Dietary Sulfate Regulates the Expression of the Renal Brush Border Na/S₁ Cotransporter NaS₁-1

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Abstract. Dietary inorganic sulfate (S₄) intake is an important factor in the regulation of renal proximal tubular sodium-dependent S₄ transport (Na/S₄ cotransport). The purpose of the present study was to determine whether modulation of Na/S₄ cotransport activity by dietary S₄ is mediated through regulation of the renal expression of the recently cloned NaS₁-1 protein located in the apical brush border membrane (BBM) of the proximal tubule. It was found that rats fed a high S₄ diet had a marked increase in the renal excretion of S₄ and a concomitant decrease in BBM Na/S₄ cotransport activity when compared with rats on a control S₄ diet. The 43% decrease in BBM Na/S₄ cotransport activity was associated with a 33% decrease in BBM NaS₁-1 protein abundance, as determined by Western blotting, and a 2.7-fold decrease in cortical NaS₁-1 mRNA abundance, as determined by Northern blotting. Furthermore, cortical mRNA from rats fed a high S₄ diet when injected into Xenopus laevis oocytes led to a 2.2-fold decrease in Na/S₁ cotransport activity compared with mRNA isolated from control S₄ diet rats. This study indicates that adaptation to a high S₄ diet is accompanied by a decrease in renal cortical NaS₁-1 mRNA abundance, which results in reduced expression of the NaS₁-1 protein at the level of the proximal tubular BBM.

The kidney plays a major role in the regulation of sulfate homeostasis. Inorganic sulfate (S₄) is freely filtered by the glomerulus and then actively reabsorbed in the proximal convoluted tubule of the kidney. Transport studies with in vivo and in vitro perfused tubules and purified proximal tubular apical brush border membrane (BBM) vesicles have demonstrated that S₄ transport across the proximal tubular BBM is secondary active and involves a sodium gradient-dependent transport process (Na/S₄ cotransport) (1–3). The plasma S₄ concentration is strictly maintained within a narrow range. At the physiologic serum S₄ concentration of 0.7 to 1.0 mM in the rat (4–6), renal S₄ clearance is approximately 10 to 30% of the GFR. Renal S₄ clearance increases with increasing serum S₄ concentration, approaching almost 100% of the GFR. Conversely, renal S₄ clearance decreases with decreasing serum S₄ concentration (7). Recent studies have suggested that dietary S₄ content regulates renal S₄ transport (8–11). In response to a low S₄ diet, there is a decrease in the urinary S₄ excretion and an increase in the proximal tubular BBM Na/S₄ cotransport activity. The cellular mechanisms that mediate the adaptive response in Na/S₄ cotransport activity have not been determined; however, it has been postulated that the apical (brush border) Na/S₄ cotransport system may play an important role in regulating plasma S₄ concentration (9).

We have recently isolated a cDNA coding for a sodium-dependent S₄ transport protein (NaS₁-1) by expression cloning (12). Expression of the NaS₁-1 protein in Xenopus laevis oocytes revealed that the kinetic parameters of expressed Na/S₁ cotransport are identical to those with purified proximal tubular BBM (2,13,14). Northern blotting with the NaS₁-1 cDNA showed strong hybridization with rat kidney cortex mRNA (12), and reverse-transcription (RT)-PCR experiments using microdissected nephron segments from rat kidney showed NaS₁-1-related products in the proximal convoluted tubule (15). Furthermore, immunohistochemical and immunoblotting studies using an antibody against the NaS₁-1 protein indicated that the NaS₁-1 protein is expressed in the apical (brush border) membranes of the proximal tubule (16).

The purpose of the present study was to determine the potential role of the NaS₁-1 protein and mRNA in renal tubular adaptation to changes in dietary S₄ intake. We show that in rats fed a high S₄ diet, there is an increase in the urinary excretion of S₄, which is paralleled by decreases in BBM Na/S₄ cotransport activity and BBM NaS₁-1 protein abundance. In addition, there is also a decrease in renal cortical NaS₁-1 mRNA abundance and a reduced Na/S₄ cotransport activity in Xenopus oocytes injected with mRNA from rats fed a high S₄ diet when compared with controls. These results indicate that dietary S₄ modulates renal Na/S₄ cotransport activity by regulating the expression of renal cortical NaS₁-1 mRNA and renal proximal tubular apical NaS₁-1 protein.
Materials and Methods

Experimental Animals
All experiments were performed with male Sprague Dawley rats weighing 175 to 225 g. During the experiments, animals were kept in individual metabolic cages to control food and fluid intake and to collect urine for determination of S, and creatinine. Experiments were started after animals were allowed to adapt to the metabolic cages for 3 d. Control and experimental rats were pair-fed (1) control rat chow supplemented with 100 mM NaCl and distilled deionized water supplemented with 100 mM NaCl (control S diet) or (2) control rat chow supplemented with 100 mM Na_{2}SO_{4} and distilled deionized water supplemented with 100 mM Na_{2}SO_{4} (high S diet) for 7 d.

