Stimulation of renal sulfate secretion by metabolic acidosis requires $\text{Na}^+/\text{H}^+$ exchange induction and carbonic anhydrase

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$^1$Department of Physiology and Neurobiology, University of Connecticut, Storrs, Connecticut; $^2$Department of Physiology and Pharmacology, James Cook University, Cairns, Australia; $^3$Department of Biology, University of Virginia’s College at Wise, Wise, Virginia; $^4$Department of Biology, Georgia Southern University, Statesboro, Georgia; and $^5$Mount Desert Island Biological Laboratory, Salisbury Cove, Maine

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Pelis, Ryan M., Susan L. Edwards, Stan C. Kunigelis, James B. Claiborne, and J. Larry Renfro. Stimulation of renal sulfate secretion by metabolic acidosis requires $\text{Na}^+/\text{H}^+$ exchange induction and carbonic anhydrase. Am J Physiol Renal Physiol 289: F208–F216, 2005. First published March 1, 2005; doi:10.1152/ajprenal.00468.2004.—The acute effect of metabolic acidosis on SO$_4^{2-}$ secretion by the marine teleost renal proximal tubule was examined. Metabolic acidosis was mimicked in primary cultures of winter flounder renal proximal tubule epithelium (fPTCs) mounted in Ussing chambers by reducing interstitial pH to 7.1 (normally 7.7). fPTCs with metabolic acidosis secreted SO$_4^{2-}$ at a net rate that was 40% higher than in paired isohydric controls (pH 7.7 on interstitium). The stimulation was completely blocked by the carbonic anhydrase inhibitor methazolamide (100 μM). Although Na$^+/\text{H}^+$ exchange (NHE) isoforms 1, 2, and 3 were identified in fPTCs by immuno blotting, administering EIPA (20 μM) to the interstitial and luminal bath solutions had no effect on net SO$_4^{2-}$ secretion by fPTCs with a normal interstitial pH of 7.7. However, EIPA (20 μM) blocked most of the stimulation caused by acidosis when applied to the lumen but not interstitium, demonstrating that induction of brush-border NHE activity is important. In the intact flounder, serum pH dropped 0.4 pH units (pH 7.7 to 7.3, at 2–3 h) when environmental pH was lowered from 7.8 to ~4.3. Whereas serum [SO$_4^{2-}$] was not altered by acidosis, renal tubular SO$_4^{2-}$ secretion rate was elevated 200%. Thus metabolic acidosis strongly stimulates renal sulfate excretion most likely by a direct effect on active renal proximal tubule SO$_4^{2-}$ secretion. This stimulation appears to be independent on inducible brush-border NHE activity.

Proximal tubule; epithelial transport; metabolic alkalosis; transport metabolon; marine teleost

PLASMA SULFATE CONCENTRATION is controlled by reabsorptive and secretory processes in the renal proximal tubule. Reabsorption predominates in terrestrial vertebrates; however, in marine teleosts, active secretion eliminates the plasma SO$_4^{2-}$ burden that would otherwise occur from the continuous ingestion of SO$_4^{2-}$-rich (~25 mM) seawater. At least two electro-neutral anion exchangers facilitate transepithelial SO$_4^{2-}$ secretion (interstitium-to-lumen). Movement of SO$_4^{2-}$ across the basolateral membrane into the cell is pH dependent, suggesting SO$_4^{2-}$/OH$^-$ exchange (34). Exit of SO$_4^{2-}$ into the lumen occurs on an anion exchanger in the brush-border membrane with affinities for both luminal HCO$_3^-$ and Cl$^-$ but not OH$^-$ (35). It has been suggested that both filtered HCO$_3^-$ and Cl$^-$ can serve as substrates in the early proximal tubule and that reduced [HCO$_3^-$] due to reabsorption requires that Cl$^-$ facilitates secretion in later segments (33). A large fraction (~50%) of tubular SO$_4^{2-}$ secretion is dependent on intracellular carbonic anhydrase (CA) activity (33). The proposed function of CA is to accelerate the formation of OH$^-$ from HCO$_3^-$ (HCO$_3^-$ $\leftrightarrow$ CO$_2$ + OH$^-$) consequently supplying the basolateral SO$_4^{2-}$/OH$^-$ exchanger with substrate (33). Basolateral SO$_4^{2-}$/OH$^-$ exchange is an intracellular acid-loading process. Therefore, under conditions where filtered Cl$^-$ is the substrate at the brush-border membrane, there should be a tendency for intracellular acidification, thus requiring acid/base transporters to maintain intracellular pH (pH$_r$). Imposition of a Na gradient does not stimulate SO$_4^{2-}$ uptake into flounder renal basolateral or brush-border membrane vesicles; yet, SO$_4^{2-}$ uptake across the basolateral membrane of intact flounder proximal tubule cells is dependent on the Na$^+$ gradient (32). The obvious importance of pH$_r$ regulation in setting intracellular [HCO$_3^-$] and [OH$^-$], along with the observation that SO$_4^{2-}$ secretion is Na$^+$ gradient dependent, has led to the inclusion of Na$^+/\text{H}^+$ exchangers (NHEs) into the model of tubular SO$_4^{2-}$ secretion.

