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Expression and Localization of the Na^+/H^+ Exchanger Isoform NHE3 in the Rat Efferent Ducts

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ABSTRACT: The efferent ducts reabsorb most of the fluid released with spermatozoa from the testis. This absorptive capacity results in a severalfold increase in sperm concentration in the proximal epididymis and is partly responsible for maintenance of the optimal microenvironment for the sperm maturation. The fluid absorption is coupled to active Na^+ transport and is inhibitable by amiloride, both of which suggest a role for a Na^+/H^+ exchanger (NHE). NHE3 is an apical membrane NHE responsible for sodium absorption in renal proximal tubule and intestinal epithelium. In the present study, we examined the expression of NHE3 messenger RNA (mRNA) and protein in the rat efferent ducts by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting and the localization of NHE3 by indirect immunofluoresce. RT-PCR indicated the expression of NHE3 mRNA, and Western blotting showed an NHE3 protein in the efferent duct membrane homogenate. By immunofluorescence, NHE3 was localized to the apical membrane of the nonciliated cells in the efferent duct epithelium, which also expressed aquaporin-1 water channel protein. These results suggest that NHE3 potentially plays an important role in the fluid reabsorption in the efferent ducts.

Key words: Absorption, fluid, immunohistochemistry, reverse transcription-polymerase chain reaction, Western blot.

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The testicular efferent ducts transport fluid and spermatozoa from the rete testis to the epididymal duct. They reabsorb up to 96% of fluid released with spermatozoa, thus resulting in increased sperm concentration (Crabo, 1965; Jones and Jurd, 1987; Clulow et al, 1994). The fluid absorption in the efferent occurs at high rates without significant changes in the composition and osmolality of the luminal fluid. The correlation between Na^+ and fluid reabsorption suggests that the fluid reabsorption in the efferent duct is dependent on Na^+ transport across the epithelium (Clulow et al, 1994).

The molecular mechanisms of water and electrolyte absorption in the efferent duct are still largely unknown. Na^+-K^+-adenosine triphosphate (ATPase) present in the basolateral membrane may establish the sodium gradient, which serves as a driving force for apical sodium-coupled ion transporters (Byers and Graham, 1990; Ilio and Hess, 1992). The identity of those luminal ion transporters has remained undefined so far. Recently, Hansen et al (1999) reported that luminal amiloride decreased fluid absorption in a dose-dependent manner by up to 70% in in vivo microperfused rat efferent duct, which suggests an important role for Na^+/H^+ exchange.

Electroneutral exchange of Na^+ and H^+ ions across cell membranes is mediated by a family of Na^+/H^+ exchangers (NHEs) (Orlowski and Grinstein, 1997; Wakabayashi et al, 1997). To date, 6 NHE isoforms have been identified, NHE1–NHE6, which are selectively expressed in various tissues and involved in diverse cellular functions, including intracellular pH regulation, transepithelial ion transport, cell volume regulation, and cell growth (Orlowski and Grinstein, 1997; Wakabayashi et al, 1997). NHE3 is expressed in the apical membrane of the renal and intestinal epithelium, where it mediates the vectorial Na^+ and H^+ transport (Biemesderfer et al, 1993; Amemiya et al, 1995; Hoogerwerf et al, 1996; Schultheis et al, 1998). Recently, we (Kaunisto et al, 2001) and other laboratories (Pushkin et al, 2000; Bagnis et al, 2001) have demonstrated the presence of NHE3 in the rat epididymal duct, where it is believed to be involved in the luminal acidification. To address whether NHE3 is linked to fluid absorption in the efferent duct, we examined here the expression of NHE3 messenger RNA (mRNA) and protein in the rat efferent duct by reverse transcription-polymerase chain reaction (RT-PCR), Western blotting, and immunofluorescence techniques.

