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Expression of rat, renal NHE2 and NHE3 during postnatal developmental

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Abstract

The current studies were designed to characterize the expression of sodium–hydrogen exchangers NHE2 and NHE3 during rat, renal ontogeny. NHE2 mRNA and immunoreactive protein were more highly expressed at 2 and 3 weeks of age, with declining levels into adulthood. In situ hybridization of NHE2 mRNA localized the message to the renal inner cortex and outer medullary regions and suggested higher mRNA levels in suckling animals as compared to adults. Immunohistochemical analysis of rat kidney with the NHE2 antiserum showed specific staining of the distal convoluted tubules. In contrast, NHE3 mRNA expression was lowest in 2-week animals and higher in older rats, while NHE3 immunoreactive protein showed constant expression levels during development. Additionally uptake experiments utilizing HOE694 showed no change in NHE2 or NHE3 functional protein expression in 2-week-old rats versus adults. We conclude that the developmental increase in NHE2 mRNA and immunoreactive protein expression cannot be detected by functional assays, which suggests that NHE2 does not play a role in sodium absorption by the renal tubules (as has been previously suggested). Additionally, molecular changes seen in NHE3 mRNA expression do not affect functional protein activity, suggesting increased mRNA translational efficiency or protein stability in suckling rats.

Keywords: Sodium–hydrogen exchanger; Kidney; Ontogeny; HOE694

1. Introduction

The sodium–hydrogen exchangers (NHE) are ubiquitous, integral membrane proteins that mediate the exchange of extracellular Na^+ for intracellular H^+. Several mammalian isoforms of NHE have been identified over the past decade (NHE1 through NHE6) and the physiological role of many of the isoforms has been postulated from functional studies, analysis of tissue distribution, and gene inactivation studies. Four NHE isoforms are known to exist in the mammalian kidney, NHE1–4 [1–4]. Previous studies suggested that NHE1 and NHE4 are expressed on the basolateral membrane (BLM) in cultured cells of renal origin and in the renal tubules. NHE1 was shown to be on the BLM in rabbit, renal collecting duct (RC.SV3) cells [5], in various renal tubular segments from rabbit and rat kidney [6–8], and in OK7a [9] and LLC-PK1 cells [10,11]. However, another recent report exemplified NHE1 targeting to both basolateral and brush-border membranes

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(BBM) in MDCK, HT-29 and OK cells [12]. NHE4 has also been localized to the BLM in the epithelium of the rat, renal tubules [13].

Additional previous investigations showed that NHE2 and NHE3 are exclusively BBM proteins in the mammalian kidney. NHE2 was found on the BBM in RC.SV3 cells [5], in LLCPK-1 cells [10], and in the apical membrane of the cortical and medullary thick ascending limbs, the distal convoluted tubules, and the connecting tubules of rat and mouse kidney [14–16]. NHE3, meanwhile, was shown to be apically expressed in the rat renal tubules [7,17–19], in OK and MDCK cells [12] and in rabbit and dog kidney [20,21]. Overall, with respect to these studies concerning the renal, apical isoforms, NHE2 was shown to be expressed in the renal medulla, while NHE3 was shown to be expressed in the thick and thin loop of Henle of the renal cortex and outer medulla. Based upon these localization studies and previous studies by other groups, it seems likely that NHE3 plays a physiological role in transepithelial Na^+ absorption, while the role of NHE2 is more likely related to regulation of cell volume in response to the hyperosmolar conditions found in the renal medulla.

Our group has previously characterized the expression of the NHE1 and NHE4 isoforms in the mammalian kidney and intestine during ontogeny [22]. These investigations showed that NHE1 and NHE4 mRNA are expressed at varying levels during maturation in the rat (although no NHE4 was detected in intestine). We concluded that these NHE isoforms most likely play a role in differential BLM NHE activity observed during development in the kidney but not in the intestine [22]. Other previous investigations looked at the expression of NHE2 and NHE3 in the developing rat small intestine [23,24]. These studies showed ontogenic changes in expression and they further demonstrated that NHE2 and NHE3 contribute almost equally to basal NHE activity in suckling and weanling rats, but in older animals, NHE3 is the predominant isoform. Since these studies, concerning developmental expression of the NHE isoforms in the mammalian intestine and kidney, have revealed important information concerning possible regulatory mechanisms and potential physiological roles, we felt it was important to document the ontogenic expression of NHE2 and NHE3 in the rat kidney. Therefore, the current investigation was undertaken to precisely define the developmental expression of the apical NHEs in the rat kidney at a functional and molecular level.

2. Materials and methods

2.1. Animals

Sprague–Dawley rats were used in groups of at least four animals for all experiments. Male rats of the following ages were used for all studies: 2 week (sucklings), at 14 days of age; 3 week (weanlings), at 21 days of age; 6 week (adolescents), at 42–45 days of age; and adult, at 4–6 months of age. Animals were maintained in overhanging cages with food and water supplied ad libitum. Animals were subjected to CO_2 narcosis, and killed by cervical dislocation.