NHE activity is ubiquitously expressed in animal cells where it performs important functions including regulation of pH$_r$ and cell volume (46). In mammalian proximal tubule cells, NHE activity is responsible for a portion of transeellular Na$^+$ reabsorption and H$^+$ excretion (4, 6, 28). NHE activity has been demonstrated in renal brush-border membrane vesicles from seawater adapted eels (Anguilla anguilla) and NHE isoform 3 (NHE3) mRNA has been identified in the kidneys of the acid-tolerant Osorezan dace (Trioblodon hakonensis) (15, 43, 48). Although there is evidence suggesting that NHE is present in the marine teleost kidney, low urine flow rates appear to preclude a significant renal participation in acid/base balance (8, 20).

Metabolic acidosis manifests as a decrease in serum pH and [HCO$_3^-$] without a change in P$_{CO_2}$. The overproduction of acid (e.g., lactic acid), ingestion of acid, and interference with branchial acid excretion (e.g., exposure of fish to acidic water) can all elicit metabolic acidosis. In rats, metabolic acidosis (>24 h) increases renal SO$_4^{2-}$ excretion by effectively reducing Na-dependent SO$_4^{2-}$ reabsorption (29). The purpose of the current study was to determine the short-term effect of metabolic acidosis and the role of NHE activity in proximal tubular SO$_4^{2-}$ secretion. The findings reported here indicate that metabolic acidosis acutely stimulates the rate of renal proximal tubular SO$_4^{2-}$ secretion both in vitro and in vivo. Furthermore, both CA and brush-border NHE activity appear to be required for the high level of SO$_4^{2-}$ secretion during acidosis.

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METHODS

Animals. Winter flounder (Pseudopleuronectes americanus) were obtained by otter trawl in Long Island Sound, CT. Animals (250–700 g) were held in Living Stream Units (Toledo) filled with artificial seawater (Utikem) at 12°C. Animal use followed the newest guiding principles for research (1). The animal research reported here adhered to APS’s Guiding Principles in the Care and Use of Animals. All investigations involving animals reported in this study were conducted in conformity with these principles, and the animal protocol was approved by the University of Connecticut IACUC (protocol no. A04-107).

Solutions and chemicals. Ouabain, methazolamide, and EIPA were obtained from Sigma (St. Louis, MO). Modified medium 199 with Earle’s salts (M199, Sigma) was supplemented with (in mM) 30.0 NaCl, 4.2 NaHCO₃ (air equilibrated), 1.0 L-glutamine, 25.0 HEPES, Earle’s salts (M199, Sigma) was supplemented with (in mM) 30.0 KH₂PO₄, pH 7.3.

Investigations involving animals reported in this study were conducted by increasing the concentration of the above ingredients, except NaCl was added to the pH 7.7 FS to balance osmolality. Final osmolality of FS was obtained (in mM) 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.5 NaH₂PO₄, 4.2 NaHCO₃, 25.0 HEPES, 5.5 glucose, 0.3 ethylene-diaminetetraacetic acid, 14.75 NaOH (pH 7.5), and 20 mg/ml tetracycline. Flounder saline (FS) contained (in mM) 150.0 NaCl, 4.0 KCl, 0.5 NaH₂PO₄, 4.2 NaHCO₃, 25.0 HEPES, 5.5 glucose, 0.3 ethylene-diaminetetraacetic acid, 14.75 NaOH (pH 7.5), and 20 mg/ml tetracycline. Flounder saline (FS) contained (in mM) 150.0 NaCl, 4.0 KCl, 2.0 CaCl₂, 1.0 MgSO₄, 0.4 NaH₂PO₄, 8.0 NaHCO₃ (gassed with 1% CO₂), 25.0 HEPES, 5.5 glucose, 1.0 L-glutamine, and titrated to pH 7.7 with NaOH, 340 mosmol/kgH₂O. High-osmolality FS was made by increasing the concentration of the above ingredients, except glucose and L-glutamine, by 6% (360 mosmol/kgH₂O). PBS contained (in mM) 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.3.

Preparation and primary culture of flounder renal proximal tubule epithelium. The procedure used here was initially described by Dickman and Renfrø (9) and later modified by Gupta and Renfrø (13). Briefly, kidneys were perfused with modified M199 and removed from the animal. Renal tubules were teased apart and hemapoietic and lymphoid tissues were removed by 45-min incubation of tubules in CMF containing 0.2% trypsin at 22°C. Epithelial cells were released from tubule fragments by 3-day incubation (5°C) in M199 containing 0.05% trypsin. Cells were washed, suspended in culture medium, plated on native rat-tail collagen, and the collagen gels (35-mm diameter) were released after 4 days. Transport studies were conducted on the contracted epithelial monolayers 12 days after releasing the collagen gels (12–17-mm diameter). Flounder renal proximal tubule epithelium (fPTCs) were devoid of extrarenal tissues (e.g., hemapoietic tissue) and express differentiated properties of proximal tubule cells (e.g., Na-dependent glucose transport).

