Effect of Metabolic Acidosis on Neonatal
Proximal Tubule Acidification

Katherine Twombley¹, Jyothsna Gattineni¹, Ion Alexandru Bobulescu²,
Vangipuram Dwarakanath¹ and Michel Baum¹, ²

Department of Pediatrics¹ and Internal Medicine²
University of Texas Southwestern Medical Center at Dallas
Dallas, Texas 75235-9063

Running Head: Neonatal Proximal Tubule Response to Acidosis

Send Reprint Requests and Correspondence to:

Michel Baum, M.D.
Department of Pediatrics
U.T. Southwestern Medical Center
5323 Harry Hines Blvd.
Dallas, Texas 75390-9063
PH: (214) 648-3438
FAX: (214) 648-2034
Michel.Baum@UTSouthwestern.edu

Copyright © 2010 by the American Physiological Society.
ABSTRACT

The serum bicarbonate in neonates is lower than adults due in large part to a lower rate of proximal tubule acidification. It is unclear if the neonatal proximal tubule is functioning at maximal capacity or if the proximal tubule can respond to metabolic acidosis as has been described in adult proximal tubules. We find that neonatal mouse brush border membranes have a lower NHE3 protein abundance (neonate 0.11 ± 0.05 vs adult 0.64 ± 0.07; P<0.05) and a higher NHE8 protein abundance (neonate 1.0 ± 0.01 vs adult 0.13 ± 0.09; P<0.001) compared to adults. To examine if neonates can adapt to acidosis, neonatal mice were gavaged with either acid or vehicle for four days resulting in a drop in serum bicarbonate from 19.5 ± 1.0 to 8.9 ± 0.6 mEq/l (P<0.001). Proximal convoluted tubule Na⁺/H⁺ exchanger activity (dpH/dt) was 1.68 ± 0.19 pH units/min in control tubules and 2.49 ± 0.60 pH units/min in acidemic neonatal mice (P<0.05) indicating that the neonatal proximal tubule can respond to metabolic acidosis with an increase in Na⁺/H⁺ exchanger activity. Similarly, brush border membrane vesicles from neonatal rats had an increase in Na⁺/H⁺ exchanger activity with acidemia that was almost totally inhibited by 10⁻⁶ M 5-(N-ethyl-n-isopropyl)-amiloride, a dose that has little effect on NHE3 but inhibits NHE8. There was a significant increase in both NHE3 (vehicle 0.35 ± 0.07 vs acid 0.73 ± 0.07; P<0.003) and NHE8 brush border membrane protein abundance (vehicle 0.41 ± 0.05 vs acid 0.73 ± 0.06; P<0.001) in acidemic mouse neonates compared to controls. A comparable increase in NHE3 and NHE8 was found in neonatal rats with acidosis. In conclusion, the neonatal proximal tubule can adapt to metabolic acidosis with an increase in Na⁺/H⁺ exchanger activity.

Key Words: acidification, NHE3, NHE8, Na⁺/H⁺ exchanger, renal development
INTRODUCTION

The proximal tubule reabsorbs the majority of filtered bicarbonate. Two-thirds of luminal proton secretion (6; 36) and virtually all the luminal acidification in the neonate is due to the Na\(^+/\)H\(^+\) exchanger (6). A small fraction of proximal tubule proton secretion in the adult is the result of a luminal H\(^+\)-ATPase (6; 29; 36). In the adult proximal tubule, luminal Na\(^+/\)H\(^+\) exchange is predominantly mediated by NHE3 (12; 37; 43; 44). In adult animals proximal tubule luminal bicarbonate absorption, Na\(^+/\)H\(^+\) exchange and NHE3 protein abundance are upregulated in response to metabolic acidosis (3; 19; 35; 41; 44).

Neonates have a lower serum bicarbonate level, which is due to a lower bicarbonate threshold and are more prone to acid base disturbances than adults (21). The lower bicarbonate threshold is predominantly due to the lower rate of bicarbonate reabsorption in the neonatal proximal tubule compared to the adult proximal tubule (9; 38). The maturational increase in proximal tubular acidification is largely due to the maturational increase in the apical membrane Na\(^+/\)H\(^+\) exchanger (NHE3) (5-7; 10). Examination of neonatal proximal tubular acidification mechanisms demonstrated that prior to weaning there was Na\(^+/\)H\(^+\) exchange activity despite a paucity of NHE3 suggesting another NHE isoform (39). The presence of another proximal tubule Na\(^+/\)H\(^+\) exchanger was confirmed in NHE3 null mice (17).

