanisms responsible for the increased renin content. Significant and chronic activation of ERK1/2 was observed in MD cells of NHE2\(^{-/-}\) kidneys. Removal of salt or addition of NHE inhibitors to cultured mouse MD-derived (MMD1) cells caused a time-dependent activation of ERK1/2. In conclusion, the NHE2 isoform appears to be important in the MD feedback control of renin secretion, and the signaling pathway likely involves MD cell shrinkage and activation of ERK1/2, COX-2, and mPGES, all well-established elements of the MD-PGE2-renin release pathway.

macula densa; cell volume; renin release; mitogen-activated protein kinases; cyclooxygenase-2

THE JUXTAGLOMERULAR APPARATUS (JGA) represents a major structural component of the renin-angiotensin system and is one of the most important regulatory sites of renal salt and water conservation and blood pressure maintenance (36, 37). The JGA consists of a tubular component (the macula densa, MD), the extraglomerular mesangium, and a vascular component that includes the terminal part of the afferent arteriole containing the renin-producing juxtaglomerular (JG) cells (2). Two major regulatory functions are performed by the JGA: the high distal tubular NaCl concentration ([NaCl])-induced afferent arteriolar vasoconstriction (tubuloglomerular feedback, TGF) and the low tubular [NaCl]-induced renin release (3, 5, 22, 23, 36–38). MD-mediated renin release signaling include, at least, the low tubular salt-induced activation of p38 and ERK1/2 MAP kinases, cyclooxygenase-2 (COX-2), and microsomal prostaglandin E synthase (mPGES) expression, indicating MD-specific mechanisms responsible for the increased renin content. Significant and chronic activation of ERK1/2 was observed in MD cells of NHE2\(^{-/-}\) kidneys. Removal of salt or addition of NHE inhibitors to cultured mouse MD-derived (MMD1) cells caused a time-dependent activation of ERK1/2. In conclusion, the NHE2 isoform appears to be important in the MD feedback control of renin secretion, and the signaling pathway likely involves MD cell shrinkage and activation of ERK1/2, COX-2, and mPGES, all well-established elements of the MD-PGE2-renin release pathway.

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TGF responses under control conditions were not different in NHE2-deficient mice compared with wild type (24). The present studies aimed to determine whether NHE2 participates in the MD control of renin synthesis and release.

MATERIALS AND METHODS

Animal breeding and genotyping. The generation and characterization of NHE2−/− mice have been previously described (19, 39). Heterozygotes were obtained from an established colony and bred to produce NHE2 wild-type (NHE2+/+) and null (NHE2−/−) mice. All mice were genotyped at the age of 3 wk by PCR, using DNA from tail biopsies as previously described (19), using the following primers: NHE2-wild-type (wt) forward, 5′-CATCTTATACAAATGTGCCCAATCCTG-3′; NHE2-wt reverse, 5′-GTGACTGCTGTTGAGACAGTGCTG-3′; and NHE2-mutant (mt), 5′-GACAATAGGACGATCGTGCT-3′. Mice were fed standard chow (0.3% NaCl) or salt-deficient diet (113760; Dyets, Bethlehem, PA) for 1 wk. Wild-type and null mice were age matched in all experiments. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Southern California and the University of Paris.

Antibodies. Two different renin antibodies were used. One rabbit anti-mouse renin antibody was a kind gift from Dr. Inagami (Vanderbilt University, Nashville, TN), and the other was purchased from Dr. E.J. Brandt (Technology Transfer Office, St. Louis University, St. Louis, MO). A rabbit polyclonal antibody against mPGES was purchased from Cayman Chemical (Ann Arbor, MI). The goat polyclonal COX-2 and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal GAPDH antibody was purchased from Ambion (Austin, TX). A rabbit polyclonal anti-phosphorylated ERK1/2 (pERK1/2) antibody was purchased from Cell Signaling Technology (Danvers, MA). The NHE3 antibody was purchased from Chemicon (Temecula, CA). These antibodies have been previously characterized (4, 11, 27, 34, 35, 45).