Determination of transepithelial electrical properties and SO₄²⁻ fluxes by fPTCs. fPTCs were mounted in Ussing chambers. Aperture size was 0.332 cm², fluid volume was 1.2 ml/emichamber, and temperature was maintained at 20°C with water circulated on the outside surface of the chambers by a Lauda RM6 electronic water bath. FS inside the chambers was vigorously stirred with magnetic stir bars turned by external stir-plates. Interstitial pH was 7.1 or 7.4 (metabolic acidosis), 7.7 (isohydric control), or 8.0 (metabolic alkalosis), and luminal pH was always 7.7. The pH 7.1 (and 7.4) and 8.0 FS were titrated with HCl and NaOH, respectively, to the pH 7.7 FS to balance osmolality. Final osmolality of FS was 340 mosmol/kgH₂O. FS inside the chambers was insufflated with humidified 99% O₂-1% CO₂. Continuous gassing with 1% CO₂ resulted in pHs of 7.1 (and 7.4), 7.7, or 8.0 in FS.

Ag/AgCl electrodes connected to the luminal and interstitial bath solutions with 3 M KCl-2% agar bridges were used to determine transepithelial potential difference (TPD) and as short-circuiting electrodes. Electrode asymmetry was corrected at the beginning and end of each experiment with compensation for fluid resistance. Transepithelial resistance (TER) was determined from the change in TPD produced by a brief 10-μA pulse controlled by the voltage clamps (DVC 1000; World Precision Instruments, Sarasota, FL).

During flux determination, fPTCs were not short-circuited when asymmetrical salines bathed either side of the epithelium. Unidirectional tracer fluxes began with the addition of 1.0–2.0 μCi/100 μl to the appropriate hemichamber. Duplicate 50-μl samples were taken from the unlabeled side and replaced with 100 μl of unlabeled solution at 30-min intervals over a period of 1.5 h. The specific activity of the labeled solution was determined before and after each experiment. The difference between the unidirectional secretory and reabsorptive fluxes represents net flux. fPTCs used in a given experiment were prepared from the same culture batch. Proximal tubule-like function and tissue integrity were assessed by measurement of TPD, TER, and Na-dependent phlorizin-sensitive glucose current (i₆₆). SDS-PAGE and immunoblotting. fPTCs were placed in Kaman buffer (2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.5% saturated bromophenol blue, 62.5 mM trizma base, pH 7.1), centrifuged at 11,000 g for 90 s, and the supernatant was boiled for 5 min. SDS-PAGE was conducted with 7.5% polyacrylamide gels. Following SDS-PAGE, proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Milipore, Bedford, MA). PVDF membranes were incubated in blocking buffer (PBS containing 0.05% polyoxymethylene-sorbitan monolaurate (Tween 20), 0.01% antifoam A, 0.02% NaNO₃, and 10% nonfat dry milk) overnight at 4°C followed by 1-h incubation at room temperature with primary antibodies diluted 1:500 in blocking buffer. Primary antibodies included mouse anti-porcine NHE1 (MAB3140, Chemicon International, Temecula, CA), rabbit anti-shark NHE2 (produced by JB Claiborne), and rabbit anti-Osorezan dace NHE3 (compliments of S. Hirose, Tokyo Institute of Technology). Membranes were washed three times with PBS (10 min each), once in phosphate-free buffer (150 mM NaCl, 10 mM Tris base, 40 mM Tris-HCl, pH 7.5), and incubated for 1 h at room temperature with secondary antibodies diluted 1:1,000 in phosphate-free buffer containing 10% nonfat dry milk. Secondary antibodies included alkaline phosphatase-conjugated goat anti-mouse and goat anti-rabbit IgG (StressGen, Victoria BC, Canada). Membranes were washed four times in phosphate-free buffer followed by signal detection with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma).

Immunolocalization of NHEs and Na⁺-K⁺-ATPase to intact flounder proximal tubules. The method for immunohistochemistry in intact fish proximal tubules has been reported (21). Briefly, kidneys were removed and tubules were teased apart yielding clumps of tubules. Tubules were fixed for 10 min in 2% formaldehyde and 0.1% glutaraldehyde and washed in PBS. Tubules were permeabilized in 1% Triton X-100, washed in blocking buffer (PBS containing 1% BSA), and incubated with primary antibodies including mouse anti-porcine NHE1, rabbit anti-shark NHE2, rabbit anti-Osorezan dace NHE3, or rabbit anti-eel Na⁺-K⁺-ATPase (compliments of S. Hirose, Tokyo Institute of Technology) diluted 1:100 in blocking buffer for 90 min at 37°C. Tubules were washed in blocking buffer and incubated with fluorescein isothiocyanate-labeled goat anti-rabbit or anti-mouse IgG diluted 1:50 (final concentration of 40 μg/ml) in blocking buffer for 60 min at 37°C. Tubules were washed in blocking buffer before viewing on a confocal microscope (Leica TCS SP2). All washing steps were done at room temperature and consisted of 4× 5-min washes followed by a 15-min wash.