NHE8 was cloned by Goyal et al. (24) and localized to the apical membrane of all segments of the proximal tubule (11; 23). NHE8 is a Na\(^+\) dependent proton exchanger that is sensitive to 5-(N-ethyl-n-isopropyl)-amiloride (EIPA) (46). Our laboratory has shown that NHE8 is a developmental NHE isoform that is highly expressed on the apical
brush border membranes in neonatal rat tubules and that expression decreases with age (11).

Metabolic acidosis has been shown to increase apical brush border membrane NHE3 protein expression and Na\(^+\)/H\(^+\) exchange activity in adult rats (2; 3; 28; 44). Since renal acidification in the neonate is less than that of the adult, it is unclear if the neonate can adapt to metabolic acidosis, as seen in the adult, with an increase in Na\(^+\)/H\(^+\) exchange activity. The purpose of this study was to examine if metabolic acidosis increases proximal tubule Na\(^+\)/H\(^+\) exchange activity and if NHE8 expression is increased by metabolic acidosis \textit{in vivo}.
Methods

Animals and gavage:

C57BL/6 mice were born at our institution. Starting at 5 days of age, mice were gavaged orally with 1 mMol/100 grams of body weight with a volume of 1ml/100 grams body weight with either ammonium chloride or sodium chloride (control) twice daily for 7 doses. This protocol is similar to one that had previously been used to study metabolic acidosis in adult rats (18; 35) and one used in neonatal rats starting as young as 2 days of age to produce metabolic acidosis (27). The age chosen was at a point during postnatal development where there is a paucity of NHE3 and NHE8 is normally highly expressed on the apical membrane of the proximal tubule (11). The kidneys were harvested for brush border membrane vesicle (BBMV) isolation, renal cortical protein analysis, RNA for cDNA synthesis, or for proximal tubule isolation for in vitro microperfusion. The studies performed were in accordance with the APS’s Guiding Principles in the Care and Use of Animals and were approved by the IACUC at the University of Texas Southwestern Medical Center.

Blood collection and analysis:

Mice were sedated with Inactin (1mg / 10g of body weight). 100 μl of whole blood was collected via intracardiac puncture and placed in heparinized tubes for analysis of serum electrolytes, pH, pCO₂ and bicarbonate levels using a Stat Profile Critical Care Express blood gas machine (Nova Biomedical, Waltham, MA).

Brush Border Membrane Vesicle Isolation:

Brush border membrane vesicles were prepared as previously described (13; 20; 39). Intraperitoneal Inactin (1 mg/10 g) was administered to mice or rats gavaged with
saline (control) and NH₄Cl prior to sacrifice. Kidneys were removed quickly and placed in 6 ml of isolation buffer containing 300mM mannitol, 16 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Hepes), and 5mM ethylene glycol-bis (B-aminoethyl ether) N,N,N’N’-tetraacetic acid (EGTA) that was titrated to a pH of 7.4 with Tris. The isolation buffer contained phenyl-methyl-sulfonyl fluoride (PMSF; 100µg/mL) and 1 µL/mL protease inhibitor cocktail (Sigma, Saint Louis, MO). The kidneys were homogenized with 15 strokes of a Teflon-glass homogenizer at 4°C. Briefly, the kidney was homogenized in ice-cold isolation buffer and crude membranes were obtained by centrifugation at 48,000 g at 4°C for 1 h (Beckman J2–21M; JA-20 rotor; Beckman Coulter, Fullerton, CA). The pellets were resuspended, homogenized in a Dounce glass homogenizer, and subjected to Mg²⁺ precipitation by addition of MgCl₂ at a final concentration of 15 mM at 4°C for 20 min. The precipitated membranes were removed by centrifugation at 3,000 g for 10 min at 4°C and the supernatant was subjected to two additional rounds of Mg²⁺ aggregation. This was followed by centrifugation at 48,000 g for 30 min at 4°C and the resulting pellet enriched in BBMV was used for Na⁺/H⁺ exchange activity experiments or immunoblotting. All protein fractions were assayed using the Bradford method with bovine serum albumin as the standard (15). The brush border membrane enrichment compared to homogenate of leucine amino peptidase in neonatal rats was 10.6 ± 0.6 and that of adults was 9.8 ± 0.7 (P=0.38) and neonatal mice was 9.4 ± 0.9 and 8.7 ± 1.0 in adult mice (P=0.61).

