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Original Paper

Upregulation of Intestinal NHE3 Following Saline Ingestion

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Key Words
Na^+/H^+ exchanger • Osmolarity • Fluid intake • Water • Salt

Abstract
Background: Little is known about the effect of salt content of ingested fluid on intestinal transport processes. Osmosensitive genes include the serum- and glucocorticoid-inducible kinase SGK1, which is up-regulated by hyperosmolarity and cell shrinkage. SGK1 is in turn a powerful stimulator of the intestinal Na^+/H^+ exchanger NHE3. The present study was thus performed to elucidate, whether the NaCl content of beverages influences NHE3 activity. ***Methods:*** Mice were offered access to either plain water or isotonic saline ad libitum. NHE3 transcript levels and protein abundance in intestinal tissue were determined by confocal immunofluorescent microscopy, RT-PCR and western blotting, cytosolic pH (pH_i) in intestinal cells from 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) fluorescence and Na^+/H^+ exchanger activity from the Na^+ dependent realkalinization following an ammonium pulse. ***Results:*** Saline drinking significantly enhanced fluid intake and increased NHE3 transcript levels, NHE3 protein and Na^+/H^+ exchanger activity. ***Conclusions:*** Salt content of ingested fluid has a profound effect on intestinal Na^+/H^+ exchanger expression and activity.

Introduction

Intestinal epithelia are exposed to alterations of Na^+ concentration and osmolarities depending on the ingested food and beverages [1]. Changes of extracellular osmolarity result in the osmotic gradients and water fluxes across the cell membranes and thus lead to the respective alterations of cell volume [2, 3]. Hyperosmolarity leads to cell shrinkage, hypoosmolarity to cell swelling [3]. Alterations of cell volume modify in turn a wide variety of functions

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including gene expression [3]. Genes up-regulated by cell shrinkage include the serum- and glucocorticoid-inducible kinase SGK1 [4], a gene originally cloned as a glucocorticoid-inducible gene [5, 6] and later found to be up-regulated by mineralocorticoids [7-9]. SGK1 is activated by insulin and insulin-like growth factor 1 through phosphatidyl-inositide 3 (PI3) kinase and phosphoinositide-dependent kinase PDK1 [10].

SGK1 is strongly expressed in intestinal epithelia [4, 11, 12] and contributes to the regulation of intestinal transport [13]. SGK1 up-regulates a wide variety of epithelial channels and transporters [13], including the Na^+/H^+ exchanger NHE3 [14, 15]. Ussing chamber experiments confirmed the *in vivo* significance of SGK1 sensitive intestinal absorption [16, 17].

The present study explored whether the NaCl content of ingested fluid influences the activity of the intestinal Na^+/H^+ exchanger. To this end, animals were allowed access to either plain water or isotonic saline and NHE transcript levels, NHE3 protein abundance and Na^+/H^+ exchanger activity determined.

Materials and Methods

Animals

Experiments were performed in sex and age matched mice of 3 months of age. All animal experiments were conducted according to the German law for the care and use of laboratory animals and were approved by local authorities. The mice (8 mice in each group) were fed a control diet (C1314, Altromin, Lage, Germany, NaCl content 0.49%) and had access to either plain water or isotonic saline ad libitum. To determine food and fluid intake, the mice were placed individually in metabolic cages (Techniplast, Hohenpeissenberg, Germany). To obtain blood, mice were anaesthetized with diethylether (Roth, Karlsruhe, Germany) and blood specimens (50 - 200 µl) were withdrawn into capillaries containing EDTA by puncturing the retro-orbital plexus. To obtain tissue, the animals were anaesthetized with diethylether and sacrificed by cervical dislocation.