Renal clearance determination. The method for measuring renal clearance in winter flounder has been reported (33). Briefly, winter flounder (300–700 g) were anesthetized with MS-222 (1:2,000 wt/vol) followed by insertion of a heparinized polyethylene tube (PE-10) into the renal bladder and urine was sampled with a syringe attached to a PE-50 tube inserted into the bladder through the PE-90 catheter. Each animal was maintained in 50 liters of recirculating seawater at 4°C for 24 h and the initial 50 vol% followed by insertion of a heparinized polyethylene tube (PE-10) into the renal bladder and urine was sampled with a syringe attached to a PE-50 tube inserted into the bladder through the PE-90 catheter. Each animal was maintained in 50 liters of recirculating seawater at 4°C for 24 h and the initial 50 vol%
12°C. Animals were allowed to recover for 3 days before reducing the water pH from 7.8 to a mean pH of 4.2 by the addition of concentrated HCl (1.2 mM). The same protocol has been used to produce metabolic acidosis in rainbow trout (Onchorhynchus mykiss) and carp (Cyprinus carpio) (22, 42, 45). Inulin was injected (200 mg/kg body wt) intramuscularly 24 h before clearance measurements. Blood and urine were sampled 2–3 h before and after reducing water pH. Plasma and urine inulin concentration were determined by the indole acetic acid colorimetric method (3) and inorganic cations and anions by ion chromatography (DX-120 ion chromatograph; Dionex, Sunnyvale, CA).

Results. Experimental results are presented as means ± SE. One-way ANOVA with a repeated-measures design was used to test the effect of multiple treatments and was followed by Tukey’s HSD test for pairwise comparisons. Paired comparison of sample means was done using a paired t-test. Regression analyses were used to test the effects of serum [H+] and osmolality on tubular SO4\(^{2-}\) secretion rate in vivo. All statistical analyses were done using Statistica (StatSoft, Tulsa, OK) and deemed significant when P < 0.05.

Influence of metabolic acidosis on SO4\(^{2-}\) secretion by fPTCs. Figure 1A is a representative plot demonstrating SO4\(^{2-}\) transport by control fPTCs (pH 7.7 FS on interstitium and lumen) in Ussing chambers. Unidirectional SO4\(^{2-}\) fluxes were initiated at t = 0 h and net transport reached steady state at t = 1 h, reflecting the time for the label to equilibrate with intracellular SO4\(^{2-}\) pools. The magnitude of the unidirectional reabsorptive flux was ~5% of the unidirectional secretory flux (at t = 1.5 h). In the example shown, reducing interstitial pH to 7.1 stimulated net SO4\(^{2-}\) secretion by increasing the unidirectional secretory flux (Fig. 1B). The pH 7.7 and 7.1 FS solutions were continuously gassed with 1% CO2 resulting in HCO3\(^-\) concentrations of 8 and 2 mM, respectively. Thus metabolic acidosis was mimicked in the Ussing chambers by reducing interstitial pH to 7.1.

Net SO4\(^{2-}\) secretion in control fPTCs was 80.7 ± 7.74 nmol/cm\(^2\)-h\(^{-1}\), and inhibition of CA with methazolamide (100 μM) reduced net secretion 54% (Fig. 2). Metabolic acidosis stimulated net secretion 41%, and the effect was prevented by 100 μM methazolamide. It should be noted that with methazolamide present the magnitude of the stimulatory effect of interstitial pH 7.1 was almost identical to its effect in the absence of methazolamide.

At least in early proximal tubule segments, luminal pH should approximate interstitial pH. However, in the experiments shown in Figure 2 and see Figure 4, only pH of the interstitial side was altered in an attempt to examine direct effects of changes in pH at the basolateral membrane with little or no expected change in [HCO3\(^-\)] of the luminal side. When both luminal and interstitial sides were acidified in combination, there was a 61% increase in net SO4\(^{2-}\) secretion by fPTCs (182 ± 17.1 vs. 113 ± 14.0 nmol/cm\(^2\)-h\(^{-1}\), n = 3, P < 0.05, paired t-test), which was similar to the effect caused by interstitial acidification alone. Metabolic alkalosis, which was mimicked by increasing interstitial pH to 8.0 at constant CO2 (1%), reduced net SO4\(^{2-}\) secretion 40% (Fig. 2). CA inhibition had no effect on SO4\(^{2-}\) transport during metabolic alkalosis. The magnitude of net secretion was the same for fPTCs with an interstitial pH of 7.7 and 8.0 when CA was inhibited. All of the effects on SO4\(^{2-}\) secretion were due to changes in the unidirectional secretory flux (unidirectional fluxes not shown). TER, TPD, and I\(_{th}\) in control fPTCs were 45 ± 8.0 Ω·cm\(^{-2}\), −0.19 ± 0.06 mV (lumen negative), and −1.5 ± 0.10 μA/cm\(^2\), respectively. Transepithelial electrical properties were unaffected by metabolic acidosis, alkalosis, or methazolamide treatment (data not shown).