*SDS- PAGE and immunoblotting:*
BBM (15 µg) cortical homogenates were diluted in 2.5X loading buffer [2.5 mM Tris HCl (pH 6.8), 2.5% β-mercaptoethanol, 25% glycerol and 2.5% SDS] (15 µg/lane) and heated at 85°C for 5 min (NHE3) or 37°C for 5 min (NHE8). The proteins were then separated on an 8% polyacrylamide gel using SDS-PAGE as previously described (20) and transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore, Billerica, MA) at 400 mAmperes at 4°C for 1 hour (8; 14; 39). The blots were blocked with Blotto (1% nonfat milk and 0.05% Tween-20 in PBS, pH 7.4) for at least one hour prior to incubation with primary antibodies to either NHE3 or NHE8. The NHE8 antibody was the monoclonal antibody 7A11 at a 1:5 dilution from a hybridoma supernatant and the NHE3 antibody was a rabbit anti-rat polyclonal antiserum 1568 against amino acids 809-822 of NHE3 at a 1:1000 dilution (4; 23) (gifts from Orson W. Moe and Peter Aronson, respectively). The blots were washed in Blotto followed by the addition of the secondary antibody, horseradish peroxidase-conjugated donkey anti-mouse antibody at a 1:10,000 dilution. Enhanced chemiluminescence was used to detect bound antibody (Amersham Biosciences, Piscataway, NJ). Equal loading of the samples was verified using an antibody to β-actin at 1:15,000 dilution (Sigma Biochemicals and Reagents, St. Louis, MO). Relative NHE3, NHE8 or β-actin protein abundance was quantitated using densitometry.

**cDNA synthesis and real-time PCR:**

Total cellular RNA was isolated from whole kidneys of 8 day old mice using the GenElute Mammalian Total RNA Miniprep Kit per the manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO). The quantity and purity of total RNA was assayed using an LKB Ultra-spec III spectrophotometer at 260 and 280 nm wavelengths. Total RNA (2
µg) was first treated with DNAse I (Invitrogen, Carlsbad, CA) and the product was used to synthesize cDNA with random hexamer primers and reverse transcriptase (Stratagene, La Jolla, CA) at an annealing temperature of 25ºC for 10 minutes, extension at 42ºC for 50 minutes, and termination at 70ºC for 15 minutes.

Real time PCR was performed using an iCycler PCR Thermal Cycler (BioRad, Hercules, CA) to quantify relative mRNA abundance. Primers for NHE3 and NHE8 (Table 1) were mixed with cDNA and SYBR green master mix per the manufacturer's instructions (Bio-Rad). The PCR conditions were denaturation at 94ºC for 30 seconds, annealing at 61ºC for 20 seconds, and extension at 72ºC for 20 seconds for 40 cycles. The housekeeping gene 28s was used to normalize the relative expression of NHE3 and NHE8 using the method described by Vandesompele et al. (42).

*In vitro pHi studies:*

Proximal tubules were dissected from 8 day old mice that were either gavaged for 4 days with normal saline (control) or with ammonium chloride as described above. After sacrifice the kidney was quickly placed in ice cold Hanks’ solution that contained (in mM) 137 NaCl, 5 KCl, 0.8 MgSO$_4$, 0.33 Na$_2$HPO$_4$, 0.44 KH$_2$PO$_4$, 1 MgCl$_2$ 10 tris (hydroxymethyl) amino methane hydrochloride, 0.25 CaCl$_2$, 2 glutamine, and 2 L-lactate at 4ºC. Proximal convoluted tubules were dissected free hand from the mid-cortical and juxtamedullary region of the kidney. The dissected tubules were transferred in cold solution to a bathing chamber on a Nikon Eclipse TE 2000-U inverted epifluorescent microscope with a Photometrics Cascade model 512F camera (Ottobrunn, Germany) and Lambda DG-4/DG-5 illumination system (Sutter Instrument Co. (Novato, CA) to measure cell pH (pHi). The tubules were perfused using concentric glass pipettes with
an ultrafiltrate-like solution that contained (in mM) 115 NaCl, 25 NaHCO₃, 4.0 Na₂HPO₄, 10 Na acetate, 1.8 mM CaCl₂, 1 MgSO₄, 5 KCl, 8.3 glucose, 5 alanine, 2 lactate, 2 glutamine and bathed with the same solution preheated to 37°C. The bath solution was changed to one containing 5x10⁻⁶ M BCECF-AM (Molecular Probes, Eugene, OR) to load the tubule with the pH sensitive dye BCECF. To estimate cell pHi a nigericin calibration curve was generated comparing the ratio of fluorescence at (F₅₀₀/F₄₅₀) to pH as previously described (1; 5; 17; 39).

After the tubular cells were loaded with BCECF for ~2 minutes, the bathing solution was changed to solution A containing 1 mM 4-acetamido-4'-isothiocyanostilbene-2,2' disulfonic acid (SITS), to inhibit the sodium dependent bicarbonate cotransporter on the basolateral membrane, the major regulator of cell pH in the proximal tubule (1; 1; 5; 36). The solution had a bicarbonate concentration of 5 mM and a pH of 6.6 to compensate for the cell alkalinization caused by SITS (solution A-Table 2). The sodium containing luminal solution perfusing the tubule (solution B-Table 2) was rapidly changed to one without sodium (solution C-Table 2) and the rate of change in cell pH was measured. The initial change in cell pH after sodium removal was used to measure dpHi/dt. The luminal solution was then changed back to the original sodium containing solution. This protocol has been used previously to study the activity of the luminal Na⁺/H⁺ exchanger (1; 17; 20; 36).