Immunofluorescence labeling of kidney tissue. Mice were anesthetized with 100 mg/kg Inactin, and their kidneys were fixed in situ by perfusion of 4% paraformaldehyde in PBS. Coronal kidney sections were then post-fixed overnight at 4°C in 4% paraformaldehyde and perfusion of 4% paraformaldehyde in PBS. Coronal kidney sections were labeled in parallel.

Sections were rehydrated through graded alcohol-fixed paraffin-embedded tissue, the sections were heated in a microwave for 20 min (boiling) and then allowed to cool in PBS for 40 min. To reduce nonspecific binding, sections were incubated with 5% nonfat dry milk, immunoblotting was performed with a polyclonal antibody to renin (1:250 dilution), a rabbit polyclonal antibody to pERK1/2 antibodies overnight at a dilution of 1:100 for 1 h. Sections were labeled with an Alexa Fluor 594-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR) for 1 h at a dilution of 1:500. After a wash step, sections were stained with Vectashield mounting medium containing the nuclear stain 4,6-diamidino-2-phenylindole (Vector Laboratories) and examined with a Leica TCS SP2 confocal microscope. All sections were labeled in parallel.

Immunofluorescence staining. Kidney tissue was labeled by incubating the plasma at pH 8.5 in the presence of an excess of rat angiotensinogen as previously described (26).

Cell culture. Mouse macula densa-derived (MMDD1) cells were a kind gift of Dr. Jürgen Schnermann (National Institutes of Health, Bethesda, MD). These cells were successfully used before to study the activation of p38 and ERK1/2 MAP kinases, well-established elements of the MD renin release signal, by using various physiological stimuli, including isosmotic reduction of medium Cl− concentrations (45). MMDD1 cells were cultured as described previously (45).

RT-PCR. Total RNA was purified from whole mouse kidney samples or confluent MMDD1 cells by using a Total RNA mini kit in accordance with the manufacturer’s instructions (Bio-Rad, Hercules, CA). RNA was then quantified using spectrophotometry and reverse-transcribed to single-stranded cDNA using avian reverse transcriptase and random hexamers according to the manufacturer’s instructions (Thermoscript RT-PCR system; Invitrogen). cDNA (2 µl) was amplified using a master mix containing Taq polymerase (Invitrogen) and the following primers: NHE2-wt forward and NHE2-wt reverse (listed above), β-actin sense, 5′-GGTGTGATGGGTGGGAATGG-GTC-3′, and β-actin antisense, 5′-ATGCCGTAGGAGGAGCAGACAGACG-3′ as published earlier (25), each at a final concentration of 200 µM. The PCR reaction was carried out for 45 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The PCR product was analyzed on a 2% agarose gel stained with ethidium bromide to identify fragments of ~455 bp for NHE2 and 350 bp for β-actin.

Western blotting. Mice were anesthetized with 100 mg/kg Inactin, and kidneys were removed. Slices of cortex were manually dissected, and tissue was homogenized with a rotor-stator homogenizer in a buffer containing 20 mM Tris·HCl, 1 mM EGTA, pH 7.0, and a protease inhibitor cocktail (BD Bioscience, San Jose, CA). Samples were centrifuged at low speed to pellet cellular debris, and supernatant was collected and assayed for protein concentration by using a modified Bradford method (Quick Start Bradford protein assay; Bio-Rad). Forty micrometers of protein were run per lane, separated on either a 7.5 or 4–20% SDS-polyacrylamide gel, depending on the protein of interest. The samples were then transferred to a polyvinylidene difluoride membrane (Bio-Rad). After the membrane was blocked in 5% nonfat dry milk, immunoblotting was performed with a polyclonal antibody to renin (1:250 dilution), a rabbit polyclonal antibody to mPGES (1:200 dilution), or a goat polyclonal COX-2 antibody (1:200 dilution). Reactivity was detected using a horseradish peroxidase-labeled goat anti-rabbit (1:1,000 dilution; Santa Cruz Biotechnology) or donkey anti-goat secondary antibody (1:1,000 dilution; Santa Cruz Biotechnology). An enhanced chemiluminescence kit (Amersham Biosciences, Little Chalfont, UK) was used to visualize the secondary antibody. The blot was stripped and reprobed with a goat polyclonal antibody to actin (1:200 dilution; Santa Cruz Biotechnology) to test for protein loading and quality of transfer.