2.2. Chemicals and reagents

Poly(A)$^+$ RNA was isolated utilizing the FastTrack kit from Invitrogen (La Jolla, CA). ^{22}Na (100–2000 Ci (3.70–74.0 Tbq)/g) for uptake studies, and α-[^{32}P]dCTP (3000 Ci/mmol) for Northern blot analyses were purchased from New England Nuclear (Boston, MA). Radioactive probes for Northern blot analyses were generated by random prime labeling using the RediPrime system from Amersham Life Science (Piscataway, NJ). Nitrocellulose membranes (Nitroplus) were from Micron Separations (Westboro, MA). HOE694 was kindly provided by Dr. H.J. Lang, Hoechst Marion Roussel Pharmaceuticals (Frankfurt am Main, Germany). DNA fragments were gel purified utilizing the GeneClean kit from BIO101 (Vista, CA). Protein was quantitated by a Bradford assay utilizing the Bio-Rad Protein Assay reagent (Hercules, CA). All other chemicals and reagents were purchased from Fisher Biotechnology (Pittsburgh, PA), or Sigma (St. Louis, MO).

2.3. Northern blot analysis

Poly(A)$^+$ RNA was isolated from kidneys of rat groups utilizing a commercially available kit. Northern blots were carried out as previously described

using 5 μg poly(A)$^+$ RNA per gel lane [25,26]. NHE isoform-specific cDNA fragments [24] were used as templates to generate radiolabeled probes. High stringency washes were performed at 65°C with 0.1×SSC, 0.1% SDS, and blots were placed to a phosphorimaging screen or film. Northern blots were stripped and subsequently reprobed with 1B15 (encoding rat cyclophilin [24]) specific probes. Quantitation of hybridization signals was done by phosphorimage analysis utilizing volume integration (GS 525 Molecular Imager; Bio-Rad, Hercules, CA). Northern blot experiment was performed multiple times with poly(A)$^+$ RNA samples isolated from different groups of animals, with hybridization intensities being averaged from the different experiments. NHE hybridization intensities were normalized for 1B15 levels on the same blot.

2.4. In situ hybridization

RNA probes were produced from the 5′ and 3′ ends of NHE2, regions that show no nucleotide sequence similarity with the other NHE isoforms. Probes were generated in the presence of ^{35}S-labeled UTP, and 5×10^5 cpm per μl of reaction solution were used for hybridizations to paraformaldehyde fixed, paraffin embedded tissue sections. The entire experimental protocol has been described previously [27], and it was followed exactly in the present investigation.

2.5. Brush-border membrane vesicle (BBMV) preparation

Brush-border membrane vesicles (BBMV) were prepared from groups of at least four animals by the $MgCl_2$ precipitation technique essentially as previously described [28,29]. The final BBMV pellets were resuspended in either preincubation buffer for no pH gradient condition ($pH_i/pH_o = 7.5/7.5$) (100 mM TMA-gluconate, 85 mM HEPES, 45 mM Tris/HCl (pH 7.5)), or pre-incubation buffer with outwardly directed pH gradient ($pH_i/pH_o = 5.2/7.5$) (100 mM TMA-gluconate, 90 mM MES, 40 mM HEPES/Tris (pH 5.2)), and placed at 25°C for 1 h. Protein was quantitated by a Bradford protein assay. The purity and enrichment of membrane preparations was assessed by the measurement of alkaline phosphatase activity as previously described [30]. Membrane preparations were used on the day of preparation for uptake analysis or Western blot analyses and were never frozen.

2.6. Western blot analysis of rat renal BBM proteins with NHE2 and NHE3 specific antiserum

Kidneys were harvested, decapsulated and renal BBMV were purified by a $MgCl_2$ precipitation method as described previously. Twenty micrograms of protein was placed in a 2-fold excess of Laemmli solubilization buffer plus 2 mM β-mercaptoethanol (β-ME), boiled for 4 min and placed on ice. Protein samples were fractionated by 4–12% gradient SDS polyacrylamide gel electrophoresis (SDS/PAGE), and transferred onto nitrocellulose membranes. Blots were processed as previously described with the Renaissance chemiluminescent system (NEN/Dupont; Boston, MA) with NHE2 (at 1:500 dilution) or NHE3 specific (at 1:4000 dilution), polyclonal antisera. NHE2 antiserum was raised in rabbits against a multiple antigen peptide (MAP) specific for amino acids 652–661 of the putative rat NHE2 protein and has been characterized elsewhere [23]. NHE3 antiserum was similarly raised in rabbits against an NHE3 fusion protein from the COOH-terminal portion of the putative NHE3 protein and has likewise been extensively characterized elsewhere [22,24]. Membranes were stripped and subsequently reacted with β-actin antiserum (Sigma, St. Louis, MO) at 1:5000 dilution. NHE2 and NHE3 specific band intensities were determined by densitometric analysis (utilizing GS-700 Imaging Densitometer and Molecular Analyst software (Bio-Rad, Hercules, CA)) and were normalized for β-actin band intensities on the same blot. Experiments were repeated multiple times with protein samples isolated from different groups of animals.

2.7. Immunohistochemical analysis of rat kidney with NHE2 antiserum

Renal tissue was harvested from 3-week-old rats, fixed in paraformaldehyde, embedded in paraffin, and sections were cut and affixed to slides. Slides were blocked by overnight incubation with 5% goat serum at room temperature in a humidified chamber.

Slides were processed utilizing an immunoperoxidase staining system (ABC Elite system; Vector, Burlingame, CA) exactly as previously described [31]. NHE2 antiserum was reacted with sections for 1 h at 1:100 dilution in 0.1% normal goat serum. Some sections were reacted with pre-immune serum (1:100) and immunogenic peptide pre-treated serum (400 mg/ml at 25°C overnight) at 1:100 dilution. Slides were subsequently visualized and photographed by standard light microscopy and were analyzed by two 'blinded' observers to gain independent opinions about staining intensity and localization.