Immunofluorescence

Tissue samples were cut into 8 µm frozen sections from mouse jejunum and subsequently fixed in 4% paraformaldehyde for 15 min at room temperature. The sections were blocked with 5% milk in PBS 0.3% Triton 100 for 1 hour and incubated with a rabbit anti-NHE3 antibody (1:100 dilution; Novus Biologicals, USA) overnight at 4°C. Secondary FITC-conjugated goat anti-rabbit IgG (Invitrogen, Karlsruhe, Germany) was used in a 1: 1000 dilution. Nuclei were stained with DRAQ5™ (Biostatus Limited, Leicestershire, UK). Slides were mounted using the ProLang® Gold Antifade reagent (Invitrogen). Confocal microscopy was performed with Zeiss LSM 5 EXCITER confocal laser-scanning module (Carl Zeiss, Oberkochen, Germany) and images were analyzed with the software of the instrument.

Real-Time Reverse transcription polymerase chain reaction (RT-PCR)

To determine NHE3 transcript levels, total RNA was extracted from intestinal tissue in Trizol (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. After DNAse digestion reverse transcription of total RNA was performed using random hexamers (Roche Diagnostics, Penzberg, Germany) and SuperScriptII reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Polymerase chain reaction (PCR) amplification of the respective genes were set up in a total volume of 20 µl using 40 ng of cDNA, 500 nM forward and reverse primer and 2x iTaq Fast SYBR Green (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Amplification of the house-keeping gene Tbp (TATA box binding protein) was performed to standardize the amount of sample RNA. Cycling conditions were chosen as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 68°C for 20 sec. For the amplification the following primers were used (5`->3`orientation):

fw GTCACCCAGGATGTAGCCTCTG rev GGTGGCACCCTGGATAGGAT;

Western blotting

Mice were sacrificed by cervical dislocation under ether anaesthesia, and the abdomen was opened. The intestine was then longitudinally cut and cleaned with PBS. A piece of 0.5 g of intestinal tissue was

added to 1 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 1 mM NaF, 1 mM Na_3VO_4, 0.4% β-mercaptoethanol) containing protease inhibitor cocktail (Sigma, Schnelldorf, Germany). The tissue was then homogenized with a Dounce homogenizer on ice for 30 min. Samples were centrifuged at 17,000 rpm for 20 min, and supernatants were collected. After measurement of the total protein concentration (Bradford assay), 80 µg of tissue protein were solubilized in Laemmli sample buffer at 95ºC for 5 min and resolved by 10% SDS-PAGE. For immunoblotting proteins were electro-transferred onto a PVDF membrane and blocked with 5% nonfat milk in TBS-0.10% Tween 20 at room temperature for 1 h. The membrane was then incubated with a rabbit anti-NHE3 antibody (1:1000; 84 kDa, Novus biologicals, USA) at 4°C overnight. After washing (TBST) and subsequent blocking the blot was incubated with secondary anti-rabbit antibody (1:2000, Cell Signaling) for 1 h at room temperature. After washing, antibody-binding was detected with the ECL detection reagent (Amersham, Freiburg, Germany). For loading control the blot was stripped in stripping buffer (Roth, Karlsruhe, Germany) at 56°C for 30 min. After washing with PBST the blot was blocked with TBST + 5% milk for 1 h at room temperature. The blot was then incubated with an anti GAPDH antibody (1:1000, 37 kDa, Cell Signaling) at 4°C overnight. After washing with PBST and incubation with anti-rabbit antibody (GAPDH, 1:2000, Cell Signaling), antibody-binding was detected. Bands were quantified with Quantity One Software (Biorad, Munich, Germany).

