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Nucleotide Sequence of the Na^+/H^+ Exchanger-8 in Patients With Congenital Sodium Diarrhea

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Abstract

Sodium absorption by the intestine is mediated by brush border Na^+/H^+ exchangers, which include the NHE3 and NHE8 isoforms. We demonstrated a maturational decrease in NHE8 and increase in NHE3 in mouse intestine mRNA abundance and brush border membrane protein abundance, indicating a developmental switch of isoforms. Congenital sodium diarrhea is a rare autosomal recessive disorder characterized by polyhydramnios, hyponatremia, metabolic acidosis, and diarrhea with a high sodium content. Previous studies using intestinal brush border membrane vesicles from patients with this disorder have demonstrated a decrease in Na^+/H^+ exchanger activity. Because some patients with congenital sodium diarrhea improve with age and knowing the developmental switch from NHE8 to NHE3, NHE8 may be a candidate gene for this disorder. We sequenced NHE8 from 5 patients with this disorder and found no disease-causing homozygous mutations. Although brush border membrane Na^+/H^+ exchange activity may be decreased, exonic mutations in NHE8 cannot account for this disorder in these subjects.

Keywords

congenital diarrhea; intestine; Na^+/H^+ exchanger

Congenital sodium diarrhea is a rare inherited disorder characterized by metabolic acidosis, dehydration, hyponatremia, and profuse secretory diarrhea that begins at birth (1-5). The stools have an alkaline pH and a high sodium content (3,4). Sodium absorption from the intestine is largely mediated by the apical membrane Na^+/H^+ exchanger (6). Studies from

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intestinal biopsies have shown that brush border membrane vesicles from these patients had impaired Na^+/H^+ exchanger activity (1,2,4), whereas sodium-dependent glucose transporter activity was intact (4). Thus, gene-inactivating mutations of the apical membrane Na^+/H^+ exchangers are logical underlying candidates for this disorder. However, previous studies using homozygosity mapping and multipoint linkage analysis had excluded NHE1, NHE2, NHE3, and NHE5 in the patients studied (5).

Recently, NHE8 has been cloned and shown to be a functional Na^+/H^+ exchanger (7,8). NHE8 is expressed in several organs, including the intestine and the kidney (7,9-13). As in the kidney, NHE8 expression is higher in the neonatal intestine than in the adult (12,13). Adult NHE3 $^{-/-}$ mice have impaired proximal tubule acidification and diarrhea with intestinal alkali loss consistent with presence and importance of NHE3 in the intestine (14). The role of NHE3 in the intestine is further testified to by the relatively mild phenotype when the NHE3 $^{-/-}$ mice were rescued by intestinal transgenic expression of NHE3 (15) and direct measurements of Na absorption rates in isolated jejuna from NHE3 $^{-/-}$ mice, which is about one-third that of wild-type controls (16). Adult NHE3 $^{-/-}$ mice also have evidence of volume depletion with increased plasma aldosterone and renal renin mRNA (14). Renal compensation includes increased renal α-ENaC (epithelial sodium channel) mRNA (renal β- and γ-subunits were unaffected) and anion exchanger-1 (14). In the colon, there was upregulation of colonic H^+-K^+-ATPase, colonic β- and γ-subunit-ENaC mRNA (not α-ENaC), and SLC 26A Cl^-/HCO_3^- exchanger expression (14,17).

Congenital sodium diarrhea has been shown to remit in some patients when they reach the first year of life (2). NHE8 is highly expressed in neonates in both the kidney and intestine and decreases in importance in mediating Na^+/H^+ exchange with the maturational increase in NHE3 (9,12). The present study examined whether congenital sodium diarrhea was caused by a mutation in NHE8.

MATERIALS AND METHODS

Animal Studies

Small intestines were harvested from 7-day-old and adult mice (2–3 months old). Upon harvest, the intestines were flushed with normal saline to remove intestinal contents. In adult mice, the intestines were then inverted and the brush border membranes were removed with gentle scraping with a scalpel blade. In neonatal mice, the entire small intestine was harvested. The adult intestinal scrapings and whole neonatal intestines were then placed in isolation buffer, which contained 300 mmol/L mannitol, 16 mmol/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, and 5 mmol/L ethylene glycol-bis (B-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid, which was titrated to a pH of 7.4 with Tris 1 μL/mL of protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO) and 100 μg/mL of phenyl-methyl-sulfonyl fluoride (Calbiochem, La Jolla, CA) also were added to the isolation buffer just before the intestinal samples were added. Brush border membrane vesicles (BBMVs) were isolated by differential centrifugation and magnesium precipitation, as previously described (18). The BBMVs were resuspended in radioimmunoprecipitation assay buffer (150 mmol/L NaCl, 50 mmol/L Tris, 5 mmol/L EDTA, 1% Triton X-100, 0.5%

deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]), and quantification of the final proteins was determined using the Bradford method (19).