**Na⁺/H⁺ exchange activity in BBMV.**

Due to the small amount of renal cortex available from mice, rat renal cortical BBMV were prepared as above. Neonatal rats were gavaged starting at 10 days with
vehicle or acid twice daily as above form mice and sacrificed on day 13 of age. $\text{Na}^+$/H$^+$ exchange activity was measured using freshly prepared BBMV using the acridine orange fluorescence quenching technique (13). The BBMV pellet obtained as above was rehomogenized in intravesicular solution containing 280 mM mannitol, 5 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.5 with a Potter-Elvehjem Teflon-glass homogenizer, equilibrated at 4°C for 2 h, centrifuged at 48,000 g, and resuspended with a 27-gauge syringe needle. The final protein concentration was 10 mg/ml. The extravesicular solution contained 120 mM N-methyl-D-glucamine (NMDG)-gluconate, 20 mM HEPES, pH 7.5 with acridine orange (Invitrogen) added to a final concentration of 6 µM. This solution was placed into a cuvette with a magnetic stirrer. Acridine orange fluorescence was monitored with a QM-8/2003, Photon Technology Spectrofluorometer (International, London, ON, Canada) using ($\lambda_{\text{excitation}} = 493$ nm, $\lambda_{\text{emission}} = 530$ nm). Fluorescence was quenched by the addition of acid-loaded BBMV (a volume containing 150 µg protein) in the absence of Na$^+$. Addition of extravesicular Na$^+$ (Na$^+$-gluconate to a final concentration of 60 mM) resulted in Na$^+$/H$^+$ exchange and an increase in fluorescence. In some experiments 10$^{-6}$ M ethylisopropylamiloride (EIPA), an inhibitor of Na$^+$/H$^+$ activity was added before sodium. NMDG-gluconate used to monitor Na$^+$-independent quenching, which was <1/10th of sodium dependent activity. Na$^+$/H$^+$ exchange activity was estimated as the initial rate of increase in fluorescence on addition of Na$^+$-gluconate minus the sodium independent increase in fluorescence. Adult rat Na$^+$/H$^+$ exchange activity was examined for comparison.

Statistical Analysis:
All data are expressed as mean ± SEM. Comparisons were made using an unpaired Student’s t test or ANOVA when more than two groups were studied. A P value of < 0.05 was considered significant.
RESULTS

In our first series of experiments we examined the ontogeny of NHE3 and NHE8 mRNA and brush border membrane protein abundance in the mouse. In figure 1 we find that at 1, 7 and 14 days of age there was less NHE3 mRNA abundance than in 24 day old and adult mice. The abundance of NHE8 mRNA was comparable at all ages except NHE8 mRNA abundance was higher at 24 days than at any other age. In Figure 2 we show the ontogeny of NHE3 and NHE8 brush border membrane protein abundance. There was a maturational increase in NHE3 protein abundance and decrease in NHE8 protein abundance.

We next examined the effects of acid gavage on serum electrolytes, pH and pCO₂. Neonatal mice and rats were gavaged with NH₄Cl or vehicle for four days. The results of the electrolytes and blood gases are shown in Table 3. Neonates appeared healthy but developed a significant hyperchloremic metabolic acidosis with acid gavage compared to controls as well as a higher blood potassium levels and blood urea nitrogen (BUN).

We next examined whether the acidemic neonatal mice had changes in NHE3 and NHE8 mRNA and protein abundance. As shown in Figure 3, metabolic acidosis did not have an effect on NHE3 or NHE8 mRNA abundance in neonatal mice. In neonatal rats there was no change in NHE8 mRNA abundance/28s [6.8 ± 1.0 control vs 4.8 ± 0.9 acid (n=6), P=0.16] and a decrease in NHE3 mRNA abundance/28s [3.9 ± 0.6 control (n=11) vs 2.0 ± 0.2 acid (n=10), P=0.009]. In mice there was an increase in NHE3 total protein abundance but no change in NHE8 total protein abundance with metabolic acidosis as shown in Figure 4. Similarly, in rats total cellular NHE8/β-actin was 0.58 ±
0.16 in controls which was not different than 0.70 ± 0.09 measured in acidemic neonatal rats (P=0.53), while total cellular NHE3 was higher in acidemic rats [0.33 ± 0.03 vs 0.73 ± 0.09, (n=6) P=0.002]. Finally, we examined the effect of metabolic acidosis on brush border membrane NHE3 and NHE8 protein abundance in mice and rats. As shown in Figure 5, there was an increase in both NHE3 and NHE8 brush border membrane protein abundance in mice. Similarly, metabolic acidosis in neonatal rats resulted in an increase in both NHE3 [0.70 ± 0.04 vs 0.90 ± 0.04 (n=5), P=0.002] and NHE8 [0.59 ± 0.04 vs 0.78 ± 0.05 (n=11), P=0.009] brush border membrane protein abundance (not shown). Thus, the neonatal proximal tubule can respond to metabolic acidosis with an increase in brush border membrane NHE8 protein abundance in mice and rats demonstrating that NHE8 may play a role in the adaptation to metabolic acidosis.