MMDD1 cells were grown to confluence on plates as previously described (45). In some experiments, the cells bathed in Krebs-Ringer solution were incubated with a NaCl-free isosomotic, modified Krebs-Ringer solution [NaCl was replaced with N-methyl-d-glucamine chloride as described previously (31), 100 µM furosemide, 100 µM dimethyl amiloride (DMA) or 100 µM ethylisopropyl amiloride (EIPA), 20 mM NH4Cl, 10 µM nigericin, or 500 mosmol/kgH2O modified Krebs-Ringer solution (by adding mannitol) for 0, 2, 5, or 15 min. All reagents were purchased from Sigma. After treatment, cells were lysed using CellLytic-M lysis buffer (Sigma) according to the manufacturer’s instructions and centrifuged at maximum speed for 20 min. The supernatant was collected and assayed as previously described. Samples were run on 4–20% gels and probed with anti-PERK1/2 antibodies overnight at a dilution of 1:500 and imaged as
described above. Blots were then probed for 1 h with GAPDH (1:4,000; Ambion), which served as a loading control.

Densitometric analysis of blots was performed using National Institutes of Health ImageJ software. The data were then normalized against the control sample, and an average for each group was calculated.

The pHi of MMDD1 cells in culture was measured using the pH-sensitive fluorochrome BCECF as described previously (31). Briefly, MMDD1 cells grown on glass coverslips were loaded with the dye by adding 10 μM BCECF-AM to the culture medium at 37°C. Coverslips were inserted in a cuvette-based spectrofluorometer (Quantamaster-8; PTI) and superfused with Krebs-Ringer solution containing either 20 mM NH4Cl (alkalinization) (45) or 10 μM nigericin (cell acidification) (30). BCECF fluorescence was detected continuously (5 points/s) at 530 nm in response to excitation at 500 and 440 nm. pH was determined as the excitation ratio signal (500 nm/440 nm).

Cell volume changes of MMDD1 cells in culture were measured using fluorescence detection of calcein self-quenching as described previously (14). Briefly, MMDD1 cells grown on glass coverslips were loaded with the dye by adding 10 μM BCECF-AM to the culture medium at 37°C. After calcein fluorescence stabilized, the cells were superfused with a coverslip-containing cuvette was superfused with Krebs-Ringer solution, and fluorescence was detected at 530 nm, and fluorescence was detected at 530 nm. The coverslip containing either 20 mM NH4Cl (alkalinization) (30) or 10 μM nigericin (cell acidification) (30). BCECF fluorescence was detected continuously (5 points/s) at 530 nm in response to excitation at 500 and 440 nm. pH was determined as the excitation ratio signal (500 nm/440 nm).

RESULTS

Renin immunohistochemistry. Kidneys from NHE2+/+ (Fig. 1A) and NHE2−/− mice (Fig. 1B) were paraffin-embedded, sectioned, and stained in parallel with a renin antibody. Intense renin immunolabeling was detected in cells of the terminal JG segment of afferent arterioles in both NHE2+/+ and NHE2−/− mice. Importantly, the number of positively labeled renin-producing JG cells per afferent arteriole was ~2.5-fold higher in NHE2−/− compared with NHE2+/+ mouse kidneys (Fig. 1C). The average number of JG cells per afferent arteriole was 3.2 ± 0.5 in NHE2+/+ and 7.6 ± 0.6 in NHE2−/− kidneys (P < 0.05). The number of afferent arterioles analyzed was n = 10 in the NHE2−/− and n = 5 in the NHE2+/+ group from 5 separate kidneys in each group.