Intestinal NHE3 activity

For the isolation of ileal villi, animals were fasted for 6 hours prior to the experiments. After sacrificing the animals the terminal 2 cm of the ileum were removed and cut longitudinally. After washing with standard Hepes solution the intestine was sliced into 0.3 cm^2 sections. The tissues were transferred onto the cooled stage of a dissecting microscope and the individual villi were detached from the intestine by snapping off the ileal base using sharpened microdissection tweezers. Care was taken not to touch the apical part of the villi. The villi were attached to a glass coverslip precoated with Cell-Tak adhesive (BD Biosciences, Heidelberg, Germany). For quantitative digital imaging of pH_i isolated individual villi were incubated in a HEPES-buffered Ringer solution containing 10 µM 2',7'-Bis-(carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethylester (BCECF-AM Molecular Probes, Leiden, The Netherlands) for 15 min at 37°C. After loading, the chamber was flushed for 5 min with Ringer solution to remove any deesterified dye sticking to the outside of the villi [18]. The perfusion chamber was mounted on the stage of an inverted microscope (Zeiss Axiovert 135, Göttingen, Germany), which was used in the epifluorescence mode with a 40x oil immersion objective (Zeiss Neoplan, Göttingen, Germany) [19]. BCECF was successively excited at 490/10 and 440/10 nm, and the resultant fluorescent signal was monitored at 535/10 nm using an intensified charge-coupled device camera (Proxitronic, Bensheim, Germany) and specialized computer software (Metafluor, Puchheim, Germany). Individual cells from the brush border of the villi were outlined and monitored during the course of the measurement. Intensity ratio data (490/440) were converted into pH values using the high-K^+/nigericin calibration technique [20, 21]. To this end, the cells were perfused at the end of each experiment for 5 minutes with standard high-K^+/nigericin (10 µg/ml) solution (pH 7.0). The intensity ratio data thus obtained were converted into pH values using the r_{max}, r_{min}, pK_a values previously generated from calibration experiments to generate a standard nonlinear curve (pH range 5 to 8.5).

For acid loading, cells were transiently exposed to a solution containing 20 mM NH_4Cl leading to initial alkalinization of cytosolic pH (pH_i) due to entry of NH_3 and binding of H^+ to form NH_4^+ [22] . The acidification of cytosolic pH upon removal of ammonia allowed calculating the mean intrinsic buffering power (ß) of the cells [23]. Assuming that NH_4^+ and NH_3 are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH_3:

$$ß = \Delta[NH_4^+]_i/\Delta pH_i,$$

where ΔpH_i is the decrease of cytosolic pH (pH_i) following ammonia removal and $\Delta[NH_4^+]_i$ is the decrease of cytosolic NH_4^+ concentration, which is identical to the concentration of $[NH_4^+]_i$ immediately before the removal of ammonia. The pK for NH_4^+/NH_3 is 8.9 [24] and at an extracellular pH (pH_o) of 7.4 the NH_4^+ concentration in extracellular fluid ($[NH_4^+]_o$) is 19.37 mM [$20/(1+10^{pHo-pK})$]. The intracellular NH_4^+ concentration ($[NH_4]_i$) was calculated from:

$$[NH_4]_i = 19.37 \cdot 10^{pHo-pHi} \text{ mM}$$

To calculate the ΔpH/min during re-alkalinization, a manual linear fit was placed over a narrow pH range (pH 6.7 to 6.9) which could be applied to all measured cells. The solutions were composed of (in mM): standard Hepes: 115 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 NaH_2PO_4,10 glucose, 32.2 Hepes; sodium free Hepes:

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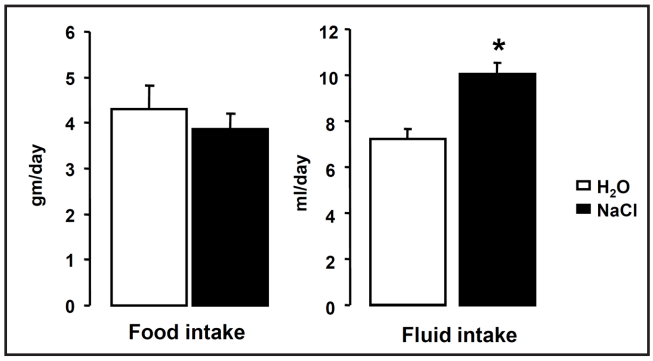

Fig. 1. Food and fluid uptake of animals drinking water or saline. Arithmetic means ± SEM (n = 8-12) of food (left panel) and fluid (right panel) uptake of animals drinking water (H_2O, white bars) or saline (NaCl, black bars).