In the next experiments, we examined if neonatal mice can respond to metabolic acidosis with an increase in Na⁺/H⁺ exchange activity. Na⁺/H⁺ exchange was assayed in control and acidemic neonatal mice in vitro as the rate of change in pHi with luminal sodium removal. The steady state pH in these experiments is shown in Table 4. Figure 6 shows that Na⁺/H⁺ exchange activity was faster in neonatal mice proximal convoluted tubules that received acid gavage. Thus, the neonatal proximal tubule can adapt comparable to the adult segment with an increase in Na⁺/H⁺ exchange in response to acidosis (35; 41; 44).

In the last series of experiments, we examined the effect of metabolic acidosis on brush border membrane Na⁺/H⁺ exchange activity. Due to the paucity of tissue from neonatal mice we performed these studies using 13 day old neonatal rats that were gavaged with vehicle or acid. As shown in Figure 7, Na⁺/H⁺ exchange was increased in
brush border membranes from acid gavaged rats compared to control rats. There was a significant reduction in Na\textsuperscript{+}/H\textsuperscript{+} exchange activity with 10\textsuperscript{-6} M EIPA in the acidemic rats. As a comparison, we examined Na\textsuperscript{+}/H\textsuperscript{+} exchange activity in adult animals. Na\textsuperscript{+}/H\textsuperscript{+} exchange was 7 fold higher in the adults demonstrating that this dose of EIPA causes a very small decrease in Na\textsuperscript{+}/H\textsuperscript{+} in the adult as shown by others (44).
DISCUSSION

The present study examined if neonates could respond to metabolic acidosis with an increase in proximal tubule acidification. We demonstrate that neonates can respond to metabolic acidosis by an increase in proximal tubule sodium dependent proton secretion. This increase in Na⁺/H⁺ exchange is accompanied by an increase in both brush border membrane NHE3 and NHE8 protein abundance. The increase in NHE8 protein abundance with acidosis and the effect of 10⁻⁶M EIPA to inhibit the increase in brush border membrane vesicle Na⁺/H⁺ exchange in neonates demonstrates that NHE8 isoform is regulated and likely plays an important physiologic role in neonates.

There have been 10 sodium-hydrogen exchangers identified in humans to this date (16; 30; 33). These NHEs differ in cellular location and function. NHE8 has its phylogenic origin in slime mold (16). From its gene sequence, NHE8 is predicted to be an intracellular NHE isoform and it shares relatively little sequence similarity with other mammalian NHEs (16; 32; 33). Indeed, NHE8 is an intracellular NHE in the mosquito Malpighian tubule (34). In mammals NHE8 has been localized to the Golgi where it likely plays a role in pH regulation and ionic composition (32). NHE8 mRNA has a ubiquitous distribution with the highest expression in the kidney, skeletal muscle, testes and liver (24; 32). In addition to its intracellular location, NHE8 has also been localized to the apical membrane of the proximal tubule and intestine (11; 22-24; 45). Colocalization studies with megalin suggest that NHE8 may also be present in subapical coated pits and potentially be involved in recycling (23). The function of NHE8 has only recently been examined (22).
There is a maturational increase in serum glucocorticoids and thyroid hormone levels. We have shown that the increase in NHE3 parallels the maturational increase in these levels and that we could prematurely increase NHE3 protein abundance and Na+/H+ exchanger activity by administering glucocorticoids or thyroid hormone to neonates (7; 8). Furthermore, we could prevent the maturational increase in NHE3 by preventing the maturational increase in glucocorticoids or thyroid hormone (8; 25; 26). We have recently shown that apical membrane NHE8 persists into adulthood in rats made hypothyroid from birth (22). Administration of thyroid hormone to neonates prior to the normal increase that occurs at the time of weaning, resulted in a premature decrease in brush border membrane NHE8 protein abundance (22).