Methods

Extraction of RNA and RT-PCR

Total cellular RNA was extracted from isolated efferent duct using TRIzol reagent (Gibco-BRL, Gaithersburg, Md), according to the manufacturer's instructions. The concentration and purity of RNA were determined by spectrophotometry at 260 and 280 nm. RT and PCR amplifications were performed by a single-step

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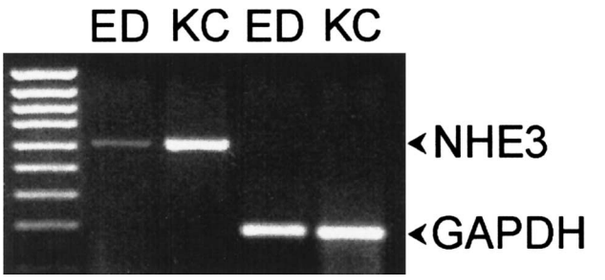

Figure 1. Detection of an apical membrane Na^+/H^+ exchanger (NHE3) and glyceraldehyde-6-phosphate dehydrogenase (GAPDH) mRNA by reverse transcription-polymerase chain reaction (RT-PCR) with 10 μg total RNA from rat efferent ducts (ED) and kidney cortex (KC) as a template. Molecular weight markers (100-bp ladder) are on the far left lane.

method (Aatsinki et al, 1994; Kaunisto et al, 2001). Primers were chosen on the basis of published rat NHE3 sequence (Orlowski et al, 1992) and were located near the 3′ end of the coding region, where there is low sequence homology between the NHE isoforms. The 2 primers used for amplifying NHE3 were 5′-GGACAATATGGTCAATGTGGACTTCAGCACA-3′ (nucleotides 1682–1712) and 5′-GATAGGATGCTTGGGTCCA-GATCCTCGTCAG-3′ (nucleotides 2267–2297), which generated a 601-bp PCR product. Primers for rat glyceraldehyde-6-phosphate dehydrogenase (GAPDH) (Holappa et al, 1999) were used to monitor the quality of the RNA samples.

Ten micrograms of total RNA was used as a template for subsequent RT and PCR reactions. RT was performed at 42°C for 1 hour, followed by denaturation at 95°C for 2 minutes. The PCR cycling protocol consisted of 30 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, followed by final extension at 72°C for 5 minutes. The PCR products were analyzed by electrophoresis on 1.5% agarose gels containing 1% ethidium bromide.

Antibodies

Two antibodies against NHE3 were used. For immunofluorescence, we used MAB3136 (Chemicon International, Temecula, Calif), which is a mouse monoclonal antibody against a maltose-binding protein coupled to carboxy terminus of rabbit NHE3. This antibody has been characterized extensively and found to cross-react with rat NHE3 (Biemesderfer et al, 1997). A rabbit polyclonal antibody against a peptide corresponding to amino acids 809–831 of rat NHE3 was a generous gift of Dr Mark Knepper, National Institutes of Health (NIH; Bethesda, Md), and it has also been previously characterized (Kim et al, 1999). A polyclonal antibody against human AQP1, provided by Dr Bruce Baum, NIH (Delporte et al, 1996), was used in double immunofluorescence labeling. For immunofluorescence microscopy, goat anti-mouse immunoglobulin G (IgG) coupled to Alexa Fluor 488 and goat anti-rabbit IgG coupled to Alexa Fluor 568 (Molecular Probes, Eugene, Ore) were used; for Western blotting, horseradish peroxidase-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, Pa) was used.