On the seventh day of the study, a 24-h urine collection was performed to measure S, and creatinine. On the eighth day, the animals were anesthetized with intraperitoneal pentobarbital, and an aortic puncture was performed to obtain blood for measurement of S, and creatinine. Both kidneys were then rapidly removed, and one-half of each kidney was used for BBM isolation and the other half of each kidney was used for RNA isolation. We used at least six rats (n = 6 BBM or RNA samples) from the control and experimental (high S diet) group.

Urine and Blood Chemistry
Urine and blood samples were analyzed for sulfate content by ion chromatography, using the Dionex Ion Chromatograph (Sunnyvale, CA) and creatinine (Autoanalyzer). The total urinary excretion of sulfate and the fractional excretion of sulfate were calculated by standard clearance formulas.

Isolation of Total Cortical Homogenate and BBM
Thin slices from the rat kidney superficial cortex were cut at 4°C and homogenized with a Polytron in a buffer consisting of (in mM): 300 mannitol, 5 ethylene glycol-bis-(aminoethyl ether) N,N.N,N-tetra-acetic acid, 1 phenylmethylsulfon fluoride, 16 Hepes, and 10 Tris (hydroxymethyl) aminomethane (Tris), pH 7.50. From the resulting total cortical homogenate, apical BBM vesicles were prepared by differential centrifugation after Mg^{2+} aggregation, as described previously (17-20). The final pellet was resuspended in a buffer of 300 mM mannitol, 16 mM Hepes, and 10 mM Tris, pH 7.50. This procedure has previously been shown to result in a highly enriched BBM fraction. There was a 10.8-fold enrichment for the BBM marker enzymes maltase and leucine aminopeptidase, whereas a 1.2-fold enrichment for the basolateral membrane marker enzyme Na-K-ATPase was observed. (17-20). Protein concentrations of the final BBM preparations were determined according to Lowry (21).

Na/S_{i} Cotransport Activity Measurements
Transport measurements were performed in freshly isolated BBM vesicles by radio tracer uptake of Na_{2}SO_{4} (Dupont-New England Nuclear Research Products, Boston, MA) and an inwardly directed sodium gradient (120 mM NaCl) followed by rapid filtration (22). Uptake was terminated after 10 s, representing the initial linear rate.

Western Blot Analysis
BBM were denatured for 2 min at 95°C in 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.5 mM ethylenediaminetetra-acetic acid (EDTA), and 95 mM Tris-HCl, pH 6.8 (final concentrations), and 10 µg of BBM protein per lane were separated on 9% polyacrylamide gels according to the method of Laemmli (23) and electrotransferred onto nitrocellulose paper (24). After blockage with 5% nonfat milk powder with 1% Triton X-100 in Tris-buffered saline (20 mM, pH 7.3), Western blots were performed with antiserum against NaS_{1}-1 (16) at a dilution of 1:4000. Primary antibody binding was visualized using enhanced chemiluminescence (Pierce, Bradford, IL), and the signals were quantified in a PhosphorImager with chemiluminescence detector and densitometry software (Bio-Rad, Richmond, CA).

RNA Isolation and Northern Blot Analysis
Thin slices from kidney superficial cortex were cut on an ice-cold glass dish and homogenized with a Polytron in a denaturing solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. Sequentially, 0.1 vol of 2 M sodium acetate, pH 4.0, 1 vol of water-saturated phenol, and 0.2 vol of chloroform-isomyl alcohol mixture (49:1) were added to the homogenate. Total RNA was isolated as described previously (25) and then purified to poly(A) RNA (mRNA) through oligo(dT) columns as described previously (12,14). A total of 5 µg of mRNA per lane was electrophoresed through a 1% agarose-formaldehyde gel and transferred to nylon membranes by a vacuum-blotting device (Bio-Rad). Prehybridization (4 h at 42°C) and hybridization (18 h at 42°C) of the RNA blots were performed with a buffer consisting of 5× saline-sodium phosphate-EDTA (0.75 M NaCl, 50 mM Na_{2}PO_{4}, and 5 mM EDTA, pH 7.40), 5× Denhardt’s solution (0.1% Ficol 400, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum [albumin fraction V]), 0.1% SDS, 100 µg/ml denatured salmon sperm DNA, and 50% deionized formamide, as described previously (26). A full-length cDNA probe of NaS_{1}-1 (12) was labeled by random priming (Pharmacia) using [a-32P] dCTP (Dupont-New England Nuclear). Blots were washed twice for 15 min in 0.1× SSC, 0.1% SDS at 37°C, and twice for 15 min in 0.1× SSC, 0.1% SDS at 50°C. Hybridization signals were quantified by a PhosphorImager analyzing system (Bio-Rad, Richmond, CA) and normalized for loading using a full-length mouse β-actin probe (27).