We have previously shown that there was a maturational increase in NHE3 mRNA and brush border membrane protein abundance and a maturational decrease in NHE8 protein abundance in the rat (11). NHE8 mRNA abundance did not change during postnatal development in the rat (11). In the present study examining the ontogeny of neonatal mouse kidney NHE3 and NHE8, we found a maturational increase in NHE3 mRNA with the highest expression at 24 days and no significant difference in NHE8 mRNA at any age except in at 24 days where it was higher than at all other ages. The reason for the higher expression of NHE3 and NHE8 mRNA at 24 days is unknown. In the mouse, there was a maturational increase in NHE3 protein expression and decrease in NHE8 protein expression that was comparable to that previously described in the rat (11).

The current study addressed a different and physiologically important question, if it is possible to alter neonatal proximal tubular acidification in response to metabolic
acidosis, a common clinical problem. We have shown that when neonatal mice are exposed to an acidic environment by acid gavage, they adapt by increasing the BBM expression of both NHE3 and NHE8. Since there was no increase in NHE8 or NHE3 mRNA in response to acidosis, it is assumed that the increase in Na⁺/H⁺ exchangers was secondary to post translational mechanisms. Similar findings have been shown for the effect of chronic metabolic acidosis on NHE3 mRNA and brush border protein abundance in adult rats (3). The regulation of NHE8 appears somewhat different from NHE3. While there was an increase in both total cellular and brush border membrane NHE3, there was an increase in brush border membrane NHE8 but no change in total cellular NHE8 abundance. This suggests that NHE8 is regulated by acid solely by trafficking. This will have to be studied further using in vitro techniques.

We demonstrate that neonatal mice can increase proximal tubule acidification with metabolic acidosis. This study shows that there can be an increase in proximal tubule Na⁺/H⁺ exchange activity with a concomitant increase in NHE3 as has been shown in adults. Most importantly, there was an increase in Na⁺/H⁺ exchanger activity that was responsive to 10⁻⁶ M EIPA, a dose that we confirm to have a minimal effect in adult Na⁺/H⁺ activity which is due primarily to NHE3, a relatively EIPA insensitive exchanger (44). It was somewhat surprising that the control Na⁺/H⁺ exchange activity was not inhibited by 10⁻⁶ M EIPA. This may be due to the fact that NHE3 has a significant contribution to basal activity or that the level of Na⁺/H⁺ exchange was so low that we could not detect a change in activity.

In conclusion, we confirm that there is a maturational increase in brush border membrane NHE3 and maturational decrease in NHE8 protein abundance. This study
shows that in neonates, NHE8 can also play an adaptive role in response to acidosis. The cellular and molecular mechanisms for the regulation of NHE8 by acidosis are unclear at present and will require further *in vitro* studies.
Perspectives

The study of postnatal development has brought about surprising findings. While initial studies looking at protein and mRNA abundance of transporters characterized in adult animals showed that there were changes in expression during postnatal development, these studies did not explain all of the transport data (17; 39; 40). Thus far developmental isoform changes have been found for both the Na+/H+ exchanger (11) and the sodium dependent phosphate transporter (31). Whether there are other isoform changes that occur during the course of pre and postnatal development is unknown. In addition, NHE8 is predominantly an intracellular transporter and the total abundance of the transporter does not change in the cell during development, however there are marked changes in abundance on the apical membrane (11). The reason for the isoform change, what is mediating this change from NHE8 to NHE3, as well as the other factors that regulate NHE8 will be of interest and physiologic importance. In addition there may be circumstances in adults where NHE8 plays an important role in acidification outside the neonatal period. We are only beginning to understand the importance of this developmentally regulated Na+/H+ exchanger.
1. **Developmental Changes in Mouse NHE8 and NHE3 mRNA Expression:**
cDNA from mouse total kidney was analyzed by real-time RT-PCR normalized to 28s mRNA. NHE3 mRNA expression increased from day 1 to 7 days of age when it reached the level seen in adults. There was no significant change in NHE8 mRNA expression except at 24 days.

2. **Developmental Changes in Mouse Brush Border Membrane NHE3 and NHE8 Protein Abundance:**
Brush border membrane vesicles were harvested from mice from 1 day of age to adults. The relative protein abundance of NHE3 and NHE8 at the ages shown were compared relative to β-actin. As can be seen, there is a developmental increase in NHE3 and a decrease in NHE8.

3. **Effect of metabolic acidosis on NHE3 and NHE8 mRNA abundance:**
NHE3 and NHE8 cDNA from total mice kidney was analyzed by real time PCR normalized to 28s mRNA. There was no significant difference in the control verses acid groups for either NHE3 or NHE8 mRNA expression.