Renin immunoblotting. Renal cortical tissue samples were removed from NHE2−/− (n = 6) and NHE2+/+ mice (n = 5) fed a control diet and immunoblotted for renin (Fig. 2A). Probing the blots with a GAPDH antibody confirmed equal protein loading (Fig. 2A). The blots were then analyzed using densitometry (Fig. 2B). Renin expression was 20% higher in NHE2−/− mice compared with NHE2+/+ mice on normal salt diet (P < 0.05). As expected, no-salt diet for 1 wk significantly increased renin content in NHE2−/− mice, but this response was blunted in NHE2−/− mice. #P < 0.05, low-salt NHE2+/+ vs. low-salt NHE2−/− (n = 3 each).

Plasma and kidney tissue renin activity. Renal cortical tissue renin activity was assayed using spectrofluorometry. Tissue homogenates were prepared and mixed with the fluorogenic renin substrate and assay buffer. The emitted FRET signal, an index of ANG I generation (renin activity), was measured and plotted as a function of time (Fig. 3A) and quantified (Fig. 3B). Renin activity was threefold higher in the renal cortex from NHE2−/− compared with NHE2+/+ mice (NHE2−/−: 30.4 ± 6.6, n = 5; NHE2+/+: 99.5 ± 16.2, n = 6, P < 0.01). No-salt diet for 1 wk increased renal tissue renin activity sixfold in NHE2+/+ mice (179.8 ± 0.6, P < 0.01), but this response was significantly blunted in NHE2−/− mice (132.2 ± 33.9, not significantly different from NHE2−/− mice on normal diet; Fig. 2B).

To determine whether the higher intrarenal renin content and activity in NHE2−/− mice are reflected in the serum, we measured PRC in NHE2−/− (n = 11) and NHE2+/+ mice (n = 12) and found that PRC was significantly higher in NHE2−/− mice (11.9 ± 4.8, n = 11) than in NHE2+/+ mice (2.2 ± 0.3, n = 12, P < 0.05).
increased renin content in NHE2 knockout mice

8). PRC was significantly increased (Fig. 3C), more than twofold in NHE2−/− compared with NHE2+/+ mice (NHE2+/+: 0.83 ± 0.18 μg ANG I·h−1·ml−1, NHE2−/−: 1.92 ± 0.68 μg ANG I·h−1·ml−1, P < 0.05).

NHE3 expression in NHE2−/− mice. To evaluate the possibility that the loss of NHE2 function in MD cells of NHE2−/− mice is compensated for by an increase in the other main apical NHE isoform NHE3, we analyzed expression of NHE3 using immunohistochemistry. Sections from NHE2+/+ and NHE2−/− mouse kidneys were incubated with an NHE3 antibody and imaged. Similar to wild-type controls (Fig. 4A), NHE2−/− mice did not show any signs of NHE3 expression at the apical membrane of MD cells (Fig. 4B), whereas the adjacent cells of the thick ascending limb were labeled as in controls (Fig. 4, A and B).

Renal COX-2 and mPGES expression. To test whether the observed increases in renal renin content and activity in NHE2−/− mice are possibly due to MD-specific mechanisms, we compared the expression of renin, COX-2, and mPGES in the same homogenates of renal cortex using immunoblotting (Fig. 5). Expression levels were quantified by densitometry and then normalized to wild-type controls. Parallel with the increase in renal cortical renin content as established above, both COX-2 (1.62 ± 0.05) and mPGES expression (1.24 ± 0.05) were significantly increased in NHE2−/− mice compared with NHE2+/+ (62 and 24% increases, respectively, P < 0.05).
MAPK activation in macula densa cells of NHE2 \(^{-/-}\) mice. Since the activation of ERK1/2 MAP kinases are well-established elements of the MD renin release signal (8, 9, 45), immunofluorescence studies were performed on NHE2 \(^{+/+}\) and NHE2 \(^{-/-}\) mouse kidney sections using a pERK1/2 antibody (Fig. 6). Specific immunolabeling was found in cells of the MD and the adjacent cortical thick ascending limb in both genotypes (Fig. 6, A and B). However, the number of positively labeled tubular epithelial cells per JGA was \(\approx\) 3.5-fold higher in NHE2 \(^{-/-}\) compared with NHE2 \(^{+/+}\) mice (Fig. 6C). The average number of pERK1/2-positive cells per JGA was 2.6 \pm 0.4 in NHE2 \(^{+/+}\) and 9.0 \pm 0.9 in NHE2 \(^{-/-}\) kidneys (\(P < 0.05\)).