Western Blotting

Efferent duct and renal cortex from adult male Sprague-Dawley rats (n = 4) were dissected out, rapidly frozen in liquid nitrogen, and homogenized in ice-cold buffer containing (in mM) 10 NaCl, 50 Tris-SO4 (pH 7.0), and 1 EDTA plus 1 tablet of Complete Protease Inhibitor (Boehringer Mannheim, Mannheim, Germany) per 50 mL buffer. The homogenates were first centrifuged at 1000 × *g* for 10 minutes at 4°C to remove nuclei and cell debris, followed by centrifugation of the supernatants at 70 000 × *g* for 90 minutes at 4°C. The pellets were resuspended in a small volume of isolation buffer, and protein concentrations were measured using the Bio-Rad protein assay kit (Hercules, Calif). The samples were then solubilized at 60°C for 15 minutes in Laemmli sample buffer, separated by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (50 μg protein per lane), and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked overnight at 4°C in PBST buffer (0.9% NaCl in 10 mM sodium phosphate buffer, 0.05% Tween-20, pH 7.4) containing 10% cow colostral whey (Hi-Col, Oulu, Finland), followed by incubation with polyclonal anti-NHE3 (diluted 1:500 with PBST containing 1% bovine serum albumin [BSA]) at room temperature for 2–3 hours. The membranes were then washed with several changes in PBST and incubated for 1 hour at room temperature with peroxidase-conjugated donkey anti-rabbit IgG diluted 1:25 000 in BSA-PBST. After several washes in PBST, antibody binding was visualized by enhanced chemiluminescence (ECL; Amersham, Little Chalfont, United Kingdom).

Immunofluorescence Microscopy

Efferent ducts from 4 adult male Sprague-Dawley rats were dissected free from fat and fixed in 4% paraformaldehyde for 6 hours, cryoprotected overnight in 20% sucrose–phosphate-buffered saline (PBS), and rapidly frozen in liquid nitrogen-cooled isopentane. Sections were cut at 5 μm using a Microm Cryo-Star microtome (Microm, Walldorf, Germany), dried onto Superfrost Plus microscope slides (Menzel, Braunschweig, Germany), and incubated with PBS containing 20% cow colostral whey for 40 minutes. The sections were then incubated for 2 hours with monoclonal anti-NHE3, MAB 3136, diluted 1:50 in 1% BSA-PBS, washed 3 times for 10 minutes in PBS, and incubated for 1 hour with Alexa 488-coupled goat anti-mouse IgG, diluted 1:100 in PBS. For double immunofluorescence staining, anti-AQP1 antibody (dilution 1:100) was applied simultaneously with anti-NHE3 and visualized with Alexa 568-conjugated goat anti-rabbit IgG. After four 5-minute washes in PBS, slides were mounted in Immu-Mount (Shandon, Pittsburgh, Pa) and examined with a conventional epifluorescence microscope (Nikon Corporation, Tokyo, Japan) or a confocal laser scanning microscope (Zeiss, Göttingen, Germany).

Results

Detection of NHE3 mRNA by RT-PCR Analysis

To examine if the rat efferent ducts expressed NHE3 mRNA, total RNA was isolated from rat efferent ducts and kidney cortex (positive control) and subjected to RT-PCR using NHE3-specific primers. RT-PCR using primers for rat GAPDH was also performed to monitor the integrity of the RNA samples. Figure 1 shows a gel elec-

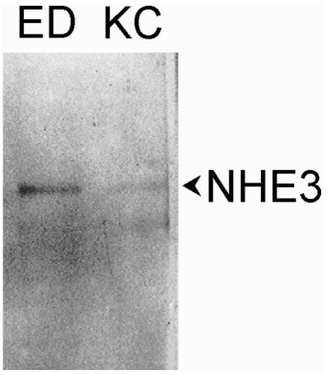

Figure 2. Western blot analysis of an apical membrane Na^+/H^+ exchanger (NHE3). Solubilized proteins (30 μg) of crude membrane particles from rat efferent ducts (ED) and kidney cortex (KC) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted on polyvinylidene difluoride (PVDF) membranes, and probed with polyclonal NHE3 antibody.

trophoresis of RT-PCR products from rat efferent ducts and kidney cortex RNAs. Amplification products of expected size were obtained from both RNA samples, 601 bp for NHE3 and 300 bp for GAPDH.