Role of NHE activity in SO4\(^{2-}\) secretion by fPTCs. NHEs are an important defense against intracellular acidification and are a proposed requirement for tubular SO4\(^{2-}\) secretion (26, 33). Figure 3 shows the effects of NHE inhibition (20 or 100 μM EIPA applied to both the interstitium and lumen) and Na\(^+\)-K\(^+\)-ATPase inhibition (0.1 mM ouabain) on the unidirectional secretory, unidirectional reabsorptive, and net SO4\(^{2-}\) fluxes by fPTCs in Ussing chambers with pH 7.7 FS bathing both sides of the epithelium. The low dose of EIPA did not alter SO4\(^{2-}\) secretion (Fig. 3A), whereas the high dose reduced secretion...
68% (Fig. 3B). The effect on net secretion was due to a significant decrease in the unidirectional secretory flux and increase in the unidirectional absorptive flux. Ouabain treatment completely abolished net secretion by reducing the unidirectional secretory flux (Fig. 3C). The low dose of EIPA had no effect on transepithelial electrical properties generated by fPTCs while the high dose caused a significant decrease in $I_{\text{glu}}$ (Table 1). There were sharp reductions in TER, TPD, and $I_{\text{glu}}$ with ouabain treatment. Both amiloride and the pyrazine-ring-substituted amiloride derivatives (e.g., EIPA) are capable of inhibiting Na$^+$-K$^+$-ATPase activity through competition with Na$^+$ and K$^+$ for the cation transport site (10, 39, 47). In our hands, treatment of rat basolateral membranes, enriched with Na$^+$-K$^+$-ATPase, with 100 µM EIPA caused a 40% reduction in ouabain-sensitive Na$^+$-K$^+$-ATPase activity (data not shown). These data indicate that the effect of 100 µM EIPA on $SO_4^{2-}$ transport is nonspecific through inhibition of Na$^+$-K$^+$-ATPase activity.

Figure 4 shows the effect of administering 20 µM EIPA to either the interstitium or lumen on net $SO_4^{2-}$ secretion by fPTCs with metabolic acidosis. Net $SO_4^{2-}$ secretion in control fPTCs was $94.0 \pm 20.3$ mmol·cm$^{-2}$·h$^{-1}$ and increased to $152.0 \pm 14.0$ mmol·cm$^{-2}$·h$^{-1}$ following metabolic acidosis. Although it had no effect under isohydric conditions (see Fig. 3A), addition of 20 µM EIPA to the lumen but not interstitium prevented the majority of stimulation caused by metabolic acidosis. Compared with controls, metabolic acidosis alone and in combination with EIPA (interstitium or lumen) had no effect on TER, TPD, and $I_{\text{glu}}$ (Table 2). TER was significantly higher when EIPA was on the lumen compared with the interstitium. Taken together, these data indicate that induction of brush-border NHE activity is required for the full stimulation in $SO_4^{2-}$ secretion during metabolic acidosis.

### Immunolocalization of NHEs

Immunoblots were performed to determine whether NHE isoforms are present in the flounder

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**Table 1. Effects of EIPA and ouabain on transepithelial electrical properties generated by fPTCs**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>TER, Ω·cm$^2$</th>
<th>TPD, mV</th>
<th>$I_{\text{glu}}, \mu$A·cm$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>42 ± 5.1</td>
<td>-0.3 ± 0.16</td>
<td>-1.8 ± 0.94</td>
</tr>
<tr>
<td>20 µM EIPA</td>
<td>3</td>
<td>41 ± 5.8</td>
<td>-0.4 ± 0.06</td>
<td>-1.7 ± 0.45</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>31 ± 3.3</td>
<td>-0.3 ± 0.44</td>
<td>-2.6 ± 0.75</td>
</tr>
<tr>
<td>100 µM EIPA</td>
<td>3</td>
<td>38 ± 8.9</td>
<td>-0.1 ± 0.13</td>
<td>-1.0 ± 0.44*</td>
</tr>
<tr>
<td>Ouabain</td>
<td>3</td>
<td>49 ± 7.1</td>
<td>-0.5 ± 0.08</td>
<td>-2.0 ± 0.32</td>
</tr>
<tr>
<td>Ouabain</td>
<td>3</td>
<td>16 ± 4.0</td>
<td>-0.0 ± 0.01*</td>
<td>-0.4 ± 0.05*</td>
</tr>
</tbody>
</table>

Values are means ± SE and were obtained at $t = 1.5$ h. EIPA was added to both the interstitial and luminal bath solutions at 20 or 100 µM. Ouabain (0.1 mM) was added to the interstitial bath solution alone. TER, transepithelial resistance; TPD, transepithelial potential difference; $I_{\text{glu}}$, phlorizin-sensitive glucose current; fPTCs, winter flounder renal proximal tubule epithelium.

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**Table 2. Effect of metabolic acidosis and EIPA on transepithelial electrical properties generated by fPTCs**

<table>
<thead>
<tr>
<th>Intestinal pH</th>
<th>20 µM EIPA</th>
<th>TER, Ω·cm$^2$</th>
<th>TPD, mV</th>
<th>$I_{\text{glu}}, \mu$A·cm$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.7</td>
<td>38 ± 3.5*</td>
<td>-0.3 ± 0.08</td>
<td>-2.3 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>7.1</td>
<td>35 ± 3.6*</td>
<td>-0.4 ± 0.07</td>
<td>-2.4 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>7.1</td>
<td>31 ± 4.2*</td>
<td>-0.3 ± 0.04</td>
<td>-2.5 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>Lumen</td>
<td>43 ± 4.7*</td>
<td>-0.4 ± 0.06</td>
<td>-2.0 ± 0.20</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE ($n = 6$) and were obtained at $t = 1.5$ h. EIPA was not present (-) or was added to either the interstitial or luminal bath solution at a final concentration of 20 µM. *Significant differences among treatments ($P < 0.05$, Tukey’s HSD).
proximal tubule (Fig. 5). When probed with an anti-porcine NHE1 antibody, a faint band centered at 100 kDa was present from fRBCs. A more intense band with an approximate molecular mass spanning from 90–110 kDa was observed from fPTCs and rat renal cortical basolateral membranes (positive control). Total protein varied among samples and different band intensities likely reflect differences in protein loading rather than NHE1 expression (typically a “smeary” band). NHE2 (~85 kDa) and NHE3 (~98 kDa) were also identified in fPTCs using shark anti-NHE2 and anti-Osorezan dace NHE3 antibodies, respectively (Fig. 5B). NHE2 was immunolocalized to intact flounder proximal tubules and exhibited both an apical and subapical distribution (Fig. 6A). In contrast, Na⁺-K⁺-ATPase was clearly restricted to the basolateral membrane (Fig. 6B). The NHE1 antibody (MAB3140) is not recommended for immunohistochemistry (Chemicon International Technical Bulletin), and, indeed, our attempts to immunolocalize NHE1 were unsuccessful. Similarly, attempts to immunolocalize NHE3 to the intact proximal tubule were unsuccessful.