Table 1
Results of NHE2 and NHE3 Northern and Western blot experiments

(A) Northern blot results				
Age	Probe	Value	Probe	Value
2 weeks	NHE2	1.01 ± 0.74[a]	NHE3	0.84 ± 0.09[b]
3 weeks	NHE2	0.920 ± 0.12	NHE3	1.43 ± 0.16
6 weeks	NHE2	0.571 ± 0.58	NHE3	1.40 ± 0.11
Adult	NHE2	0.326 ± 0.10	NHE3	1.25 ± 0.11
(B) Western blot results				
Age	Serum	Value	Serum	Value
2 weeks	NHE2	0.296 ± 0.003[c]	NHE3	0.713 ± 0.094[d]
3 weeks	NHE2	0.283 ± 0.017	NHE3	0.659 ± 0.138
6 weeks	NHE2	0.234 ± 0.018	NHE3	0.637 ± 0.188
Adult	NHE2	0.207 ± 0.003	NHE3	0.558 ± 0.160

The data for Northern and Western blot experiments are depicted. Panel A shows Northern blot data and panel B shows Western blot data. In panel A, the column just right of the probe column shows the corresponding data, while in panel B, the column just to the right of the serum column shows the corresponding data.

[a]For NHE2 Northern blots, $n=5$ for all groups; $P=0.0036$ for 2 week versus 6 week, $P<0.0001$ for 2 week versus adult, $P=0.0158$ for 3 week versus 6 week, and $P=0.0003$ for 3 week versus adult.

[b]for NHE3 Northern blots, $n=6$ for all groups; $P=0.0026$ for 2 week versus 3 week, $P=0.0039$ for 2 week versus 6 week, and $P=0.0282$ for 2 week versus adult.

[c]For NHE2 Western blots, $n=4$ for all groups; $P=0.0049$ for 2 week versus 6 week, $P=0.0003$ for 2 week versus adult, $P=0.0185$ for 3 week versus 6 week and $P=0.0011$ for 3 week versus adult.

[d]For NHE3 Western blots, $n=13$ for 6-week samples, $n=14$ for adult samples, and $n=12$ for 2- and 3-week samples.

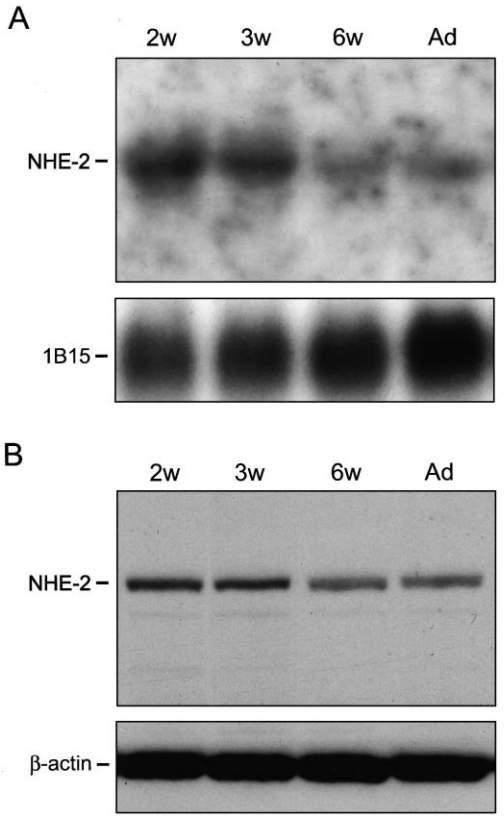

Fig. 1. Northern and Western blot analysis of NHE2. Renal mRNA and BBM proteins were purified and separated by electrophoresis. For Northern blots, NHE2 specific cDNA probes were utilized and for Western blots, NHE2-specific antiserum was used. Panel A shows one typical Northern blot experiment. NHE2 hybridization signal is shown above at ~4.4 kb and 1B15 signal is shown below at ~1.0 kb. Panel B shows a typical Western blot experiment. NHE2-specific signal is shown above at ~90 kDa and β-actin signal is shown below at ~42 kDa. In both panels, 2w is 2 weeks, 3w is 3 weeks, 6w is 6 weeks, and Ad is adult. Data are depicted graphically in Fig. 2.

2.8. Uptake analysis of renal BBMV

Uptake of radiolabeled sodium was measured by a rapid filtration technique as previously described [25,29]. All incubations were done at 25°C. Briefly, transport was initiated by adding 20 μl of the final BBMV suspension to 80 μl incubation solution (same as pre-incubation buffer (pH 7.5), with the addition of 31.25 μCi ^{22}Na/10 ml). Some studies were performed in the presence of 50 μM HOE694, a specific NHE inhibitor which allows selective inhibition of NHE2 (K_i 5 μM in PS120 cells) while NHE3 is unaffected (K_i 650 μM in PS120 cells) [32]. HOE694