Western Blotting of NHE3 in Efferent Ducts

By Western blotting, the polyclonal NHE3 antibody recognized an approximately 87-kd band in rat efferent duct membrane homogenate (Figure 2). A band of similar size, but of weaker intensity, was detected in the kidney cortex membranes, which are known to express NHE3 (Figure 2). The weak band in renal cortical homogenate is probably attributable to an unequal loading of the gel or a degraded sample. Control stainings of Western blots with normal rabbit serum or without the primary antibody showed no specific staining (data not shown).

Immunofluorescence Localization of NHE3 in Efferent Ducts

Cryosections of the rat efferent ducts stained for NHE3 with a monoclonal antibody showed a specific reaction at the apical membrane of the epithelial cells lining the efferent ducts (Figure 3A, C, and D). The staining intensity was similar in proximal and distal parts of the ducts (data not shown). Efferent ducts contain 2 cell types, ciliated cells and nonciliated cells, the latter expressing AQP1 water channel protein (Brown et al, 1993). Double immunofluorescence staining with anti-NHE3 and anti-AQP1 antibodies showed a clear apical staining for NHE3 and an apical, intracellular, and basolateral staining for AQP1 in the same cells (Figure 4), indicating that NHE3 is present in nonciliated cells. No staining was seen when a specific antibody was replaced by normal rabbit serum (Figure 3B) or when the primary antibody was omitted (data not shown).

Discussion

The efferent ducts, which connect the rete testis to the initial segment of the epididymis, efficiently absorb the fluid secreted by seminiferous tubules. Water absorption is closely coupled to transepithelial solute transport, notably sodium and chloride (Clulow et al, 1994; Ilio and Hess, 1994), but the molecular mechanisms involved in that process are still largely unknown. Basolateral membranes of the efferent ducts contain Na^+-K^+-ATPase, which may contribute in Na^+ efflux to the interstitium (Byers and Graham, 1990; Ilio and Hess, 1992). However, less is known about the ion transporters involved in luminal sodium entry. Hansen et al (1999) recently showed by use of in vivo microperfused rat efferent ducts that amiloride, an inhibitor of Na^+/H^+ exchange, inhibited fluid absorption in a dose-dependent manner. Relatively high amiloride concentrations needed for the inhibition suggested that the NHE isoform involved might be NHE3, which is more resistant to amiloride than the other apically located isoform, NHE2 (Orlowski, 1993).

Studies by Schultheis et al (1998) show that NHE3 plays an important role in Na^+ absorption in renal proximal tubule and intestinal epithelium. Recently, the expression of NHE3 has been also reported in rat epididymis, where it is probably involved in bicarbonate reabsorption (Pushkin et al, 2000; Bagnis et al, 2001; Kaunisto et al, 2001). On the basis of data from amiloride sensitivity of the fluid absorption in the efferent ducts and the role of NHE3 in other epithelia, an NHE3 isoform could thus be a good candidate for the luminal NHE also in the efferent ducts. Therefore, we used RT-PCR, Western blotting, and immunocytochemistry to determine whether NHE3 mRNA and protein are expressed in the efferent ducts. By RT-PCR, NHE3 mRNA was found to be expressed in the rat efferent ducts, and Western blotting showed an 87-kd protein corresponding to the size of NHE3 in other tissues. NHE3 was localized to the apical membrane of the efferent duct epithelium by immunofluorescence staining.

Efferent ducts are lined by 2 cell types, ciliated and nonciliated epithelial cells. Nonciliated cells resemble morphologically and functionally renal proximal tubule cells, and they have a reabsorptive function (Hermo and Morales, 1984; Robaire and Hermo, 1988). These cells have also been reported to express a water channel protein, AQP1, in the apical and basolateral membranes (Brown et al, 1993; Fisher et al, 1998), which further suggests their crucial role in water absorption. Our present finding of the colocalization of AQP1 and NHE3 in the nonciliated cells is compatible with the suggested role of these cells in water absorption. During the final preparation of this manuscript, 2 other laboratories also re-

