Stimulation of renal sulfate secretion by metabolic acidosis requires Na+/H+ exchange induction and carbonic anhydrase

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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 Na+/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 SO42− 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 SO42− 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+/H+ exchange (NHE) isoforms 1, 2, and 3 were identified in fPTCs by immunoblotting, administering EIPA (20 mM) to the interstitial and luminal bath solutions had no effect on net SO42− secretion by fPTCs with a normal interstitial pH of 7.7. However, EIPA (20 mM) 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 [SO42−] was not altered by acidosis, renal tubular SO42− secretion rate was elevated 200%. Thus metabolic acidosis strongly stimulates renal sulfate excretion most likely by a direct effect on active renal proximal tubule SO42− secretion. This stimulation appears to be independent on inducible brush-border NHE activity.

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 SO42− burden that would otherwise occur from the continuous ingestion of SO42−-rich (~25 mM) seawater. At least two electroneutral anion exchangers facilitate transepithelial SO42− secretion (interstitium-to-lumen). Movement of SO42− across the basolateral membrane into the cell is pH dependent, suggesting SO42−/OH− exchange (34). Exit of SO42− into the lumen occurs on an anion exchanger in the brush-border membrane with affinities for both luminal HCO3− and Cl− but not OH− (35). It has been suggested that both filtered HCO3− and Cl− can serve as substrates in the early proximal tubule and that reduced [HCO3−] due to reabsorption requires that Cl− facilitates secretion in later segments (33). A large fraction (~50%) of tubular SO42− secretion is dependent on intracellular carbonic anhydrase (CA) activity (33). The proposed function of CA is to accelerate the formation of OH− from HCO3− (HCO3− ← CO2 + OH−) consequently supplying the basolateral SO42−/OH− exchanger with substrate (33). Basolateral SO42−/OH− exchanger 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 (pHi). Imposition of a Na gradient does not stimulate SO42− uptake into flounder renal basolateral or brush-border membrane vesicles; yet, SO42− uptake across the basolateral membrane of intact flounder proximal tubule cells is dependent on the Na+ gradient (32). The obvious importance of pHi regulation in setting intracellular [HCO3−] and [OH−], along with the observation that SO42− secretion is Na+ gradient dependent, has led to the inclusion of Na+/H+ exchangers (NHEs) into the model of tubular SO42− secretion.

NHE activity is ubiquitously expressed in animal cells where it performs important functions including regulation of pHi and cell volume (46). In mammalian proximal tubule cells, NHE activity is responsible for a portion of transepacellular Na+ reabsorption and H+ secretion (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 (Tribolodon 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 [HCO3−] without a change in PaCO2. 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 SO42− excretion by effectively reducing Na-dependent SO42− 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 SO42− secretion. The findings reported here indicate that metabolic acidosis acutely stimulates the rate of renal proximal tubular SO42− secretion both in vitro and in vivo. Furthermore, both CA and brush-border NHE activity appear to be required for the high level of SO42− secretion during acidosis.

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METABOLIC ACIDOSIS STIMULATES RENAL SULFATE SECRETION

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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, 2.2 NaHCO3 (air equilibrated), 1.0 l-glutamine, 25.0 HEPES, 14.75 NaOH (pH 7.5), 347 mosmol/kgH2O, and 20 mg/l tetracycline. Modified M199 was supplemented with 10 μg/ml insulin, 5 μg/ml hydrocortisone, and 10% foelner serum to make the final plating medium. Ca2+- and Mg2+-free solutions (CMF) used for removing extrarenal tissues contained (in mM) 150.0 NaCl, 4.0 KCl, 0.5 NaH2PO4, 4.2 NaHCO3, 25.0 HEPES, 5.5 glucose, 0.3 ethylenediaminetetraacetic acid, 14.75 NaOH (pH 7.5), and 20 mg/l 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, 0.5 mM KH2PO4, 4.2 mM NaHCO3, 25.0 HEPES, 5.5 glucose, 0.3 ethylenediaminetetraacetic acid, 14.75 NaOH (pH 7.5), and 20 mg/l 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, 0.5 mM KH2PO4, 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 Renfro (9) and later modified by Gupta and Renfro (13). Briefly, kidneys were perfused with modified M199 and removed from the animal. Renal tubules were teased apart and hemopoietic 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- to 17-mm diameter). Flounder renal proximal tubule epithelium (fPTCs) are devoid of extrarenal tissues (e.g., hemopoietic 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. PTCs were mounted in Ussing chambers. Aperture size was 0.332 cm2, fluid volume was 1.2 ml/hemichamber, and temperature was maintained at 20°C with water circulated on the outside surface of the chambers by a Lauda RM6 electronic water temperature was maintained at 20°C with water circulated on the outside 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/μmol 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. ITPCs 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 (Igph).