MAPK activation in MMDD1 cells. This series of experiments was performed to further identify the NHE2- and ERK1/2-dependent cellular mechanism that causes the activation of the MD renin release signal. After confirming that the MMDD1 cells express NHE2 (Fig. 7A), we studied the effects of low NaCl and ion transport inhibitors on pERK1/2 levels using these cells and Western blotting (Fig. 7B). Complete removal of NaCl from the medium significantly increased the amounts of pERK1/2 in MMDD1 cells within 5 min (Fig. 7B), consistent with previous reports (8, 45). Compared with control (time 0) and averaging all time points, pERK1/2 levels were increased by 77% (Fig. 7C). The NKCC inhibitor furosemide caused a slowly progressing, time-dependent activation (2.2-fold) of ERK1/2. Inhibiting NHE by using either EIPA or DMA (not shown) or increasing osmolality of the bathing medium from 300 to 500 mosmol/kgH\(_2\)O both had effects similar to those of salt removal with the same kinetics (Fig. 7B). The amounts of pERK1/2 increased significantly by 29% in response to EIPA and by 16% in response to hyperosmolality (Fig. 7C). In contrast, well-established techniques of either cell alkalinization (by adding 20 mM NH\(_4\)Cl to the bathing medium) (45) or cell acidification (by adding 10 \(\mu\)M nigericin) (30) failed to activate ERK1/2. In fact, they caused progressive reductions in pERK1/2 levels (23% reduction with NH\(_4\)Cl and 12% reduction with nigericin, Fig. 7, B and C). Effects of NH\(_4\)Cl (rapid and significant alkalinization) and nigericin (slow but marked acidification) on MMDD1 pH, were confirmed using the pH-sensitive fluorophore BCECF as described previously (31) (Fig. 8A). Effects of EIPA and hyperosmolality on MMDD1 cell volume (both causing significant cell shrinkage) were confirmed using the calcine self-quenching technique (Fig. 8B). Mock changes of the bathing medium had no effect on pERK1/2 (not shown). All of these experiments were performed in triplicate. Equal protein loading was confirmed by probing blots with a GAPDH antibody (Fig. 7B).

DISCUSSION

This study demonstrates for the first time that the NHE isoform NHE2, which is expressed in cells of the MD, plays an important role in the control of renin synthesis and release. Multiple lines of evidence support this conclusion. Compared with wild type, NHE2 \(^{-/-}\) mice exhibit increased JGA and overall renal renin content, increased intrarenal and plasma renin activities, and high levels of enzymes and signaling molecules that constitute the MD mechanism of renin release. Similarities in MAP kinase activation by pharmacological NHE inhibition and hyperosmolality-induced cell shrinkage in a MD cell line suggest that the link between reduced MD NHE2 activity and increased JGA renin signaling may be the reduction in MD cell volume.