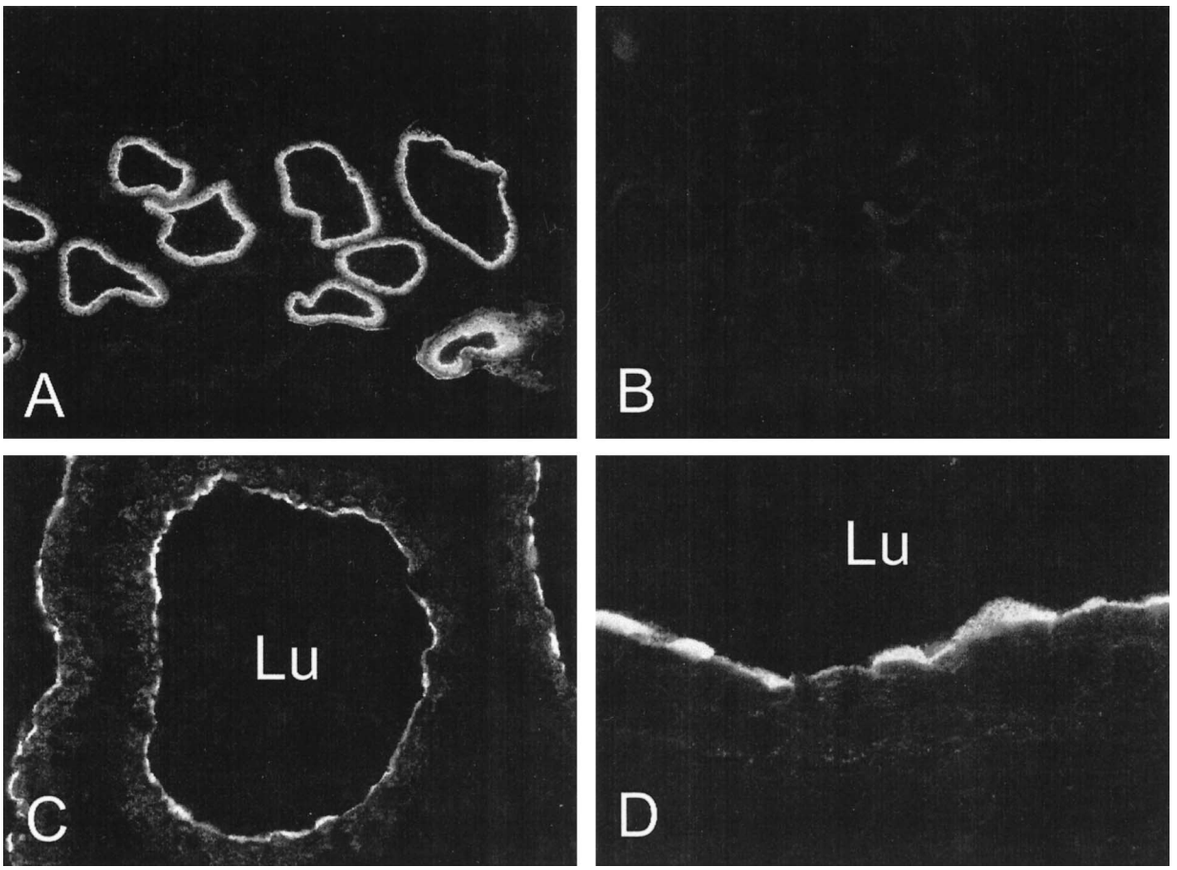

Figure 3. Immunofluorescence localization of an apical membrane Na^+/H^+ exchanger (NHE3) in the rat efferent ducts. At low magnification, several cross-sections of efferent ducts are seen with intense staining in the epithelium **(A)**. Confocal microscopy images show that the immunofluorescence staining is restricted to the apical plasma membrane of the epithelial cells **(C, D)**. When primary antibody is replaced by normal rabbit serum, no staining is observed **(B)**. Lu indicates lumen. Magnifications 50× **(A, B)**, 250× **(C)**, and 700× **(D)**.