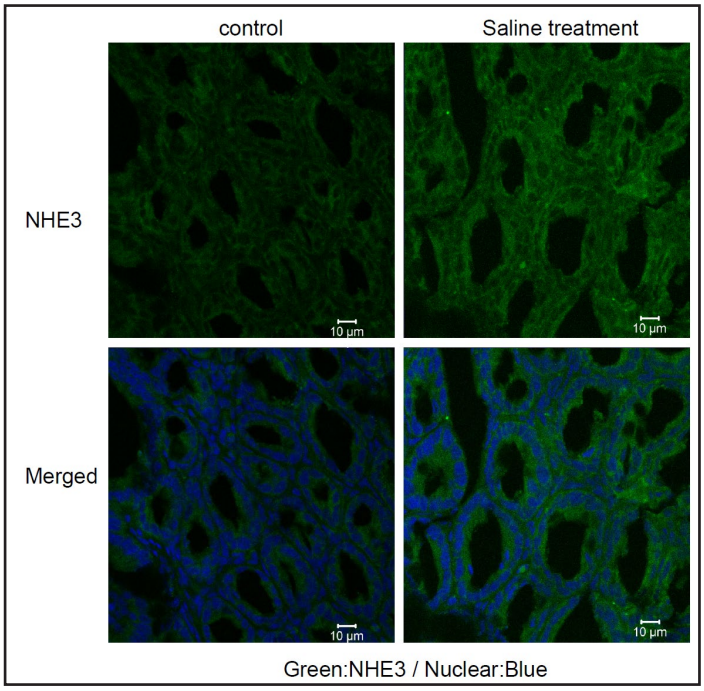

Fig. 2. NHE3 expression in intestinal tissue of animals drinking water or saline. Immunofluorescence of NHE3 protein expression (green) in intestinal tissue from animals drinking water (H_2O) or saline (NaCl).

132.8 NMDG, 3 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 KH_2PO_4, 32.2 Hepes, 10 mannitol, 10 glucose (for sodium free ammonium chloride 10 mM NMDG and mannitol were replaced with 20 mM NH_4Cl); high K^+ for calibration 105 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 32.2 Hepes, 10 mannitol, 5 µM nigericin. The pH of the solutions was titrated to 7.4 or 7.0 with HCl/NaOH, HCl/NMDG and HCl/KOH, respectively, at 37°C.

Statistics

Data are provided as means ± SEM, n represents the number of independent experiments. All data were tested for significance using Student t-test or ANOVA (Dunnet's test), where applicable, and only results with $P < 0.05$ were considered statistically significant.

Results

To determine the impact of NaCl intake on fluid and food intake, animals were placed into metabolic cages. As shown in Figure 1, the fluid intake was significantly higher in saline drinking than in water drinking animals. The food intake was not significantly different between animals drinking saline and animals drinking water (Fig. 1).

Confocal microscopy was utilized to elucidate, whether NaCl intake influences the expression of the Na^+/H^+ exchanger NHE3. As illustrated in Fig. 2, NHE3 protein abundance was indeed higher in saline drinking than in water drinking mice. To quantify the expression

Table 1. Cytosolic pH (pH_i), buffer capacity (mM/pH unit), Na^+-independent pH recovery (Δ pH units/minute) and sodium-dependent pH recovery (Δ pH units/minute) in intestinal epithelial cells from mice offered normal drinking water (H_2O) or 0.9 % saline water (NaCl)

	pH_i	Buffer Capacity	Na^+ independent pH recovery	Na^+-dependent pH recovery	Number of mice	Number of cells
H_2O	7.15 ± 0.03	29.8 ± 3.9	-0.06 ± 0.05	0.26 ± 0.06	8	111
NaCl	7.13 ± 0.02	26.0 ± 4.3	-0.01 ± 0.03	0.76 ± 0.18*	8	117

*-indicates statistically significant difference (<0.05) from H_2O drinking animals in the legend