SDS-PAGE and immunoblotting. fPTCs were placed in Kaman buffer (2.5% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.5% saturated bromphenol blue, 62.5 mM trisma 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 polyvinylidine fluoride (PVDF) membranes (Milipore, Bedford, MA). PVDF membranes were incubated in blocking buffer (PBS containing 0.05% polyoxyethylene-sorbitan monolaurate (Tween 20), 0.01% antifoam A, 0.02% NaN3, 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 (complements 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 (complements 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 (1,200 wt/vol) followed by injection of a heparinized polyethylene tube (PE-10) into the hemal vein adjacent to the caudal peduncle. When withdrawing blood, the initial 50 μl were discarded to prevent sample contamination with SO42- from the heparin. A PE-90 tube was inserted into the urinary 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
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).

**Statistics.** 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 SO$_4^{2-}$ secretion rate in vivo. All statistical analyses were done using Statistica (StatSoft, Tulsa, OK) and deemed significant when $P < 0.05$.

**RESULTS**

*Influence of metabolic acidosis on SO$_4^{2-}$ secretion by fPTCs.* Figure 1A is a representative plot demonstrating SO$_4^{2-}$ transport by control fPTCs (pH 7.7 FS on interstitium and lumen) in Ussing chambers. Unidirectional $^{35}$SO$_4^{2-}$ 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 SO$_4^{2-}$ pools. The magnitude of the unidirectional reabsorptive flux was $\sim 5\%$ of the unidirectional secretory flux (at $t = 1.5$ h). In the example shown, reducing interstitial pH to 7.1 stimulated net SO$_4^{2-}$ secretion by increasing the unidirectional secretory flux (Fig. 1B). The pH 7.7 and 7.1 FS solutions were continuously gassed with 1% CO$_2$ resulting in HCO$_3^{-}$ concentrations of 8 and 2 mM, respectively. Thus metabolic acidosis was mimicked in the Ussing chambers by reducing interstitial pH to 7.1.

Net SO$_4^{2-}$ secretion in control fPTCs was $80.7 \pm 7.74$ nmol cm$^{-2}$ h$^{-1}$, and inhibition of CA with methazolamide (100 $\mu$M) reduced net secretion 54% (Fig. 2). Metabolic acidosis stimulated net secretion 41%, and the effect was prevented by 100 $\mu$M 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 Fig. 2 and see Fig. 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 [HCO$_3^{-}$] of the luminal side. When both luminal and interstitial sides were acidified in combination, there was a 61% increase in net SO$_4^{2-}$ 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 CO$_2$ (1%), reduced net SO$_4^{2-}$ secretion 40% (Fig. 2). CA inhibition had no effect on SO$_4^{2-}$ 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 SO$_4^{2-}$ secretion were due to changes in the unidirectional secretory flux (unidirectional fluxes not shown). TER, TPD, and $I_{sh}$ in control fPTCs were 45 ± 8.0 $\Omega$ cm$^{-2}$, $-0.19 \pm 0.06$ mV (lumen negative), and $-1.5 \pm 0.10$ $\mu$A cm$^{-2}$, respectively. Transepithelial electrical properties were unaffected by metabolic acidosis, alkalosis, or methazolamide treatment (data not shown).

**Role of NHE activity in SO$_4^{2-}$ secretion by fPTCs.** NHEs are an important defense against intracellular acidification and are a proposed requirement for tubular SO$_4^{2-}$ secretion (26, 33). Figure 3 shows the effects of NHE inhibition (20 or 100 $\mu$M 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 SO$_4^{2-}$ 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 SO$_4^{2-}$ secretion (Fig. 3A), whereas the high dose reduced secretion...
Effects of EIPA and ouabain on transepithelial electrical properties generated by fPTCs

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·cm²</th>
<th>TPD, mV</th>
<th>( I_{\text{glu}} ), μA/cm²</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>100 μM EIPA</td>
<td>3</td>
<td>31 ± 3.3</td>
<td>-0.3 ± 0.44</td>
<td>-2.6 ± 0.75</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></td>
<td></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.

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·cm²</th>
<th>TPD, mV</th>
<th>( I_{\text{glu}} ), μA/cm²</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 intermittent</td>
<td>31 ± 4.2*</td>
<td>-0.3 ± 0.04</td>
<td>-2.5 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>7.1 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. *Significantly different from paired control (\( P < 0.05 \), Tukey’s HSD).