Intestinal BBMVs (20 μg/lane) were suspended in 2.5× loading buffer (2.5 mmol/L Tris HCl [pH 6.8], 2.5% β-mercap-toethanol, 25% glycerol, and 2.5% SDS) and incubated at 85°C for 5 minutes for NHE3 and 37°C for 5 minutes for NHE8. The proteins were then separated on a 7.5% polyacrylamide SDS gel and then transferred to a polyvinylidene difluoride membrane. Fresh Blotto (5% nonfat milk and 0.05% Tween 20 in phosphate buffered saline, pH 7.4) was used for blocking the membranes for 1 hour before incubation overnight at 4°C with the primary antibodies to either NHE8 or NHE3. The NHE8 antibody was the monoclonal antibody 7A11 (gift from Dr Peter Aronson) 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 to 822 of NHE3 at a 1:1000 dilution (11,20). The blots were then washed with Blotto, and the secondary antibodies, horseradish peroxidase–conjugated donkey anti-mouse immunoglobulin for NHE8 and horseradish peroxidase–labeled anti-rabbit immunoglobulin for NHE3 were added at 1:10,000 dilution. The blots were then washed with Blotto and bound antibody was detected using enhanced chemiluminescence (Amersham Life Science, Chicago, IL). An antibody to β-actin (1:15,000 dilution; Sigma, St Louis, MO) was used to confirm equal loading of the samples. Protein abundance was quantified by densitometry using the Scion Image software (Scion Corporation, Frederick, MD).

cDNA Synthesis and Real-time Polymerase Chain Reaction

RNA was isolated from the jejunum of 7-day-old and 2- to 3-month-old adult mice using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St Louis, MO). Total RNA was treated with DNAse I (Invitrogen, Carlsbad, CA) and the product was used to synthesize cDNA using random hexamer primers and reverse transcriptase (Stratagene, La Jolla, CA) at an annealing temperature of 25°C for 10 minutes, extension at 42°C for 50 minutes, and termination at 70°C for 15 minutes. An iCycler PCR (polymerase chain reaction) Thermal Cycler (Bio-Rad, Hercules, CA) was used for real-time PCR to quantify relative mRNA abundance. Primers for NHE3 (forward: 5′-TTC AAA TGG CAC CAC GTC CAG G-3′; reverse: 5′-TGA CCT TGT GGG ACA GGT GAA AG-3′) and NHE8 (forward: 5′-TGA CCT TGT GGG ACA GGT GAA AG-3′; reverse: 5′-AGC GAA GAG AGT GAT GGA ACC G-3′) were mixed with cDNA and SYBR Green master mix as per the manufacturer’s instructions (Bio-Rad). The PCR conditions were denaturation at 94°C for 30 seconds, annealing at 61°C for 20 seconds, and extension at 72°C for 20 seconds for 40 cycles. The housekeeping gene 28s (forward: 5′-TTG AAA ATC CGG GGG AGAG-3′; reverse: 5′-ACATTG TTC CAT GCC AG-3′) was used to normalize the relative expression of mRNA.

Human Subjects

The patients studied presented in the newborn period with intractable watery diarrhea with an alkaline pH and high sodium content that did not abate when oral feeds were withheld. The patients had metabolic acidosis and hyponatremia. There were no endocrine causes for the diarrhea and the diarrhea persisted throughout life. None of the patients had

characteristics of syndromic congenital sodium diarrhea such as corneal erosions, hypertelorism, or choanal or anal atresia caused by *SPINT2* loss of functional mutations (21).

Sequencing

DNA was collected from blood or saliva samples using the Oragene DNA/saliva kits. DNA was isolated using established methods recommended bythemanufacturer(http://www.dnagenotek.com). Exons and exon/intron boundaries were amplified by PCR using AmpliTaq Gold polymerase (Applied Biosystems Inc, Foster, CA). DNA sequence was obtained using an ABI Big Dye Terminator Cycle Sequencing Kit, and sequence analysis was performed on an ABI 3100 Genetic Analyzer (Applied Biosystems). All of the exons were sequenced along with approximately 50 bps of flanking intronic sequence.

The present study was approved by the institutional review board of the University of Texas Southwestern Medical Center. Informed consent was obtained from patients or their legal guardians.