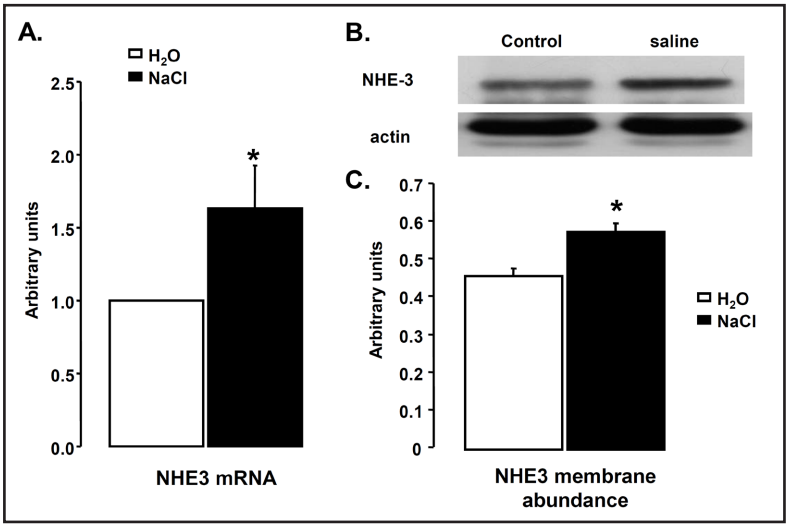

Fig. 3. NHE3 transcript and protein abundance in intestinal brush border of animals drinking water or saline. A: Arithmetic means ± SEM (n = 6) of NHE3 transcript levels (in arbitrary units, a.u.) in intestinal tissue from animals drinking water (H_2O, white bar) or saline (NaCl, black bar) * indicates statistically significant difference ($p<0.05$) from H_2O drinking animals. **B:** Original Western blot of NHE3 protein abundance in intestinal tissue from animals drinking water (H_2O, white bars) or saline (NaCl, black bars). **C:** Arithmetic means ± SEM (n = 6) of NHE3 protein density (in arbitrary units, a.u.) in intestinal tissue from animals drinking water (H_2O, white bars) or saline (NaCl, black bars). * indicates statistically significant difference ($p<0.05$) from H_2O drinking animals.

of the intestinal Na^+/H^+ exchanger, NHE3 transcript levels were determined by RT-PCR and membrane protein abundance by Western blotting. As shown in Fig. 3, the NHE3 transcript levels were significantly higher in saline drinking animals than in water drinking animals. A similar observation was made utilizing Western blotting. The NHE3 protein abundance was again significantly higher in saline drinking animals than in water drinking animals.

NHE3 activity was estimated from the pH recovery following an ammonium pulse in intestinal cells isolated from ileum of animals drinking either water or saline. Prior to exposure of the intestinal cells to ammoniumchloride, cytosolic pH (pH_i) was similar in saline and water drinking animals (Table 1). In intestinal cells from both, water and saline drinking animals, the application of 20 mM NH_4Cl was followed by cytosolic alkalinization due to entry of NH_3 with subsequent binding of intracellular H^+. The subsequent NH_4^+ removal was followed by a sharp cytosolic acidification due to exit of NH_3 with cellular retention of H^+. The alterations of pH_i following an ammonium pulse allowed calculating the cellular buffer capacity (see methods), which was again similar in intestinal cells from water and saline drinking animals (Table 1). In the absence of extracellular Na^+, cytosolic pH remained acidic pointing to lack of Na^+ independent acid extrusion. The addition of Na^+ resulted in a rapid realkalinization, reflecting Na^+/H^+ exchanger activity. The pH_i recovery in the presence of extracellular Na^+ was significantly more rapid in intestinal cells isolated from saline drinking animals than in intestinal cells isolated from water drinking animals (Figure 4). Thus, saline drinking upregulates the intestinal Na^+/H^+ exchanger activity.