was prepared for use by dissolving solid HOE694 in dimethylsulfoxide (DMSO) to make a 100-mM working stock solution, and was used within 90 min (an equivalent concentration of DMSO was added to control samples). The reactions were stopped after 10 s by the addition of 2 ml ice-cold stop solution (185 mM potassium-gluconate, 10 mM Tris, 16 mM HEPES, 0.1 mM amiloride). The vesicles were immediately collected on a cellulose nitrate filter (0.45 μm pore size) and kept under suction while they were washed with 5 ml ice-cold stop solution. The amount of radioactive substrate remaining on the filter was determined in a Beckman liquid scintillation counter, with ReadySafe (Beckman; Fullerton, CA) as the liquid scintillant. Radioactivity remaining in the filters after pipetting incubation medium into the radioactive substrate in the absence of vesicles was used as background, and was considered in all calculations. Experiments from all groups of animals were carried out on the same day. Uptake values were determined by subtracting the uptake levels with no pH gradient condition ($pH_i/pH_o = 7.5/7.5$) from those with outwardly directed pH gradient ($pH_i/pH_o = 5.2/7.5$). All values are expressed as nmol of sodium uptake per mg of vesicle protein. Graphical representation is expressed as mean ± S.E.M. of four uptake assays for each age group.

2.9. Statistical analysis of results

Data from uptake studies, and Northern blot and Western blot analyses were analyzed for statistical significance by one-way ANOVA, followed by Fischer's PLSD test, utilizing the StatView program (Abacus Concepts; Berkeley, CA), and are presented as mean ± S.E.M. with n, and P values reported.

3. Results

3.1. Northern blot analysis

Poly(A)$^+$ RNA was purified from groups of rats at various ages, fractionated by denaturing agarose gel electrophoresis and transferred to nitrocellulose membranes. Blots were probed with either NHE2 or NHE3 cDNA specific probes, then stripped and reprobed with 1B15 cDNA specific probes. Results showed that NHE2 mRNA expression was highest at 2 and 3 weeks of age and decreased in 6-week-old and adult animals (Table 1). Results with NHE3

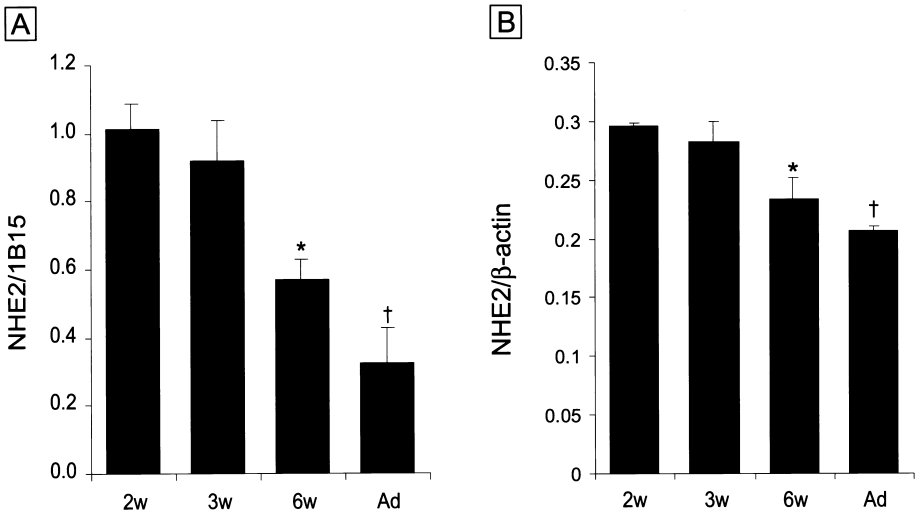

Fig. 2. Quantitation of NHE2 Northern blot and Western blot analyses. Data from all experiments were analyzed for statistical significance as described in Section 2. Panel A shows data from NHE2 Northern blot experiments ($n = 5$) and panel B shows data from NHE2 Western blot experiments ($n = 4$). Actual data points and P values are presented in Section 3. In both panels, 2w is 2 weeks, 3w is 3 weeks, 6w is 6 weeks and Ad is adult. In panel A, * indicates significance between 6w and 2w and between 6w and 3w; † indicates significance between Ad and 2w and between Ad and 3w. In panel B, * indicates significance between 6w and 2w and between 6w and 3w and † indicates significance between Ad and 2w and between Ad and 3w.

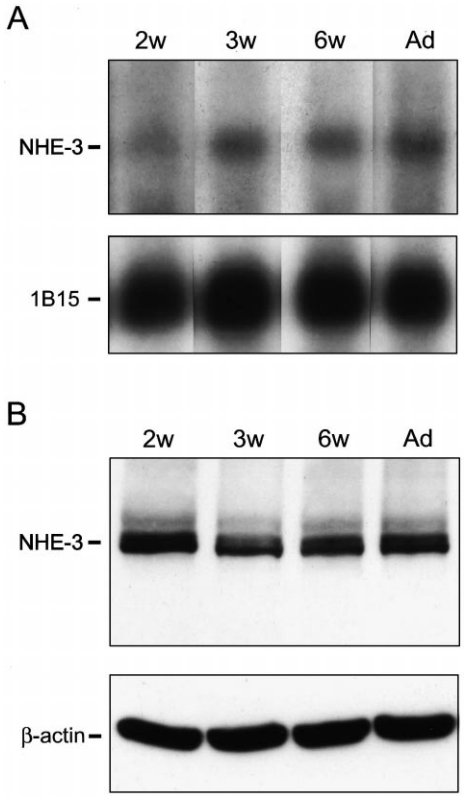

Fig. 3. Northern and Western blot analysis of NHE3. Renal mRNA and BBM proteins were purified and separated by electrophoresis. For Northern blots, NHE3 specific cDNA probes were utilized and for Western blots, NHE3-specific antiserum was used. Panel A shows one typical Northern blot experiment. NHE3 hybridization signal is shown above at ~4.4 kb and 1B15 signal is shown below at ~1.0 kb. Panel B shows a typical Western blot experiment. NHE3-specific signal is shown above at ~85 kDa and β-actin signal is shown below at ~42 kDa. In both panels, 2w is 2 weeks, 3w is 3 weeks, 6w is 6 weeks, and Ad is adult. Data are depicted graphically in Fig. 4.

specific probes showed lower mRNA expression in 2-week-old rats and higher expression in the other age groups (Table 1). A typical experiment with NHE2 specific probes is seen in Fig. 1A and with NHE3 specific probes in Fig. 3A. These data are depicted graphically in Fig. 2A (NHE2) and Fig. 4A (NHE3).