Fig. 4. Net \( SO_4^{2-} \) secretion by fPTCs in Ussing chambers in response to metabolic acidosis and treatment with EIPA. Intestinal pH was 7.7 (isohydric control) or 7.1 (metabolic acidosis). Luminal pH was 7.7. The pH 7.1 FS was titrated with HCl, and NaCl was added to the pH 7.7 FS to balance osmolality. EIPA was absent (−) or present on the interstitium or lumen at 20 μM. fPTCs were maintained under open-circuit conditions throughout the flux period. Values shown are means ± SE (n = 6) and were obtained at \( t = 1.5 \) h. Different letters indicate significant differences among treatments (\( P < 0.05 \), Tukey’s HSD).

68% (Fig. 3B). The effect on net secretion was due to a significant decrease in the unidirectional secretory flux and increase in the unidirectional reabsorptive flux. Ouabain treatment completely abolished net secretion by reducing the unidirectional secretory flux and increasing the unidirectional reabsorptive fluxes (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 \( Na^+\text{-K}^+\text{-ATPase} \) 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 \( Na^+\text{-K}^+\text{-ATPase} \), with 100 μM EIPA caused a 40% reduction in ouabain-sensitive \( Na^+\text{-K}^+\text{-ATPase} \) activity (data not shown). These data indicate that the effect of 100 μM EIPA on \( SO_4^{2-} \) transport is nonspecific through inhibition of \( Na^+\text{-K}^+\text{-ATPase} \) 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 ± 10.2 nmol·cm⁻²·h⁻¹ and increased to 152 ± 14.0 nmol·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.
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 SO\(_4^{2-}\) clearance. The substantial effect of acidosis on SO\(_4^{2-}\) 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 ± 6.3 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 [Mg\(^{2+}\)] was significantly elevated (0.39 ± 0.067 to 0.61 ± 0.064 mM) by metabolic acidosis. Serum [Ca\(^{2+}\)] (3.1 ± 0.11 mM), [PO\(_4^{3-}\)] (2.8 ± 0.43 mM), and [SO\(_4^{2-}\)] (0.20 ± 0.045 mM) did not change. Small changes in serum Na\(^+\), K\(^+\), and Cl\(^-\), together with the significant change in serum Mg\(^{2+}\), likely contributed to the significant increase in serum osmolality (16 mosmol/kgH\(_2\)O) 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 (Q\(_{\text{reabsorption}}\)) or secretion (Q\(_{\text{secretion}}\)) 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]\(_{\text{urine}}\) and [ion]\(_{\text{serum}}\) are the concentrations of the inorganic ion in the urine and serum, respectively. The fractional reabsorption of Na\(^+\), K\(^+\), Ca\(^{2+}\), and Cl\(^-\) and secretion of Mg\(^{2+}\) were not significantly altered by metabolic acidosis (Table 3). Both PO\(_4^{3-}\) and SO\(_4^{2-}\) secretion were elevated 200%, and there was a positive correlation between SO\(_4^{2-}\) 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 SO\(_4^{2-}\) secretion was examined in fPTCs with an interstitial pH 7.4, which closely matched that found in vivo. Under these conditions, net SO\(_4^{2-}\) secretion remained significantly higher (50%) than in isohydric controls at pH 7.7 (Fig. 8). There was also a positive relationship between SO\(_4^{2-}\) 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 SO\(_4^{2-}\) secretion (Fig. 8).
Table 3. Effect of metabolic acidosis on renal function in winter flounder

<table>
<thead>
<tr>
<th></th>
<th>GFR (ml/kg h⁻¹)</th>
<th>Urine Flow Rate (µmol kg⁻¹ h⁻¹)</th>
<th>Serum pH</th>
<th>Urine pH</th>
<th>Serum Osmolality (mosmol/kg H₂O)</th>
<th>Urine Osmolality (mosmol/kg H₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></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>
<td>318 ± 8.0</td>
</tr>
<tr>
<td><strong>Metabolic acidosis</strong></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>
<td>332 ± 8.9</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 animals. Values are paired and taken -3 h before (control) and -3 h after lowering of environmental pH to ~4.3 (metabolic acidosis). Q_reabsorption, reabsorption rate; Q_secretion, secretion rate; GFR, glomerular filtration rate. Significant differences determined by paired t test, *P < 0.05, †P < 0.001 compared with control.

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 that 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 alkalinization (pH 8.0) reduce (mean of 0.35 pH units, n = 4 cells) and increase (mean of 0.20 pH units, n = 2 cells) pHᵥ in pPTCs, respectively (Pelis RM and Renfro JL, unpublished observations). Intracellular [HCO₃⁻] would be expected to change in the same direction as pHᵥ (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 lumenal) 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 pHᵥ 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 CAI 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, CAI physically associates with NHE1 and the Na⁺+HCO₃⁻ cotransporter isoform 1 (37). In addition, acidic and basic pH microdomains form at sites where CAI 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. 7. Regression plots of serum [H⁺] vs. SO₄²⁻ secretion rate (A) and serum osmolality vs. SO₄²⁻ secretion rate (B; n = 5). Data were compiled from in vivo experiments (Table 3). Individual animals are represented by a different symbol. The dotted line connects individuals before (filled symbols) and after (open symbols) metabolic acidosis. The solid line is the linear regression of all data combined. Linear regression analyses indicated positive relationships between SO₄²⁻ secretion rate and serum [H⁺] (r² = 0.62, P < 0.01) and SO₄²⁻ secretion rate and serum osmolality (r² = 0.59, P < 0.01).
Fig. 8. Net $\text{SO}_4^{2-}$ 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.