RESULTS

In the first series of experiments, we examined the developmental changes in 2 intestinal Na^+/H^+ exchangers, NHE3 and NHE8, in mouse intestine. As shown in Figure 1, there is a maturational increase in jejunal NHE3 and decrease in NHE8 mRNA. In the brush border membranes, there was an increase in NHE3 protein abundance, whereas there was a decrease in NHE8 protein abundance with postnatal age. Because some patients with congenital sodium diarrhea improve with age and NHE8 is the only NHE expressed in the intestine that has not yet been examined as a cause for congenital sodium diarrhea, we sequenced NHE8 as a candidate gene in 5 patients with congenital sodium diarrhea. The patient data are shown in Table 1.

We sequenced the immediate promoter region, exons, and intron/exon boundaries of NHE8 and failed to identify any disease-causing base changes in the NHE8 gene in any of the patients. We found a heterozygous 8-bp insertion (compared with the published genome sequence) in the 5′-UTR of NHE8 in 2 patients, but we also observed a similar change in 2 of 40 normal individuals we examined (Fig. 2). We also found a heterozygous 4-bp deletion in intron 8 about 90 bp 5′ of the intron-exon boundary in 3 of 4 patients. Likewise, we found this deletion in 30 of the 40 normal individuals sequenced. Thus, we did not find mutations in NHE8 to explain the diarrhea in these patients.

DISCUSSION

Congenital sodium diarrhea has been shown to be associated with defects in brush border membrane Na^+/H^+ exchange activity (1,2,4). Although an NHE8 null mouse has not been examined, the phenotype of patients with congenital sodium diarrhea resembles mice lacking NHE3 (14). In the present study, we show that NHE8 and NHE3 are on the brush border membranes of mouse intestine and undergo maturational switch with a decrease in NHE8 and increase in NHE3 brush border protein with age. A maturational change in NHE3

and NHE8 mRNA abundance also was found. We sequenced NHE8 in 5 patients with congenital sodium diarrhea and found no homozygous mutations.

A recent study examined the genetic cause for a syndromic form of congenital sodium diarrhea. They examined 24 patients from various parts of the world and found several inactivating mutations in *SPINT2* in patients from 10 families (21). All of the patients who had a mutation in *SPINT2* had hypertelorism, corneal erosions, and either choanal or anal atresia in addition to congenital sodium diarrhea. In addition, the small bowel histology was not normal in these subjects because blunt villi and long crypts also were identified. Interestingly, none of the patients studied from 7 families without these associated abnormalities had a mutation in *SPINT2*. None of our 5 subjects had extraintestinal manifestations of the syndromic form of this disorder. How SPINT2, a serine protease inhibitor, causes these malformations and congenital sodium diarrhea is unknown at present.

A previous study had examined whether mutations NHE1, NHE2, NHE3, and NHE5 were associated with congenital sodium diarrhea (5). Although the present study used heterozygosity mapping linkage analysis to exclude these genes, all of the patients were from the same rural area of Austria and had a common ancestor 5 generations back. It is now clear that congenital sodium diarrhea is not 1 disease with mutations in a single locus (21). There is both phenotypic and genotypic heterogeneity. Some patients have isolated diarrhea and some have syndromic multisystemic features (21). There are patients with profound diarrhea who despite medical treatment die shortly after birth, whereas there are patients who improve significantly and spontaneously with age (21). Based on this latter observation and the fact that NHE8 is expressed in the intestine and has not been examined previously, we fathomed that NHE8 is a logical potential candidate gene that deserved further study. Of our patients, 1 died in infancy, but the others lived and still have diarrhea. The diarrhea of patient 2 is still as severe, but occasionally she has more solid stools; the other 3 patients have improved compared with when they were an infant.

The findings of a maturational increase in NHE3 mRNA and protein abundance confirm previous work in the rat (22). Although it has recently been shown that NHE8 is more highly expressed in a 3-week-old intestine compared with an adult rat (13), the present study showed that there is a more profound maturational decrease in brush border membrane protein abundance in NHE8 when comparing a 7-day-old mouse to an adult. We also find a maturational increase in NHE3 mRNA and decrease in NHE8 mRNA abundance. There are other potential candidate genes that could cause congenital sodium diarrhea. The fact that there is a decrease in Na^+/H^+ exchanger activity and NHE2 is expressed on the apical membrane makes it a potential candidate gene (23); however, unlike NHE3 $^{-/-}$ mice that have profound diarrhea and metabolic acidosis, NHE2 $^{-/-}$ mice do not have diarrhea nor do they have a metabolic acidosis (24). Furthermore, NHE2 has no compensatory role in mitigating the diarrhea in NHE3-deficient mice, because NHE3/NHE2 double-deficient mice have diarrhea and acid-base status comparable to NHE3 null mice (25) and direct measurements of Na flux in NHE2 $^{-/-}$ animals were comparable to wild-type controls (16). Compensatory upregulation of NHE3 may in part account for the lack of phenotype in NHE2 null animals (26). In addition, numerous proteins interact either directly or indirectly with NHE3, which regulates its function (27). Obviously, these proteins are candidate genes