4. **Effect of metabolic acidosis on total cellular NHE3 and NHE8 protein abundance:**
Total protein was assayed for NHE3 and NHE8 using immunoblot. Metabolic acidosis resulted in an increase in NHE3 protein abundance, but there was no difference in NHE8 total protein abundance normalized to β-actin. NHE3 and NHE8 had the expected size of ~80 kDa.
5. **Effect of metabolic acidosis on brush border membrane NHE3 and NHE8 protein abundance:**

Brush border membranes were harvested from 8 day old neonatal mice that were gavaged with vehicle or ammonium chloride. The acidemic mice had an increase in brush border membrane NHE8 and NHE3 protein abundance compared to control mice. NHE3 and NHE8 had the expected size of ∼80 kDa and the results shown were normalized to β-actin.

6. **Effect of In Vivo Acidosis on Na⁺/H⁺ exchanger activity in mouse proximal convoluted tubules perfused in vitro:**

Proximal convoluted tubules were perfused in vitro from 8 day old mice that were gavaged with vehicle or ammonium chloride for 4 days. Na⁺/H⁺ activity was assessed by measuring the rate of change in cell pH after luminal sodium removal. Na⁺/H⁺ activity was significantly faster in the neonatal mice that were acidemic compared to control.

7. **Effect of Metabolic Acidosis on Neonatal Rat Brush Border Membrane Vesicle Na⁺/H⁺ exchanger activity:**

A. Na⁺/H⁺ activity from border membrane vesicles from neonatal rats was measured using the acridine orange technique. Brush border membrane vesicles from neonatal acidotic rats had a higher Na⁺/H⁺ activity than control. The Na⁺/H⁺ activity was inhibited by 10⁻⁶ M EIPA.

B. **Effect of EIPA on Adult Rat Brush Border Membrane Vesicle Na⁺/H⁺ exchanger activity**
$\text{Na}^+/\text{H}^+$ exchanger activity was measured in adults in the absence and presence of EIPA. The activity was several fold greater than that in neonates and was relatively resistant to EIPA.
Acknowledgments

This work was supported by NIH grant DK41612 and DK078596 to M.B, T32 DK07257, and the O'Brien Center P30DK079328.
Table 1. Primers used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC9A3 (NHE3)</td>
<td>F: 5’-TTC AAA TGG CAC CAC GTC CAG G-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-TGA CCT TGT GGG ACA GGT GAA AG-3’</td>
</tr>
<tr>
<td>SLC9A8 (NHE8)</td>
<td>F: 5’-ACA GTT TCG CAT TTG GCT CCC TG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-TGT TGG TGA GGA CGA TGG AGA CTG-3’</td>
</tr>
<tr>
<td>28S</td>
<td>F: 5’- TTG AAA ATC CGG GGG AGA G-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-ACA TTG TTC CAT GCC AG-3’</td>
</tr>
</tbody>
</table>
Table 2:

SOLUTIONS IN CELL pH STUDIES

<table>
<thead>
<tr>
<th>Salt Concentration</th>
<th>Bath Solution</th>
<th>Luminal Na⁺ Solution</th>
<th>Luminal ONa⁺ Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>140</td>
<td>115</td>
<td>-</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>5</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>NMDG-Cl</td>
<td>-</td>
<td>-</td>
<td>115</td>
</tr>
<tr>
<td>Choline HCO₃</td>
<td>-</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td>KCl</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>-</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>-</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urea</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Heptanoic Acid</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pH</td>
<td>6.6</td>
<td>7.4</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Salt concentrations are in mM. Solutions had an osmolality of 300 mOsm/kg H₂O.
Table 3.

**Effect of acid gavage on electrolytes and blood gas in neonatal mouse and rat.**

<table>
<thead>
<tr>
<th></th>
<th>Mouse Mean ± SEM</th>
<th>Rat Mean ± SEM</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HCO₃⁻</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>19.5 ± 1.0</td>
<td>21.3 ± 0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Acid</td>
<td>8.9 ± 0.6</td>
<td>11.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.39 ± 0.02</td>
<td>7.45 ± 0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Acid</td>
<td>7.15 ± 0.02</td>
<td>7.26 ± 0.02</td>
<td></td>
</tr>
<tr>
<td><strong>pCO₂</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>32.5 ± 2.2</td>
<td>30.5 ± 0.4</td>
<td>0.005</td>
</tr>
<tr>
<td>Acid</td>
<td>24.8 ± 1.2</td>
<td>24.5 ± 0.8</td>
<td></td>
</tr>
<tr>
<td><strong>Na⁺</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>153 ± 1</td>
<td>142 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td>Acid</td>
<td>151 ± 2</td>
<td>111 ± 1</td>
<td></td>
</tr>
<tr>
<td><strong>K⁺</strong></td>
<td></td>
<td></td>
<td>&lt;0.001 for mouse t</td>
</tr>
<tr>
<td>Control</td>
<td>3.1 ± 0.1</td>
<td>3.7± 0.1</td>
<td>ns for ra</td>
</tr>
<tr>
<td>Acid</td>
<td>4.6 ± 0.2</td>
<td>4.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td><strong>Cl⁻</strong></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Control</td>
<td>127 ± 2</td>
<td>116 ± 1</td>
<td></td>
</tr>
<tr>
<td>Acid</td>
<td>139 ± 2</td>
<td>122 ± 1</td>
<td></td>
</tr>
<tr>
<td><strong>BUN</strong></td>
<td></td>
<td></td>
<td>0.03 for mouse</td>
</tr>
<tr>
<td>Control</td>
<td>26 ± 2</td>
<td>15.1 ± 1</td>
<td>0.001 for rat</td>
</tr>
<tr>
<td>Acid</td>
<td>35 ± 3</td>
<td>23.6 ± 1</td>
<td></td>
</tr>
</tbody>
</table>
Table 4