mRNA Isolation, Injection into Xenopus Laevis Oocytes, and Uptake Measurements
Kidney cortex poly(A) RNA (mRNA) was purified through an oligo(dT) column as described previously (12,14). mRNA (0.2 µg/µl) was then injected into Xenopus laevis oocytes, using a Nanoject automatic oocyte injector (Drummond Scientific, Broomall, PA). Na_{2}SO_{4} uptake measurements (12,14) in oocytes were performed on day 3 postinjection as follows. Ten oocytes per individual data point were first washed for 1 to 2 min in solution A (100 mM choline chloride, 2 mM KCl, 1 mM CaCl_{2}, 1 mM MgCl_{2}, 10 mM Hepes/Tris, pH 7.5). This solution was then replaced by 100 µl of solution B (100 mM NaCl, 2 mM KCl, 1 mM CaCl_{2}, 1 mM MgCl_{2}, and 10 mM Hepes/Tris, pH 7.5) supplemented with the 0.1 mM cold substrate (K_{2}SO_{4}, K_{2}HPO_{4}/KH_{2}PO_{4} or L-leucine) and labeled substrate Na_{2}^{35}SO_{4}, H_{3}^{32}PO_{4}, or "H-L-leucine (New England Nuclear Radiochemicals), respectively, at the specific activity of 20 µCi/ml. Oocytes were incubated at room temperature (25°C) for 30 min (within the initial linear rate of uptake). After incubation, the uptake solution was removed and the oocytes were washed 3 times with 3 ml of ice-cold stop solution (solution A). Each single oocyte was then placed in a scintillation vial, dissolved in 250 µl of 1% SDS, followed by the addition of 2 ml of scintillation fluid (Emulsifier Safe, Canberra Packard) and counted (2 min/oocyte) using liquid scintillation spectrometry.

Statistical Analyses
Results are expressed as means ± SEM (n = 6). The statistical significance of results in control (Con S) versus experimental (High S) diet rats was determined by unpaired t test. Each experiment was performed at least four times.
Results

Serum Sulfate Concentration and Urinary Sulfate Excretion Rates

Rats fed chronically (7 d) a high Si diet showed slightly increased serum Si levels (1.30 mmol/L) compared with those on a control Si diet (1.08 mmol/L) (Figure 1). Urinary Si excretion rate was markedly increased (by 12-fold) in rats fed a high Si diet (5.31 mmol/24 h) compared with control Si rats (0.44 mmol/24 h) (Figure 1). This indicates that there is a marked increase in urinary Si excretion in rats fed a high Si diet, as expected, without a major change in serum Si concentration. We also measured serum and urine creatinine values in both control and high diet rats and calculated the GFR for both groups (control rats: GFR = 404 ± 556 ml/24 h; high Si diet rats: GFR = 3731 ± 372 ml/24 h; P = NS) (data not shown).

The calculated filtered load of Si for these two rat groups was insignificant (4.37 ± 0.60 mmol/d in controls versus 4.85 ± 0.48 mmol/d in high Si diet rats; P = NS). From the filtered load and urinary excretion rates, we were able to calculate the fractional excretion of Si for the two rat groups (controls 10.6% versus high Si diet rats 136.5%) (data not shown), suggesting a net Si secretion in rats fed high Si diet.

BBM Na/Si Cotransport Activity

BBM vesicles (BBMV) were isolated from rats fed high and control Si diets, and Na/Si uptake was performed. A significant decrease in BBMV Na/Si cotransport activity was observed in rats fed a high Si diet (215 pmol Si/10 s per mg BBM protein) compared with control rats (377 pmol Si/10 s per mg BBM protein) (Figure 2). No alteration was observed for BBMV Na/Pi cotransport activity in rats fed a high Si diet (1591 ± 97 pmol Pi/10 s per mg BBM protein) versus control rats (1663 ± 127 pmol Pi/10 s per mg BBM protein; P = NS; data not shown). Under these conditions, we observed a significant reduction (43%) in BBM vesicular Na/Si uptake in rats chronically adapted to a high Si diet and showed that this adaptation impairs Na/Si cotransport with no effect on Na/Pi cotransport.

BBM NaS1-1 Protein Abundance

From the same BBMV used in the uptake assays (above), we performed Western blot analysis using a polyclonal antibody (16) directed against the NaS1-1 cotransporter (Figure 3): The 68-kD NaS1-1-specific protein band was reduced in BBMV from rats fed a high Si diet compared with controls. The NaSi-1-specific signals were quantified using densitometry (Figure 4): NaS1-1 protein abundance in rats fed a high Si diet was reduced by 33% when compared with control diet rats. As a control, Western blot analysis was performed using the same BBM proteins with a polyclonal antibody (20) raised against the NaP1-2 cotransporter: No alteration was observed in NaP1-2 protein abundance in control versus high Si diet rats (data not shown). This reduction in NaS1-1 protein abundance in BBMV closely correlates with the reduction in BBMV Na/Si cotransport activity in rats fed a high Si diet compared with control rats.