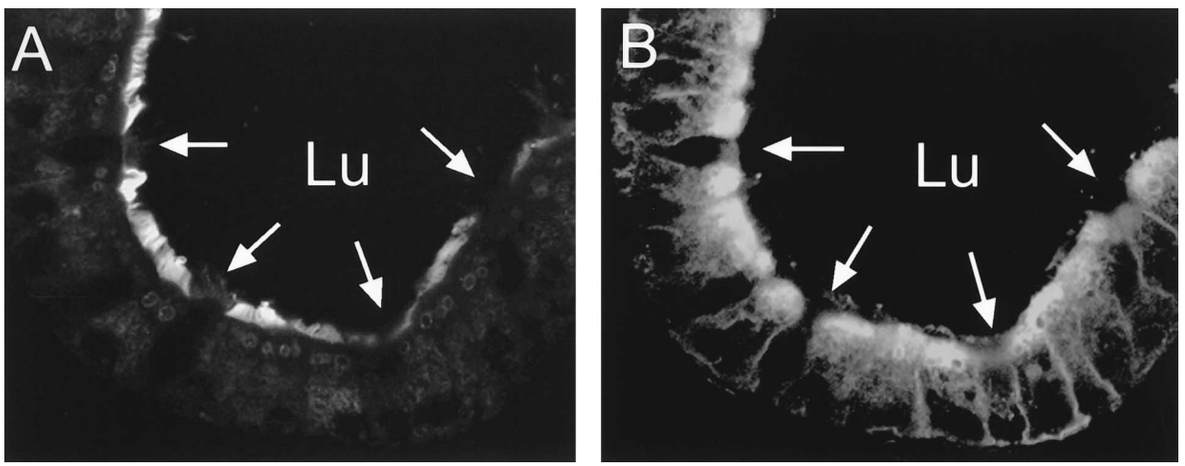

Figure 4. Double immunofluorescence staining of an apical membrane Na^+/H^+ exchanger (NHE3) **(A)** and AQP1 **(B)** in the rat efferent duct. Ciliated cells (arrows) show lack of staining for both NHE3 and AQP1, whereas adjacent nonciliated cells reveal apical labeling with anti-NHE3 **(A)** and apical, intracellular, and basolateral labeling with anti-AQP1 **(B)**. Lu indicates lumen. Magnification 250×.

ported immunohistochemical findings showing the localization of NHE3 protein in the nonciliated cells of the rat efferent ducts, which is in agreement with our present results (Bagnis et al, 2001; Leung et al, 2001). Moreover, Leung et al (2001) detected NHE3 mRNA and protein in cultured epithelial cells from rat efferent ducts.

NHE3 has been implicated in the urinary acidification in the renal proximal tubules (Orlowski and Grinstein, 1997). Moreover, the presence of NHE3 in the epididymal duct suggests its involvement also in epididymal fluid acidification (Pushkin et al, 2000; Bagnis et al, 2001; Kaunisto et al, 2001). However, a recent study by Newcombe et al (2000) has shown that luminal pH and bicarbonate levels in the rat efferent ducts are high relative to those reported in the epididymis. It seems, therefore, that there is a parallel HCO_3^- flux into the lumen, which could be provided, for instance, by a Cl^-/HCO_3^- exchanger operating together with an apical NHE. In fact, the inhibition of Na^+/H^+ exchange and fluid reabsorption in efferent ducts does not change the luminal chloride concentration, which suggests a parallel reabsorption of Cl^- (Hansen et al, 1999). The molecular identity of the putative apical bicarbonate transporter(s) remains to be established.

In conclusion, NHE isoform NHE3 is present in apical plasma membranes of the nonciliated epithelial cells of the rat efferent ducts. These findings suggest that NHE3 plays an important role in mediating fluid and electrolyte reabsorption in the efferent ducts.

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