Effect of metabolic acidosis on renal SO₄²⁻ clearance. The substantial effect of acidosis on SO₄²⁻ secretion in vitro called for further examination in the intact animal (in vivo). Winter flounder were exposed to low environmental pH to induce metabolic acidosis. The majority (>90%) of acid-base transfer between fish and the environment occurs across the gills and exposure to low environmental pH apparently causes acid loading by reducing branchial acid excretion (14, 45). Serum and urine pH dropped 0.4 units when environmental pH was reduced from 7.8 to a mean value of 4.2 (range 3.7–5.0; Table 3). Serum [Na⁺] (159 ± 2.4 to 170 ± 63 mM), [K⁺] (3.2 ± 0.58 to 5.2 ± 1.48 mM), and [Cl⁻] (153 ± 3.0 to 162 ± 7.7 mM) were slightly elevated (not significant), and serum [Mg²⁺] was significantly elevated (0.39 ± 0.067 to 0.61 ± 0.064 mM) by metabolic acidosis. Serum [Ca²⁺] (3.1 ± 0.11 mM), [PO₄³⁻] (2.8 ± 0.43 mM), and [SO₄²⁻] (0.20 ± 0.045 mM) did not change. Small changes in serum Na⁺, K⁺, and Cl⁻, together with the significant change in serum Mg²⁺, likely contributed to the significant increase in serum osmolality (16 mosmol/kgH₂O) during metabolic acidosis. NHEs subserve branchial acid excretion in teleosts, and increased branchial NHE activity during acidosis exacerbates the plasma Na⁺ load already experienced by marine teleosts (7, 17). Urine osmolality, glomerular filtration rate (GFR), and urine flow rate were unchanged by acidosis. The rate of inorganic ion reabsorption (Qreabsorption) or secretion (Qsecretion) was calculated as the difference in the quantity excreted and quantity filtered.

\[ Q = (\text{urine flow rate} \times [\text{ion}]_{\text{urine}}) - (\text{GFR} \times [\text{ion}]_{\text{serum}}) \]

Where [ion]urine and [ion]serum are the concentrations of the inorganic ion in the urine and serum, respectively. The fractional reabsorption of Na⁺, K⁺, Ca²⁺, and Cl⁻ and secretion of Mg²⁺ were not significantly altered by metabolic acidosis (Table 3). Both PO₄³⁻ and SO₄²⁻ secretion were elevated 200%, and there was a positive correlation between SO₄²⁻ secretion rate and serum [H⁺] (Fig. 7A). The pH used to mimic metabolic acidosis in vitro (pH 7.1) was much lower than observed in animals with metabolic acidosis (pH 7.3). Therefore, net SO₄²⁻ secretion was examined in fPTCs with an interstitial pH 7.4, which closely matched that found in vivo. Under these conditions, net SO₄²⁻ secretion remained significantly higher (50%) than in isohydric controls at pH 7.7 (Fig. 8). There was also a positive relationship between SO₄²⁻ secretion rate and serum osmolality (Fig. 7B). However, a 6% elevation of FS osmolality (340 to 360) bathing the interstitium of fPTCs had no effect on net SO₄²⁻ secretion (Fig. 8).
metabolic acidosis on renal function in winter flounder

<table>
<thead>
<tr>
<th>GFR</th>
<th>Urine Flow Rate</th>
<th>Serum pH</th>
<th>Urine pH</th>
<th>Serum Osmolality</th>
<th>Urine Osmolality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.37±0.067</td>
<td>0.12±0.018</td>
<td>7.7±0.01</td>
<td>6.5±0.22</td>
<td>335±4.1</td>
</tr>
<tr>
<td>Metabolic acidosis</td>
<td>0.66±0.20</td>
<td>0.23±0.055</td>
<td>7.3±0.05†</td>
<td>6.1±0.17*</td>
<td>351±6.5*</td>
</tr>
</tbody>
</table>