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

ion) at isohydric and high interstitial pH may reflect the exchange of $\text{SO}_4^{2-}$ for an intracellular anion(s) other than $\text{OH}^-$, 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 $\text{pHi}$ (in bulk solution) is higher than interstitial pH.

Uptake of $\text{SO}_4^{2-}$ 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 $\text{SO}_4^{2-}$ secretion (32, 34). However, there is no direct effect of $\text{Na}^+$ on $\text{SO}_4^{2-}$ transport in renal brush-border or basolateral membrane vesicles (34). These observations, along with the role of pH in setting $\left[\text{HCO}_3^-\right]_i$ and $\left[\text{OH}^-\right]_i$, have led to the hypothesis that the effect of the Na gradient on $\text{SO}_4^{2-}$ transport is secondary to control of $\text{pH}_i$ through NHE activity (26, 31, 33, 34). Although 20 $\mu$M EIPA (applied to lumen and interstitium) had no effect on net $\text{SO}_4^{2-}$ secretion by fPTCs under isohydric conditions, 100 $\mu$M 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 $\mu$M EIPA and ouabain of $\text{SO}_4^{2-}$ secretion, both caused significant reductions in Na-dependent phlorizin-sensitive glucose transport ($I_{\text{glu}}$), indicating a diminished plasma membrane Na gradient. Although 100 $\mu$M EIPA was probably nonspecific, the most likely explanation for inhibition of net $\text{SO}_4^{2-}$ secretion is a reduction in $\text{pHi}$, $\text{Na}^+$$-\text{K}^+$-ATPase inhibition, through dissipation of the Na gradient (i.e., reduced NHE activity), reduces $\text{pHi}$ in numerous cell types (16, 18). Reduced $\text{pHi}$ could potentially drive the basolateral $\text{SO}_4^{2-}/\text{OH}^-$ exchanger backwards and may explain why the unidirectional reabsorptive flux was elevated following ouabain and 100 $\mu$M EIPA treatment.

The present observation that metabolic acidosis stimulated net $\text{SO}_4^{2-}$ secretion and that 20 $\mu$M 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 $\text{pHi}$ 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 $\text{H}^+$ thus enhancing $\text{H}^+$ excretion following acid loading (2).

Two questions regarding the effect of EIPA on $\text{SO}_4^{2-}$ secretion remain. Assuming that NHEs are important, why is $\text{SO}_4^{2-}$ secretion by fPTCs under isohydric conditions not affected by 20 $\mu$M 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 $\mu$M EIPA, suggesting that flounder NHE1 is insensitive to this concentration (24, 36). Second, why doesn’t 20 $\mu$M EIPA prevent all of the stimulation in secretion? In addition to NHEs, other acid-extruding processes (e.g., $\text{H}^+$$-\text{ATPase}$ and $\text{H}^+$$-\text{K}^+$-ATPase) may also be required to control $\text{pH}_i$ 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, connecting 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$^+$$-\text{K}^+$-ATPase.

The significance of metabolic acidosis in fish (22, 41, 42, 44), along with its dramatic effect on $\text{SO}_4^{2-}$ secretion by fPTCs, prompted our examination of the intact animal response. Renal $\text{SO}_4^{2-}$ 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 $\text{SO}_4^{2-}$ secretion by fPTCs ~50%. Factors other than the acute effect of serum [H$^+$] may also influence $\text{SO}_4^{2-}$ 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 $\text{SO}_4^{2-}/\text{HCO}_3^-$ exchange activity, CA activity, CAII protein abundance, and CA-dependent $\text{SO}_4^{2-}$ secretion (25, 30). Transcriptional regulation of $\text{SO}_4^{2-}$ transport-related proteins by cortisol, along with the direct effect of serum [H$^+$], may thus act additively to stimulate $\text{SO}_4^{2-}$ secretion.
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 $\text{SO}_4^{2-}/\text{OH}^-$ exchange, such that $\text{SO}_4^{2-}/\text{OH}^-$ 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 $\text{pH}_i$ regulation is necessary to maintain proper driving forces for transepithelial $\text{SO}_4^{2-}$ secretion.

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