Multiple lines of evidence suggested the significant upregulation of renal renin content in NHE2 \(^{-/-}\) mice, including renin immunohistochemistry, immunoblotting, and measurements of renin activity from kidney tissue and plasma (Figs. 1–3). Although renal cortical tissue immunoblotting found only a modest, 20% increase in renin content compared with the more significant changes detected by the other techniques, Western blotting is the least sensitive and JGA-specific method of all. Increased renal renin content and plasma renin activity in NHE2 \(^{-/-}\) mice are clearly unexpected findings. Whole animal data indicate that in contrast to NHE3-deficient mice, the NHE2 \(^{-/-}\) mice are not hypovolemic (19), which would have been consistent with the activation of the renin-angiotensin system. Likewise, systemic blood pressure and plasma aldosterone levels are normal in NHE2 \(^{-/-}\) mice (19, 39). Previous studies also established that fecal salt excretion was slightly less in NHE2 \(^{-/-}\) mice maintained on a normal salt diet, and when switched to no-salt diet (which does not eliminate NaCl in the luminal contents because of the significant paracellular transport), both NHE2 \(^{-/-}\) and NHE2 \(^{+/+}\) mice reduced fecal salt excretion to essentially zero (19). These previous data clearly indicate that the known major NaCl-absorptive mechanisms (NHE3 and the epithelial sodium channel, ENaC) are sufficient to maintain salt absorption in the intestine, and therefore, NHE2 \(^{-/-}\) mice are not on a “functional” low-salt diet.

Tubuloglomerular feedback responses in NHE2 \(^{-/-}\) mice under control conditions are also not different from NHE2 \(^{+/+}\) mice (24). However, pharmacological NHE inhibition in the in vitro microperfused TGF augmented TGF responses (43). Although the present studies did not address the role of MD NHE2 in TGF, the new findings on altered MD signaling and...
increased renin content in NHE2-/- mice and these somewhat conflicting previous reports warrant further studies in this area. Even if there seems to be no physiological need for the inappropriately high renin synthesis in NHE2-/- mice, the MD mechanism of renin synthesis and release is highly active in these animals. The involvement of MD cells was suggested by the significantly increased COX-2 and mPGES expression in the cortex of NHE2-/- mouse kidneys, since these PGE_2-forming mechanisms are one of many physiological pathways that control renin release (5, 36, 37, 41). MD signaling that triggers renin synthesis and release starts with decreases in tubular [NaCl]. Consistent with this paradigm, no-salt diet in the present studies caused significant upregulation of renal renin content and activity in wild-type mice (Fig. 2B). However, this response was blunted in NHE2-/- mice, indicating that the MD feedback control of renin secretion was disrupted. These findings support the physiological importance of NHE2 in MD signaling and renin control. Previous work established that the inducible COX-2 isoform of cyclooxygenases, present in MD cells, produces prostaglandins in response to reductions in tubular [NaCl], which, upon release, acts on JG granular cells to stimulate renin release and renin synthesis (5, 8, 22, 23, 45). The underlying cellular mechanism was shown to involve MAP kinases, ERK1/2, and p38 activation that are causal in the acute release of PGE_2 as well as in the delayed rise in COX-2 mRNA and protein expression (8, 9, 45). It is still unclear what mechanism links MD apical ion flux to MAPK activation (8, 42, 45). We find it very interesting that MAP kinases are often activated by large changes in cytosolic pH and/or cell volume (12, 44, 45), cellular responses in which NHEs play a critical role (12, 31).