3.2. *In situ hybridization analysis*

Kidney tissue was harvested from 2-week-old and adult rats, fixed in paraformaldehyde, sectioned and affixed to slides. Slides were reacted with NHE2 specific probes. Results showed higher NHE2 mRNA expression in 2-week-old animals than in adults (Fig. 5). NHE2 message expression was evident in both ages in the inner cortex and outer medulla with minimal expression seen in the outer rim of the cortex and in the inner medulla.

3.3. *Western blot analysis of rat kidney BBM proteins with NHE2 and NHE3 specific antisera*

Rat kidneys were harvested and BBM proteins were purified and separated by SDS/PAGE. Proteins were then electroblotted onto membranes and membranes were reacted with polyclonal antisera specific for NHE2 or NHE3 and monoclonal antibodies specific for β-actin. Results with NHE2 serum showed higher immunoreactive protein levels in 2- and 3-week-old animals than in 6-week-old animals and adults (Table 1). Experiments with NHE3 antiserum showed no changes with samples from any of the age groups (Table 1). A typical NHE2 Western blot is seen in Fig. 1B and a typical NHE3 Western blot is seen in Fig. 3B. These data are depicted graphically in Fig. 2B (NHE2) and Fig. 4B (NHE3).

3.4. *Immunohistochemical analysis of rat kidney with NHE2 polyclonal antiserum*

Renal tissue was harvested from 3-week-old rats, fixed, sectioned and affixed to slides. Tissue was reacted with NHE2 specific polyclonal antiserum, antiserum that was pre-treated with the antigenic peptide or pre-immune serum. Results showed no staining in the peptide blocked or pre-immune samples, while specific staining of the apical membranes in the distal convoluted tubules was apparent in the unblocked sample. No staining was noted in the proximal convoluted tubules or in the glomeruli. One typical experiment is depicted in Fig. 6.

3.5. *Uptake analysis of renal BBMV*

Renal BBMV were purified from 2-week-old and adult rats, since these were the groups that showed the greatest differences in mRNA and protein expression for NHE2 and NHE3. Results showed no differences in initial rate uptake between 2-week rats or adults (2-week, 1.23 ± 0.25 nmol/mg protein/10 s; adult, 0.98 ± 0.11) and, furthermore, no effect was

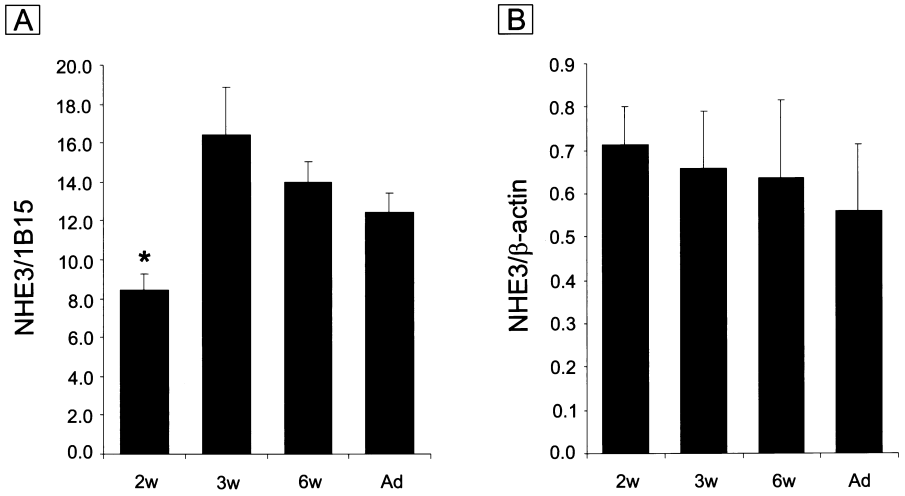

Fig. 4. Quantitation of NHE3 Northern blot and Western blot analyses. Data from all experiments were analyzed for statistical significance as described in Section 2. Panel A shows data from NHE3 Northern blot experiments ($n=6$) and panel B shows data from NHE2 Western blot experiments ($n=12$–14). Actual data points and P values are presented in Section 3. In both panels, 2w is 2 weeks, 3w is 3 weeks, 6w is 6 weeks and Ad is adult. In panel A, * indicates significance between 2w and all three other groups.