Table 3. Effect of metabolic acidosis on renal function in winter flounder

DISCUSSION

The present data indicate that changes in serum pH have dramatic acute effects on the rate of renal proximal tubular SO₄²⁻ secretion. Metabolic acidosis stimulated SO₄²⁻ secretion, whereas metabolic alkalosis inhibited it. Renal basolateral membrane vesicles isolated from southern flounder (*Paralichthys lethostigma*) exhibit pH-dependent SO₄²⁻ uptake that is sensitive to the anion exchange inhibitor DIDS, suggesting basolateral SO₄²⁻/OH⁻ exchange (intracellular OH⁻ for interstitial SO₄²⁻) (34). The in vitro effects in the present study are consistent with the vesicle data and likely reflect changes in the OH⁻ gradient across the basolateral membrane of proximal tubule cells. However, it cannot be dismissed that a change in SO₄²⁻/HCO₃⁻ exchange activity at the brush-border membrane may also contribute to the observed elevation in secretion. Preliminary data indicate that extracellular acidification (pH 7.1) and alkalization (pH 8.0) reduce (mean of 0.35 pH units, n = 4 cells) and increase (mean of 0.20 pH units, n = 2 cells) pH₈ in fPTCs, respectively (Pelis RM and Renfro JL, unpublished observations). Intracellular [HCO₃⁻] would be expected to change in the same direction as pH₈ (i.e., decreased intracellular [HCO₃⁻] during acidosis), and thus there should be a steeper inwardly directed HCO₃⁻ concentration gradient (lumen-to-cell) following interstitial acidification (but not luminal), which would likely stimulate SO₄²⁻ exit across the brush-border membrane. However, when both the lumen and interstitium were acidified to pH 7.1 (i.e., low [HCO₃⁻] on lumen and interstitium) net SO₄²⁻ secretion remained stimulated to the same or greater extent as interstitial acidification alone, suggesting that the effect of metabolic acidosis is best explained by stimulation of basolateral membrane SO₄²⁻/OH⁻ exchange.

The driving force for SO₄²⁻/OH⁻ exchange is currently unknown as estimated values for pH₈ are lower and intracellular [SO₄²⁻] are higher than in the interstitial medium, and SO₄²⁻/OH⁻ exchange is electroneutral (26). We recently hypothesized that a metabolon arrangement (physical association) of CA with the SO₄²⁻/OH⁻ exchanger or Na⁺/H⁺ exchanger creates a functional asymmetry in the SO₄²⁻/OH⁻ exchanger, perhaps by elevating local pH near the intracellular OH⁻ binding site, thus making SO₄²⁻/OH⁻ exchange energetically favorable (26). Several lines of evidence suggest that CA associates with a membrane component in the flounder proximal tubule, including the observations that CAII protein is present in fPTC plasma membranes (demonstrated by immunoblotting) and in or near the brush-border and basolateral membranes of intact flounder proximal tubule cells (demonstrated by immunocytochemistry) (25, 26). In oligodendrocytes, CAII physically associates with NHE1 and the Na⁺-HCO₃⁻ cotransporter isoform 1 (37). In addition, acidic and basic pH microdomains form at sites where CAII and NHE1 (basic domains) and CAII and NBC1 (acidic domains) colocalize, suggesting that CA may contribute to the creation of a local pH gradient near these transporters (37). If the transport metabolon theory is correct for SO₄²⁻ transport in the marine teleost proximal tubule, the CA-dependent disequilibrium pH is favorable at low and isohydric interstitial pH, and elevation of interstitial pH opposes this gradient. The rate of SO₄²⁻ transport remaining after CA inhibition (CA-independent frac-
Fig. 8. Net SO$_4^{2-}$ secretion by fPTCs in Ussing chambers in response to a 6% increase in interstitial osmolality and interstitial acidification (pH 7.4). Values shown are means ± SE (n = 4) and were obtained at t = 1.5 h. pH 7.4 FS was titrated with HCl, and NaCl was added to the pH 7.7 FS to balance osmolality.

*SSignificantly different from paired control (P < 0.05, paired t-test).

xosphate transport (at isohydric and high interstitial pH) may reflect the exchange of SO$_4^{2-}$ for an intracellular anion(s) other than OH$^{-}$, such as a metabolic intermediate (e.g., oxalate). The high level of CA-independent transport during metabolic acidosis is best explained by the reversal of the pH gradient across the basolateral membrane, such that pH$_i$ (in bulk solution) is higher than interstitial pH.

Uptake of SO$_4^{2-}$ across the basolateral membrane of intact flounder proximal tubules is inhibited by incubation in Na-free medium and ouabain treatment, suggesting involvement of the Na gradient in SO$_4^{2-}$ secretion (32, 34). However, there is no direct effect of Na$^+$ on SO$_4^{2-}$ transport in renal brush-border or basolateral membrane vesicles (34). These observations, along with the role of pH in setting [HCO$_3^{-}$] and [OH$^{-}$], have led to the hypothesis that the effect of the Na gradient on SO$_4^{2-}$ transport is secondary to control of pH$_i$, through NHE activity (26, 31, 33, 34). Although 20 µM EIPA (applied to lumen and interstitium) had no effect on net SO$_4^{2-}$ secretion by fPTCs under isohydric conditions, 100 µM significantly inhibited net secretion by reducing the unidirectional secretory flux and increasing the unidirectional reabsorptive flux, which was similar to the effect caused by ouabain treatment.