The role of salt reabsorption through various NKCC2 isoforms in MD function and renin signaling is well established (7, 22, 23, 28, 29). It appears that NHE2, perhaps coupled with Cl-/HCO_3 anion exchangers that are expressed in MD cells (17), plays a similar role. The present studies suggest that the mechanism by which NKCC2 and NHE2 activities are coupled...
to the renin release signal could be MD cell volume reduction. In fact, MD cell volume is one of the important cell parameters that undergo tremendous changes when tubular salt content is altered (18, 32). MD cell volume changes also appear to be maintained (18, 21), a sign of inefficient cell volume regulatory mechanisms that has been attributed to the sensor function of these cells (3, 18). MD cell swelling has been linked to increased tubular [NaCl]-induced afferent arteriole vasoconstriction, the TGF mechanism (32). The opposite physiological stimulus, reductions in tubular [NaCl] and MD salt reabsorption, is the classic activator of the MD renin release mechanism (5, 22, 23). Either Cl− removal or furosemide administration that triggers renin release (22, 23) can cause significant MD cell shrinkage (13, 18). The present studies confirmed that NHE inhibition with EIPA also causes MD cell shrinkage (Fig. 8B). Thus MD cell volume seems to be ideally tracking the alterations in tubular fluid composition and these opposing physiological mechanisms. It has been established that both NKCC2 and NHE2 are involved in MD apical salt entry with an estimated 80:20 ratio (3, 33). Consistent with this is the intriguing present finding that furosemide, an inhibitor of NKCC2, caused an approximately fourfold more intense activation of ERK1/2 within 15 min than did EIPA, a blocker of NHE2 (Fig. 7, A and B). Although NKCC2 is the primary player, the contribution of NHE2 to cell volume still appears to be significant (Fig. 8B). Although low MD cell volume is one possible mechanism by which NHE2 activity is coupled to renin synthesis and release, other mechanisms such as the direct interaction between NHE2 and MAP kinases or other signaling molecules cannot be ruled out. Also, it should be noted that the magnitude of change in medium osmolality (from 300 to 500 mosmol/kgH2O) applied in the present cell culture experiments is well within the range used for MD cells by others (13, 18, 21).

MMD1 cells were reported to produce cell acidification upon bath Na+ removal, a clear sign of plasma membrane Na+/H+ exchange (45). In addition, the present studies confirmed that MMD1 cells express NHE2 (Fig. 7A), suggesting that these cells are useful to study NHE2-dependent MD cell signaling mechanisms. Regulation of pHi is another key function of NHEs (31), and MD NHE2 activity, based on tubular salt content, has been shown to cause large changes in MD pHi (10, 31). Low tubular [NaCl] is associated with acidic MD pHi, and cell acidification is known to activate MAP kinases in some cell types (12, 30). However, direct and salt-independent cell acidification by nigericin in the present studies failed to activate ERK1/2, a result clearly different from the effect of NHE inhibition (Fig. 7, B and C). Thus it seems unlikely that reduced NHE2 activity generates the renin signal through cell acidification. However, it is possible that low pHi acts on molecular targets other than ERK1/2.

In contrast to previous work (8, 45) that failed to detect Na+ removal-dependent activation of MAP kinases in cells of the MD and cortical thick ascending limb, the present studies found significant and rapid phosphorylation of ERK1/2 in response to NHE inhibition. A possible explanation for these results could be the use of different experimental salt solutions. In previous work, the lowest [Na+] was 68 mM (44) or 26 mM (8), or in other words, only partial Na+ removal was used. Given that the resting [Na+] in MD cells is in the 30 mM range (33), these concentrations most likely did not significantly alter NHE activity. The present studies used pharmacological inhibitors (EIPA and DMA) at a concentration that provides maximal inhibition of MD NHE2 (31, 43). Significant and rapid activation of ERK1/2 was observed in these experiments consistent with the role of ERK1/2 in acute PGE2 release (8, 45). Although the present cell culture studies showed acute and transient activations of ERK1/2 in response to various stimuli (Fig. 7B), chronic activation of ERK1/2 was evident in the native tissue, in MD cells of NHE2−/− kidneys (Fig. 6), consistent with the chronic stimulation of COX-2 and JGA renin in NHE2−/− mice (Fig. 5).

In summary, the present studies identified another element of the MD salt-sensing mechanism that is important in the control of renin synthesis and release. Reductions in MD NHE2 activity lead to the phosphorylation of MAP kinases ERK1/2 and the activation of the PGE2 synthetic machinery including COX-2 and mPGES. NHE2 activity may be linked to renin signaling via reductions in MD cell volume. Because of the importance of renin and other elements of the renin-angiotensin-aldosterone system in many different health and disease conditions, NHE2 is a potential new target of therapeutic interventions.

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