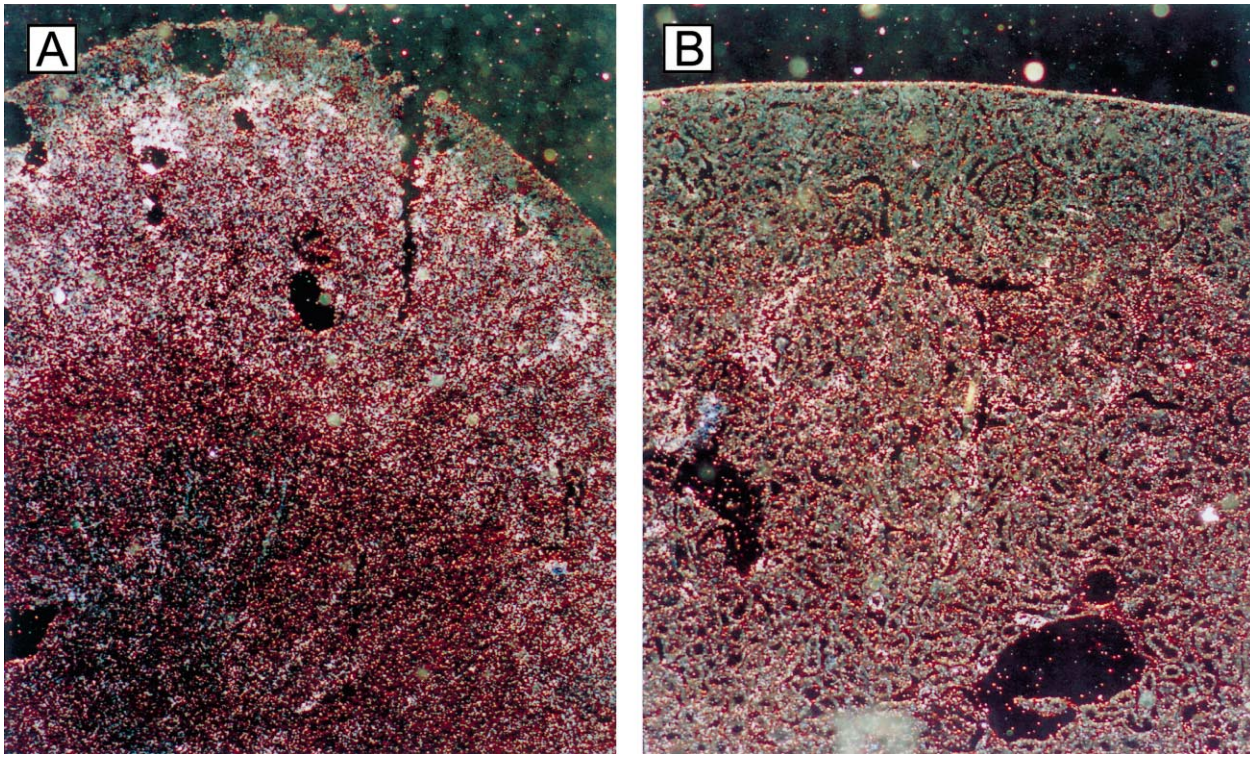

Fig. 5. In situ hybridization analysis of NHE2 mRNA in rat kidney. NHE2 mRNA was localized in 2-week (panel A) and adult rat (panel B) kidney sections. The whitish/pinkish color indicates hybridization with NHE2-specific probes. Hybridization intensity is decreased in the adult rat sample. Panel A clearly shows hybridization in the inner cortex and outer medulla only (the same pattern was observed in samples from adults). Magnification: panel A, ×75; panel B, ×150.

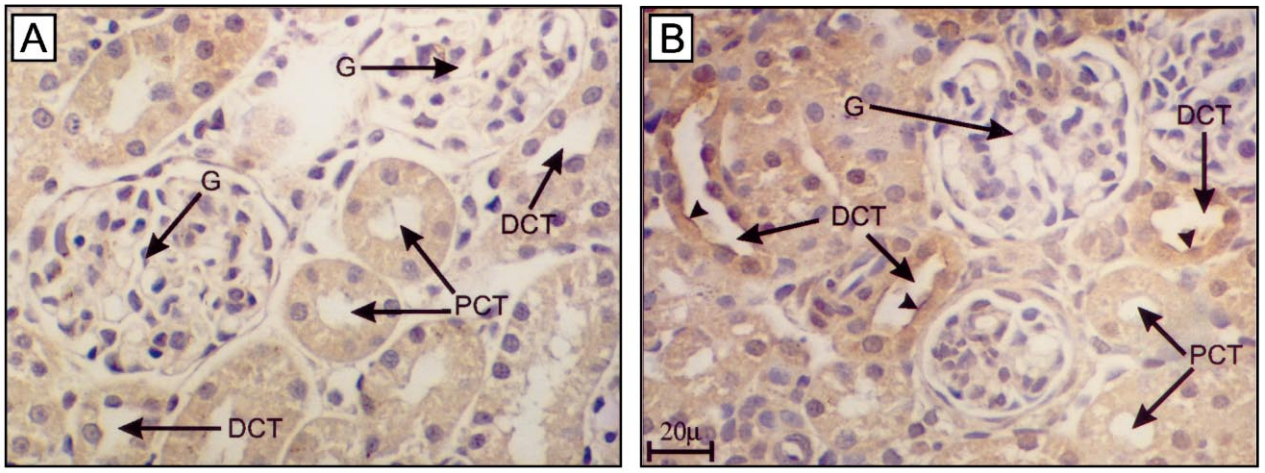

Fig. 6. Immunohistochemical analysis of NHE2 immunoreactive protein in rat kidney. NHE2 protein was localized in 3-week-old rat kidney in order to exemplify specificity of the antiserum for the NHE2 immunoreactive protein. Specific staining of the distal convoluted tubules (DCT) in the renal cortex was evident (panel B), while no staining of the proximal convoluted tubules (PCT), or the glomeruli (G) was observed. Additionally, no staining was apparent with antigenic peptide-blocked antiserum (panel A) or with pre-immune serum (not shown).

seen with 50 μM HOE694 (2-week, 1.13 ± 0.27; adult, 0.99 ± 0.11) ($n = 4$ for all groups). These data are depicted graphically in Fig. 7.