**Effect of luminal sodium removal on cell pH**

<table>
<thead>
<tr>
<th></th>
<th>Luminal Na</th>
<th>Delta pH</th>
<th>0 Na</th>
<th>Delta pH</th>
<th>Luminal Na</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control pH</td>
<td>7.39±0.07</td>
<td>0.18±0.10</td>
<td>7.03±0.05*</td>
<td>0.22±0.12</td>
<td>7.37±0.07</td>
</tr>
<tr>
<td>Acid pH</td>
<td>7.26±0.13</td>
<td>0.41±0.08</td>
<td>6.82±0.11*</td>
<td>0.30±0.06</td>
<td>7.25±0.07</td>
</tr>
</tbody>
</table>

* P<0.003 vs luminal Na

+ P<0.02 vs luminal Na
Reference List


Figure 1. Ontogeny of NHE3 and NHE8 mRNA Expression in the Mouse Kidney by Real Time RT-PCR

* 24 day > all groups  P<0.001
† Adult > 1 day and 7 day P<0.04
**Figure 2a. NHE3 BBMV Expression**

![Graph showing NHE3 expression levels across different time points (1 day, 7 day, 14 day, 24 day, Adult). The x-axis represents time points, and the y-axis represents relative NHE3 protein expression. The graphs are labeled with statistical comparisons:

* 1 day < all groups but 7 day, P<0.05
† 7 day < 24 day and adult, P<0.001
‡ 24 day > all other groups, P<0.05

**Figure 2b. NHE8 BBMV Expression**

![Graph showing NHE8 expression levels across different time points (1 day, 7 day, 14 day, 24 day, Adult). The x-axis represents time points, and the y-axis represents relative NHE8 protein expression. The graphs are labeled with statistical comparisons:

* 1 day > all other groups, P<0.05
† 7 day > 24 day and adult, P<0.05
‡ 14 day > 24 day and adult, P<0.05

**NHE3**

<table>
<thead>
<tr>
<th>β-actin</th>
<th>1D</th>
<th>7D</th>
<th>14D</th>
<th>24D</th>
<th>AD</th>
</tr>
</thead>
</table>

**NHE8**

<table>
<thead>
<tr>
<th>β-actin</th>
<th>1D</th>
<th>7D</th>
<th>14D</th>
<th>26D</th>
<th>AD</th>
</tr>
</thead>
</table>
Figure 3. Effect of In Vivo Acidosis on NHE8 and NHE3 mRNA Abundance
Figure 4a. Effect of In Vivo Acidosis on Total Cellular NHE3 Protein Abundance

![Bar graph showing relative NHE3 protein expression](image)

Control | Acid
---|---
(9) | (9)

P<0.003

Figure 4b. Effect of In Vivo Acidosis on Total Cellular NHE8 Protein Abundance

![Bar graph showing relative NHE8 protein expression](image)

Control | Acid
---|---
(9) | (9)

P<0.003

<table>
<thead>
<tr>
<th></th>
<th>NHE3</th>
<th>β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>A</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>NHE8</th>
<th>β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>A</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 5a. Effect of in Vivo Acidosis on NHE3 BBM Protein Abundance

Figure 5b. Effect of in Vivo Acidosis on NHE8 BBM Protein Abundance

P<0.01

NHE3

β-actin

C A C A

NHE8

β-actin

C A C A
Figure 6

Effect of In Vivo Acidosis on Na+/H+ Exchanger Activity in Proximal Convoluted Tubules Perfused In Vitro

![](chart.png)

P<0.05
Figure 7a.
Effect of In Vivo Acidosis and 10^{-6}M EIPA on Neonatal Rat Na^+/H^+ Exchanger Activity in Brush Border Membrane Vesicles

Figure 7b.
Effect of 10^{-6}M EIPA on Adult Rat Na^+/H^+ Exchanger Activity in Brush Border Membrane Vesicles