In addition to this inhibition by 100 µM EIPA and ouabain of SO$_4^{2-}$ secretion, both caused significant reductions in Na-dependent phlorizin-sensitive glucose transport (I$_{gph}$), indicating a diminished plasma membrane Na gradient. Although 100 µM EIPA was probably nonspecific, the most likely explanation for inhibition of net SO$_4^{2-}$ secretion is a reduction in pH$_i$, Na$^+$-K$^+$-ATPase inhibition, through dissipation of the Na gradient (i.e., reduced NHE activity), reduces pH$_i$ in numerous cell types (16, 18). Reduced pH$_i$ could potentially drive the basolateral SO$_4^{2-}$/OH$^{-}$ exchanger backwards and may explain why the unidirectional reabsorptive flux was elevated following ouabain and 100 µM EIPA treatment.

The present observation that metabolic acidosis stimulated net SO$_4^{2-}$ secretion and that 20 µM EIPA administered to the lumen, but not the interstitium, blocked this stimulation is consistent with increased activity of brush-border NHE activity following acid loading. In human embryonic kidney cells that endogenously express NHE1, NHE2, and NHE3, intracellular acidification elicits a time-dependent increase in NHE activity resulting in pH$_i$ recovery (19). NHE activity, but not NHE3 protein, is elevated in mammalian renal brush-border membrane vesicles following acute metabolic acidosis (38). This may be at least partly explained by the fact that NHEs are allosterically activated by intracellular H$^+$ thus enhancing H$^+$ excretion following acid loading (2).

Two questions regarding the effect of EIPA on SO$_4^{2-}$ secretion remain. Assuming that NHEs are important, why is SO$_4^{2-}$ secretion by fPTCs under isohydric conditions not affected by 20 µM EIPA? One possible explanation is that this concentration of EIPA is not sufficient to inhibit all of the activity generated by the NHEs in the flounder proximal tubule. Regulatory volume increase (RVI) in flounder red blood cells requires NHE1, and RVI is not inhibited by 20 µM EIPA, suggesting that flounder NHE1 is insensitive to this concentration (24, 36). Second, why doesn’t 20 µM EIPA prevent all of the stimulation in secretion? In addition to NHEs, other acid-extruding processes (e.g., H$^+$-ATPase and H$^+$-K$^+$-ATPase) may also be required to control pH$_i$ following an acid load.

Information regarding NHE expression in the marine teleost proximal tubule is restricted to activity measurements in marine eel and flounder renal brush-border membrane vesicles (11, 43, 48). In the present study, heterologous antibodies provided evidence for three NHE isoforms in the marine teleost proximal tubule. NHE1-, NHE2-, and NHE3-immunoreactive proteins were detected in fPTC lysates, with each having an apparent molecular mass similar to its mammalian counterpart. The NHE1 and NHE3 antibodies proved unsuitable for immunolocalization in flounder proximal tubule; however, NHE1 and NHE3, together with the newly demonstrated apical NHE8 (12), are localized to the basolateral and brush-border membranes of mammalian proximal tubule cells, respectively (23). Whereas NHE2 expression at the apical membrane of rat distal nephron segments including cortical and medullary thick ascending limbs, distal convoluted tubules, and macula densa cells is well established, its presence in proximal tubule cells is uncertain (5, 27, 40). Regardless, NHE2-like protein was detected in fPTCs and exhibited an apical/subapical distribution in intact flounder proximal tubule cells. This distribution was clearly different from that of the basolateral marker Na$^+$-K$^+$-ATPase.

The significance of metabolic acidosis in fish (22, 41, 42, 44), along with its dramatic effect on SO$_4^{2-}$ secretion by fPTCs, prompted our examination of the intact animal response. Renal SO$_4^{2-}$ secretion (in vivo) was increased 200% in animals with metabolic acidosis, and there was a positive correlation between the rate of secretion and serum [H$^+$]. The effect of metabolic acidosis is consistent with the in vitro data, where both nonphysiological (pH 7.1) and physiological (pH 7.4) acidic interstitial pH acutely stimulated net SO$_4^{2-}$ secretion by fPTCs ~50%. Factors other than the acute effect of serum [H$^+$] may also influence SO$_4^{2-}$ transport following metabolic acidosis. Metabolic, but not respiratory acidosis, leads to an increase in plasma cortisol levels in rainbow trout (45). In the marine teleost renal proximal tubule, cortisol increases brush-border SO$_4^{2-}$/HCO$_3^{-}$ exchange activity, CA activity, CAII protein abundance, and CA-dependent SO$_4^{2-}$ secretion (25, 30). Transcriptional regulation of SO$_4^{2-}$-transport-related proteins by cortisol, along with the direct effect of serum [H$^+$], may thus act additively to stimulate SO$_4^{2-}$ secretion.


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In conclusion, this investigation demonstrated that the rate of transepithelial $\text{SO}_4^{2-}$ secretion by the marine teleost renal proximal tubule is sensitive to acute changes in interstitial pH. These effects most likely reflect a change in the rate of $\text{SO}_4^{2-}$ uptake into proximal tubule cells via basolateral $\text{SO}_4^{2-}$/OH$^-$ exchange, such that $\text{SO}_4^{2-}$/OH$^-$ exchange activity is enhanced by interstitial acidosis and slowed by interstitial alkalosis. The high level of $\text{SO}_4^{2-}$ secretion during metabolic acidosis required both CA and brush-border NHE activity. The observation that NHE activity supports tubular $\text{SO}_4^{2-}$ secretion suggests that pH regulation is necessary to maintain proper driving forces for transepithelial $\text{SO}_4^{2-}$ secretion.

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