4. Discussion

The current studies exemplify expression of the renal NHE2 and NHE3 isoforms in the rat kidney during development. Initial experiments showed that NHE2 mRNA and immunoreactive protein were expressed at higher levels in suckling and weanling animals than in older rats. Northern blot results showed an approximate 2-fold decrease in 6-week-old rats and a 3-fold decrease in adult rats in NHE2 mRNA levels. To confirm these changes in mRNA expression, in situ hybridization was performed on kidney sections from 2-week-old and adult rats, the two ages that showed the greatest differences in NHE2 mRNA levels. Although in situ hybridization is not a highly quantitative technique, these experiments confirmed the differences in mRNA expression with the 2-week-old animal clearly, having increased NHE2 message. Furthermore, NHE2 mRNA was localized to the outer medulla and the inner cortical region with no mRNA expression in the outer rim of the cortex or in the inner medulla. These data are consistent with previous investigations which localized NHE2 immunoreactive protein to the distal convoluted tubules, the cortical thick ascending limbs and the connecting tubules [14,15].

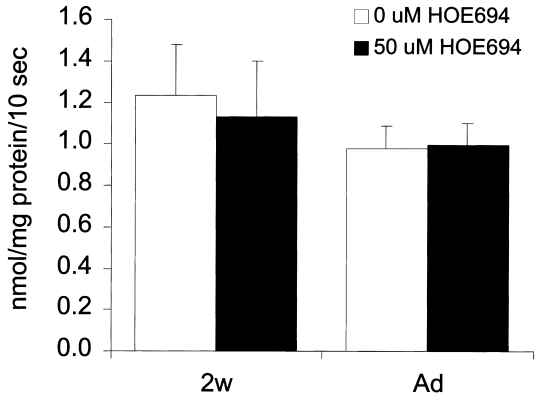

Fig. 7. pH-Dependent uptake of radiolabeled ^{22}Na with and without HOE694. Renal BBMV were assayed for NHE2 and NHE3 activity by utilizing the relatively specific NHE inhibitor, HOE694. The *y*-axis shows nmol ^{22}Na transported per mg protein per 10 s. 2w is 2-week-old rats, and Ad is adult rats. No differences were apparent between any of the four groups ($n = 4$).

Further experiments showed a decrease in NHE2 protein by immunoblot analysis in older animals as compared to sucklings and weanlings. To exemplify the specificity of our antiserum for the NHE2 protein expressed in the kidney, we performed immunohistochemical analyses of 3-week-old rat kidney cortex samples. These experiments showed specific, block-

able staining of apical membranes in the distal convoluted tubules (DCT) with no staining detected in the basolateral membranes of the DCT or in the proximal convoluted tubules or glomeruli. Additionally, no staining was detected with pre-immune serum. The decrease in immunoreactive NHE2 protein expression was to a lesser degree than that seen in mRNA expression ($\sim$20% in 6-week rats and $\sim$30% in adults rats as compared to 2- and 3-week-old animals). We have often, in the past, documented changes in mRNA expression as analyzed by Northern blot analyses that did not correspond precisely to changes seen in immunoreactive protein as analyzed by Western blots. We surmised that this may be due to differential sensitivities or other intangibles associated with the two techniques. Alternatively, these data may suggest that NHE2 is regulated at both transcriptional and post-transcriptional levels during renal ontogeny. Overall then, the immunoblot data showed decreased NHE2 protein in 6-week-old and adult rats, and immunohistochemical analyses exemplified the specificity of the antiserum for the renal NHE2 protein.

We then proceeded to look at NHE3 expression in the kidney at the molecular level during renal ontogeny. Northern blots showed that NHE3 mRNA was expressed at lower levels ($\sim$2-fold) in suckling animals as compared to the other three age groups. In situ hybridization experiments were not performed due to the smaller changes seen in NHE3 mRNA and also due to the fact that previous investigations have adequately localized NHE3 in rat kidney [8,17,18]. Additional experiments showed that there were no apparent changes in NHE3 immunoreactive protein expression as assessed by immunoblot analyses. Since protein data were somewhat inconsistent (as exemplified by the rather large standard errors), we performed 12–14 experiments to confirm this observation. We have previously characterized our NHE3 antiserum in rat kidney [22], so we did not feel it was necessary to perform immunohistochemical analyses. So, in summary, the current data showed that although NHE3 mRNA expression increases around the time of weaning, expression of NHE3 protein remains constant during development. It would therefore seem that while NHE3 gene transcription rates or mRNA stability are decreased at the young age, the overall results are similar levels of protein expression at all ages studied. Possible explanations for this observation include increased NHE3 translational efficiency or protein stability (i.e. a longer half-life).