that need to be considered in identifying the molecular basis of the nonsyndromic form of congenital sodium diarrhea.

In summary, the cause for nonsyndromic congenital sodium diarrhea remains elusive. It is clear that congenital sodium diarrhea is not the result of a single mutation in a specific gene. The specific absence of Na^+/H^+ exchange activity is an important clue in determining the cause, but our results show that in these 5 patients, NHE8 did not have any significant mutations. Perhaps newer genetic techniques will help elucidate the genetic abnormality.

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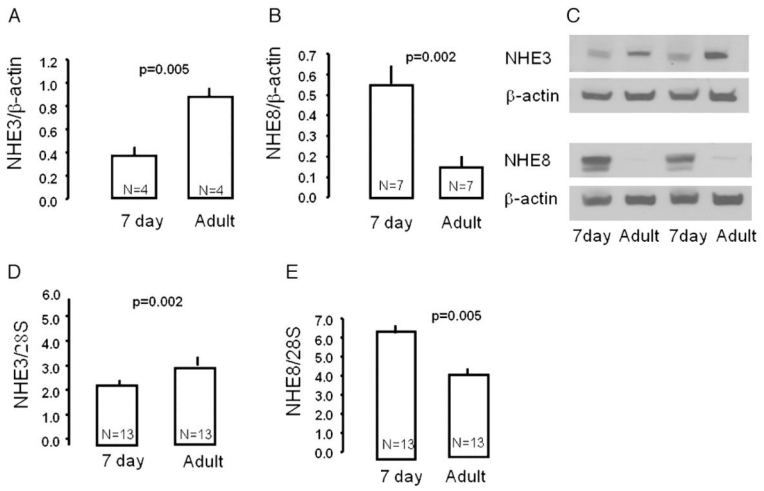

FIGURE 1.

NHE3 and NHE8 mRNA and protein abundance in neonatal and adult intestine. mRNA was obtained from neonatal and adult jejunum and quantified using real-time polymerase chain reaction. Brush border membrane vesicles were harvested from adult and 7-day-old neonatal mouse intestines. There was a maturational increase in brush border membrane NHE3 protein abundance (A) and decrease in NHE8 protein abundance (B). A typical immunoblot is shown in panel C. There was also a maturational increase in NHE3 mRNA abundance (D) and a decrease in NHE8 mRNA abundance (E).

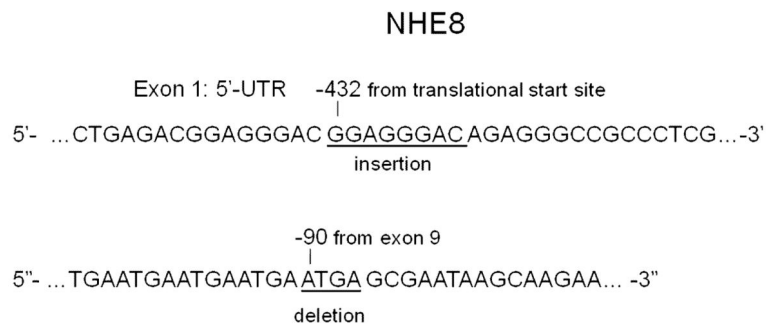

FIGURE 2.
Sequencing results of NHE8 in 5 patients and 40 normal subjects.

TABLE 1

Patient characteristics

Patient no.	1	2	3	4	5
History of polyhydramnios	Yes	Yes	Yes	Yes	Yes
Sex	F	F	F	M	F
Neonatal onset of diarrhea that persists with age	Yes	Yes	Yes	Yes	Yes
Secretory diarrhea with high sodium content	Yes	Yes	Yes	Yes	Yes
Country of birth	U.K.	Finland	Brazil	U.S.	U.S.
Associated birth defects	No	No	No	No	No
Consanguinity	No	No	No	No	No
Present age or age at death	Alive-29 y	Alive-32 y	Dead-5 mo	Alive-9 y	Alive-9 y