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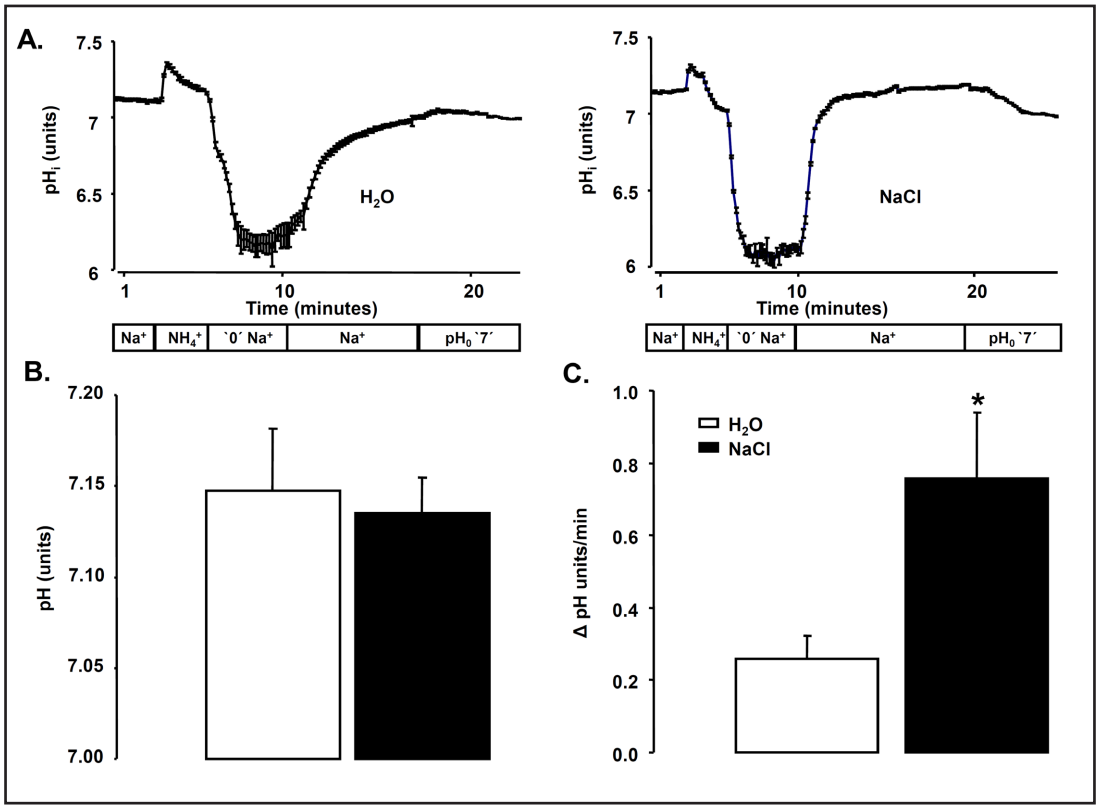

Fig. 4. pH recovery following an ammonium pulse in ileum from of animals drinking water or saline. Alterations of cytosolic pH (ΔpH) in ileal epithelial cells following an ammonium pulse. To load the cells with H^+, 20 mM NH_4Cl was added and Na^+ removed (replaced by NMDG; '0' Na^+) in a first step (see bars below each tracing), NH_4Cl removed in a second step (NH_4^+), Na^+ added in a third step and nigericin (pH_0 '7') applied in a fourth step to calibrate each individual experiment. **A:** Representative experiments showing time dependent alterations of pH in isolated intestinal villi from animals drinking water (H_2O, left panel) or saline (NaCl, right panel). **B:** Arithmetic means ± SEM (n = 8 mice) of pH_i in intestinal cells from animals drinking water (H_2O, white bars) or 0.9 % saline (NaCl, black bars). **C:** Arithmetic means ± SEM (n = 8 mice) of sodium dependent pH recovery in intestinal cells from animals drinking water (H_2O, white bars) or saline (NaCl, black bars). * indicates statistically significant difference ($p<0.05$) from H_2O drinking animals.

Discussion

The present results uncover a novel effect of beverage salt content on intestinal function. The ingestion of saline as compared to water leads to up-regulation of the intestinal Na^+/H^+ exchanger, the major carrier accomplishing intestinal Na^+ absorption.