The next logical step then was to look at functional expression of NHE2 and NHE3 during rat, renal development by BBMV uptake studies. This was accomplished by utilizing HOE694 ([3-methyl sulfonyl-4-piperidinobenzoyl]guanidine methanesulfonate), an NHE inhibitor with the unique capability of selectively inhibiting NHE2, but not NHE3, at a 50 μM concentration [32]. We previously demonstrated the usefulness of this compound for studies of NHE2 and NHE3 functional activity in rat jejunal mucosa [24]. Current results showed that there were no differences in pH-dependent sodium uptake between the 2-week and adult age groups with or without 50 μM HOE694. This suggested that there were no changes in functional NHE2 or NHE3 expression during renal ontogeny. With 50 μM HOE694, we were observing only the activity of NHE3 (K_i for HOE694-mediated inhibition in PS120 fibroblasts, 5 μM for NHE2 and 650 μM for NHE3 [32]). These results are consistent with the NHE3 immunoblot studies which showed no changes in NHE3 expression.

Furthermore, by taking the difference between 0 and 50 μM HOE694, one can approximate NHE2 activity (as described previously [24]). However, there were no detected differences between 0 and 50 μM HOE694, suggesting either that NHE2 does not contribute to basal, renal NHE activity in 2-week-old or adult rats, or that NHE2 activity cannot be detected by this experimental methodology. This finding is not surprising since it seems likely that NHE2 does not play a role in sodium absorption in the kidney due to a couple of lines of reasoning: (1) most sodium reabsorption (85%) occurs in the proximal tubules where NHE3 is the only apical NHE; and (2) NHE2 has been suggested to play a role in osmolality regulation of inner medullary collecting duct cells [33]. In fact, recent data from our laboratory has demonstrated cloning of the rat NHE2 promoter [34], and identification of genomic response elements in the rat NHE2 gene that mediate increased gene transcription in response to hyperosmolar stress in mouse inner medullary duct cells (mIMCD-3) cells [35]. Based upon these facts, it seems that BBMV uptake studies may not reflect the activity of

NHE2, but may rather detect only NHE3 activity in the rat kidney (which does not change during ontogeny). Therefore, it remains a possibility that there *are* changes in NHE2 activity during renal ontogeny, but these changes are not detectable by the current techniques (i.e. NHE2 activity is below the background levels of sodium absorption). However, to prove or disprove this, it will be necessary to develop an assay to measure functional NHE2 (perhaps by isolating BBMV from the appropriate micro-dissected renal tubules from different aged rats and performing uptake studies).

Another important aspect of the current studies is their comparison to previous data concerning expression of the other NHE isoforms in the mammalian kidney during ontogeny. We previously documented changes in basolateral NHE (i.e. NHE1 and NHE4) expression in the developing rat kidney [22]. These studies demonstrated highest BLM vesicle pH-dependent uptake of radiolabeled Na^+ (i.e. NHE activity) in 2-, 3-, and 6-week-old rats and an $\sim$2–2.5-fold decrease in activity in adults. Furthermore, these studies showed highest NHE1 and NHE4 mRNA expression in 2- and 3-week-old rats, with decreased levels in 6-week and adult rats. We concluded that NHE activity at the BLM was regulated during rat, renal development and that NHE1 and NHE4 likely play a role in this decreased BLM NHE activity seen in adult animals (although changes in NHE1 and NHE4 mRNA expression alone cannot completely explain the observed ontogenic changes). When these data are compared to the current investigation, a couple of interesting observations are noticed. First, differential NHE activity was observed in the renal BLM during development, but no such changes were apparent in BBM NHE activity. And secondly, changes in NHE1 and NHE4 mRNA expression likely underlie some ontogenic changes in the BLM, while changes in molecular NHE2 and NHE3 expression do not seem to have any effect on functional NHE activity at the renal BBM during ontogeny.

Further comparisons are important between developmental expression of NHEs in the rat kidney and small intestine. We have also previously characterized the ontogenic expression of NHE1, NHE2 and NHE3 in the developing rat intestine [22–24]. NHE1 showed no ontogenic changes in mRNA expression in the rat intestine [22]. Other studies showed that both NHE2 and NHE3 contribute to basal NHE activity at all ages studies (2, 3, and 6 weeks and adults) [24]. However, the relative contribution of each apical isoform varied from near equal contributions in 2- and 3-week-old rats (41% NHE2 and 59% NHE3) to predominantly NHE3 (92%) in 6-week-old animals. Additionally, both NHE2 and NHE3 were shown to increase in expression at the mRNA and functional protein levels around the time of weaning (i.e. 3 weeks of age). These previous data are in contrast to the current studies which showed no ontogenic changes in BBM NHE activity in rat kidney. It would therefore seem that any hormonal or other metabolic influences that lead to increased NHE2 and NHE3 expression during postnatal intestinal development have no effect on expression of the same exchanger molecules in the kidney.

Overall, the current investigation has documented the ontogenic changes in expression of the apical NHEs (NHE2 and NHE3) in the rat kidney. Initial experiments showed differential expression of NHE2 mRNA and protein and changes in NHE3 mRNA expression during development. However, further studies showed no apparent changes in the level of functional NHE2 or NHE3 protein during development. We therefore conclude that while changes are seen in NHE2 mRNA and protein expression, NHE2 activity likely cannot be detected by renal BBM uptake studies. This would imply a physiological role for NHE2 that is unrelated to transepithelial sodium absorption (as has been previously suggested [14,15,33]). Additionally, although NHE3 mRNA increased around the time of weaning, it seems that this has no effect on functional NHE3 protein expression during renal development, which remains constant.

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