Renal Cortical NaS1-1 mRNA Abundance

Messenger RNA (mRNA) was isolated from kidney cortex slices of rats fed a high or control Si diet, and Northern blots were performed with full-length NaS1-1 and β-actin probes (Figure 5). It is clearly apparent that there is a reduction in NaS1-1 mRNA signal in rats fed a high Si diet compared with control rats. The Northern blot was quantified by densitometry, and there was a 2.7-fold decrease in NaS1-1 mRNA abundance.
**Figure 3.** The effect of a high S diet on cortical BBM NaS1-1 protein abundance. Western blots were performed with NaS1-1 antisera against 10 μg of BBM protein per lane using BBM isolated from rats kept for 7 d on a control S diet (left panel) or high S diet (middle panel). For peptide protection, antigenic peptides were included at a concentration of 100 μg/ml using BBM from control rats (right panel). The identity of the antibody-reactive band that is nonsuppressible by the antigenic peptide is unknown. One representative blot is shown (n = 4).

**Figure 4.** Densitometric data of BBM NaS1-1 protein abundance. NaS1-1 protein signal intensities on Western blots of BBM protein isolated from control S (■) and high S, diet (□) rats were quantified using a Bio-Rad densitometer. Results are expressed as means ± SEM (n = 6 BBM in each group).

**Figure 5.** The effect of a high S diet on renal cortical NaS1-1 mRNA abundance. Northern blotting was performed using full-length NaS1-1 and β-actin cDNA probes against kidney cortex mRNA (5 μg/lane) isolated from rats kept for 7 d on a control S diet or high S diet. Blots were washed under high stringency. A representative blot is shown (n = 4).

**Figure 6.** Densitometric data of renal cortical NaS1-1 mRNA abundance. NaS1-1 mRNA signals on Northern blots of rats kept for 7 d on a control S diet (■) or high S diet (□) were quantified using a Bio-Rad densitometer. Results are expressed as mean ± SEM relative NaS1-1 mRNA abundance (NaS1-1/β-actin signal).

**mRNA-Induced Na/Si Cotransport in Xenopus Oocytes**

mRNA was isolated from kidney cortex rats fed a high or control S diet and injected into *Xenopus laevis* oocytes. The mRNA-induced expression of Na/Si cotransport was measured by 35S-sulfate uptake, with the transport activity being indicative of the abundance of mRNA encoding the Na/Si cotransporter (14). We observed a 2.2-fold reduction in Na/Si cotransport in oocytes injected with mRNA from rats fed a high S diet compared with controls (Figure 7). This reduction in oocyte transport rate was specific for Na/Si uptake, showing no alteration in Na/Pi cotransport or L-leucine transport activities (Figure 7). These uptake data correlate well with the Northern blot.
posttranscriptional mechanisms. It is currently unknown whether the decrease in NaS1-I mRNA abundance in rats fed a high S diet was due to a raised sodium load in the diet, because a marked decrease in NaS1-I protein was observed. The mechanisms involved in the adaptive response of the NaS1-1 transporter to chronic alterations in dietary sulfate are remarkably similar to what we have described in the adaptive responses of the type II Na/P1 cotransporter to chronic alterations in dietary phosphate concentration in the rat (20,28-29) and opossum kidney cells (30). In response to a high P1 diet, the adaptive decrease in Na/P1 cotransport activity is associated with parallel decreases in Na/P1 protein and mRNA levels (20,28-30).

In addition to alterations in dietary S intake, various other conditions have been shown to modulate Na/Si cotransport at the level of the proximal tubular BBM: acidosis (31,32), thyroid hormone (in the mouse but not in the rat) (33,34), glucocorticoids (35,36), aging (37), acetaminophen (36), nonsteroidal anti-inflammatory agents (38), and salicylic acid (39). At present, however, the role of the NaS1-1 protein in the regulation of Na/Si cotransport activity in the above-mentioned conditions remains unknown.

In conclusion, alteration in dietary S intake is an important modifier of renal Na/Si cotransport activity. We have shown that a high S diet downregulates the expression of the cloned NaS1-1 transporter by decreasing the abundance of the NaS1-1 mRNA and NaS1-1 protein in the rat proximal tubule. This is of particular physiologic and pathophysiologic relevance because it demonstrates that dietary S intake can modulate the number of NaS1-1 proteins on the BBM during times of sulfate overload or insufficiency. This would suggest that NaS1-1, at least in part, is essential for controlling circulatory S concentration.

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