The present observations did not address the mechanisms accomplishing the up-regulation of Na^+/H^+ exchanger activity in saline-drinking animals. In theory, the effect of saline ingestion on Na^+/H^+ exchanger activity could have resulted from stimulation of Na^+/H^+ exchanger activity by the serum- and glucocorticoid-inducible kinase SGK1, which is up-regulated by cell shrinkage [4] and is a powerful stimulator of the Na^+/H^+ exchanger NHE3 [14, 15, 25, 26]. SGK1 further up-regulates the Na^+/K^+-ATPase [27-30], which extrudes cytosolic Na^+ and thus establishes the driving force for secondary active transporters such as Na^+/H^+ exchangers. SGK1 has previously been shown to participate in the signaling mediating the stimulation of the intestinal Na^+/H^+ exchanger NHE3 by the glucocorticoid dexamethasone [16]. Glucocorticoids stimulate the intestinal transport of nutrients [31] and via Na^+/H^+ exchanger NHE3 the Na^+ absorption [32-34]. SGK1 enhances the cell membrane protein

abundance of a wide variety of ion channels and carriers [13, 35] including NHE3 [14, 15]. Thus, SGK1 is critically important for the effects of glucocorticoids on intestinal transport. Accordingly, the effect of glucocorticoids on NHE3 were strongly attenuated in gene-targeted mice lacking functional SGK1 [16].

Drinking water decreases but does not fully abolish Na^+/H^+ exchanger activity. Similarly, complete lack of SGK1 in the SGK1 knockout mice does not result in complete lack of Na^+/H^+ exchanger activity [16]. The residual Na^+/H^+ exchanger activity in SGK1 deficient mice may result from stimulation of the carrier by similar kinases, such as the SGK1 isoform SGK3 [36], which, similar to SGK1 stimulates several channels and transporters [13]. In contrast to SGK1, SGK3 is not genomically regulated by cell volume or glucocorticoids [13]. Accordingly, the effect of beverage osmolarity may affect the expression of SGK1 but not of SGK3. Both, SGK1 and SGK3 are activated by insulin and growth factors via phosphoinositide 3 kinase (PI3K) and phosphoinositide dependent kinase PDK1 [10].

SGK1 transcription is further up-regulated by mineralocorticoids [7-9]. The kinase stimulates the Na^+ transport not only in intestine [16] but as well in the kidney [7, 37-41]. Presumably due to its effect on NaCl homeostasis, SGK1 affects blood pressure [13]. A gain of function variant of the SGK1 gene is associated with increased blood pressure [13], which is one hallmark of metabolic syndrome or syndrome X, a condition characterized by essential hypertension, procoagulant state, obesity, insulin resistance and hyperinsulinemia [42] and associated with enhanced morbidity and mortality from cardiovascular disease [43-45].

Even though SGK1 may be a candidate for the upregulation of NHE3 following saline ingestion, the present data do not allow firm conclusions regarding the mechanisms accomplishing the altered regulation of Na^+/H^+ exchanger activity in water- and saline-drinking animals. In theory, the increase of Na^+/H^+ exchanger activity could have resulted from cytosolic acidification, which is known to stimulate Na^+/H^+ exchanger activity [46]. The Na^+/H^+ exchanger is further stimulated by cell shrinkage [2, 3], which, however, affects NHE1 rather than NHE3 [47]. NHE3 is further upregulated by inhibition of protein kinase A [48]. NHE3 could be stimulated by direct exposure to ingested saline or indirectly by local reflexes, intestinal mediators or hormones. Clearly, additional experiments are needed to elucidate the mechanisms involved in the stimulation of intestinal Na^+/H^+ exchanger activity by saline-drinking. Whatever mechanism involved, any up-regulation of NH3 activity in intestine and kidney could contribute to the development of hypertension following saline ingestion [48-55].

Conclusion

As compared to drinking water, drinking saline increases the activity of the Na^+/H^+ exchanger, which impacts on intestinal salt transport.

Conflict of Interests

The authors state that they have no conflict of interest to disclose.

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