Renal expression and localization of SLC9A3 sodium/hydrogen exchanger and its possible role in acid-base regulation in freshwater rainbow trout (Oncorhynchus mykiss)

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Ivanis G, Braun M, Perry SF. Renal expression and localization of SLC9A3 sodium/hydrogen exchanger and its possible role in acid-base regulation in freshwater rainbow trout (Oncorhynchus mykiss). Am J Physiol Regul Integr Comp Physiol 295: R971–R978, 2008. First published July 30, 2008; doi:10.1152/ajpregu.90328.2008.—Experiments were performed to assess the possible involvement of the Na+/H+ exchanger isoform 3 (NHE3; SLC9A3) in renal acid-base regulation in adult rainbow trout (Oncorhynchus mykiss). NHE3 mRNA was expressed at high levels in the kidney relative to its paralog, NHE2. The results of in situ hybridization demonstrated an abundance of NHE3 mRNA in renal tubules. The combination of immunocytochemistry and histological staining revealed that NHE3 was confined to the apical membrane of proximal tubules, where it was colocalized with the vacuolar-type H+-ATPase. Levels of NHE3 protein (assessed by Western blotting) were increased during hypercapnia, likely as a result of increased transcription, as indicated by increasing levels of NHE3 mRNA (as determined by real-time PCR). Plasma cortisol concentration was increased during hypercapnia, and administration of exogenous cortisol caused a marked increase in NHE3 mRNA and protein. Thus we speculate that the elevation of plasma cortisol during hypercapnia contributes to transcriptional activation of NHE3 that ultimately promotes acid-base regulation by stimulating H+ secretion and HCO3− reabsorption.

sodium/hydrogen exchanger; SLC9; proximal tubule; kidney; cortisol; acidosis; sodium/hydrogen exchange; bicarbonate reabsorption

THE METABOLIC REGULATION OF acid-base disturbances in fish, like in other vertebrates, relies on adjustments of plasma HCO3− levels at constant Pco2. For example, respiratory acidosis associated with external hypercapnia is compensated by gradual accumulation of HCO3− within the plasma (6). The rise in plasma HCO3− concentration reflects a marked increase in net acid excretion at the gill, which, in turn, is caused by differential modulation of the rates of branchial Cl− and Na+ net fluxes (13). Cl− uptake is linked to base efflux via Cl−/HCO3− exchange, and Na+ uptake is coupled to H+ excretion either by Na+/H+ exchange (25) or by diffusion of Na+ through channels, a process energized by vacuolar-type H+-ATPase (V-ATPase) mediated H+ secretion (2). Thus, during hypercapnic acidosis, the increase in branchial acid excretion typically reflects stimulation of Na+ uptake and inhibition of Cl− uptake (5). In rainbow trout, a reduction in the rate of branchial Cl−/HCO3− exchange is the principal adjustment underlying the increase in plasma HCO3− levels; increased Na+ uptake linked to H+ secretion is less important in this species during hypercapnia (37).

Although the gill is the major site of acid-base equivalent fluxes in fish, the kidney may also contribute significantly to net acid-base excretion during periods of pH compensation, although the extent of renal involvement varies with species, type (respiratory vs. metabolic) of acid-base disturbance, and external salinity (freshwater vs. seawater) (7, 8, 10, 12, 24, 28, 29, 36, 43, 45, 46). The prevailing view is that, during respiratory acidosis, renal acid output in freshwater teleosts, although increased (7, 37, 43, 46), is but a minor contributor to whole body acid excretion. Despite its minor role in net acid excretion, the kidney plays an essential role in acid-base regulation during respiratory acidosis by controlling the extent of HCO3− reabsorption (36, 43, 46, 47). Clearly, the accumulation of HCO3− within the plasma as a strategy to regulate hypercapnic acidosis can only succeed if the accumulating HCO3− filtered at the kidney is reabsorbed [reviewed by Perry and Gilmour (35)]. In mammals, the renal reabsorption of HCO3− involves acidification of the filtrate predominantly at the proximal tubule (40). Acidification of the filtrate is accomplished by H+ pumping via the V-ATPase (reviewed by Ref. 31) and electroneutral Na+/H+ exchange. In the mammalian proximal tubule, the specific Na+/H+ exchanger (NHE) thought to mediate the bulk of filtrate acidification is SLC9A3 or NHE3 (14, 23). Many of the components required for HCO3− reabsorption in the mammalian proximal tubule have been identified in rainbow trout. Trout Na-HCO3 cotransporter 1 (34, 38), V-ATPase (32, 33), cytoplasmic carbonic anhydrase [tCAc, the functional equivalent of carbonic anhydrase (CA) II] (11), as well as CA IV (tCA IV) (16), have been identified in rainbow trout kidney. Moreover, increased expression of Na-HCO3 cotransporter 1, V-ATPase, tCAc, and tCA IV occurs in trout exposed to hypercapnia, presumably to increase HCO3− reabsorption (16, 32–34).

One key component required for HCO3− reabsorption (at least in the mammalian model) yet to be identified in trout kidney is luminal (apical) NHE. Recently, two of the apical isoforms, NHE2 and NHE3, were cloned and demonstrated to be present in gill and kidney (20) (GenBank accession nos. EF446606 and EF446605). To complete the model of HCO3− reabsorption in trout kidney, the primary goal of the present study was to establish whether, by analogy to the mammalian proximal nephron, the NHE3 isoform is localized to the apical membrane of proximal tubule cells and whether its expression increases during hypercapnia concurrently with increased renal
HCO\textsubscript{3}\textsuperscript{-} reabsorption. Because glucocorticoids have been implicated in the regulation of mammalian NHEs (3, 4) and renal acidification (reviewed by Ref. 18), a final objective was to determine whether renal NHE3 is transcriptionally regulated by cortisol.

**MATERIAL AND METHODS**

**Experimental Animals**

Adult rainbow trout (*Oncorhynchus mykiss*) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). Fish were maintained on a 12:12-h light-dark photoperiod in circular fiberglass water tanks supplied with flowing, aerated, and dechlorinated City of Ottawa tap water at 13°C. Animals were fed daily with a commercial trout diet and were acclimated for at least 2 wk before any experiments were performed. Food was withheld for 48 h before experimentation. All experiments were approved and performed according to the University of Ottawa institutional guidelines, which comply with those of the Canadian Council on Animal Care.

**RNA and Protein Extractions**

Fish were killed by a blow to the head, and dissected tissues were ground on dry ice with a mortar and pestle and stored at −80°C until needed. Total RNA was extracted from 100-mg aliquots of frozen tissue samples using TRIzol reagent (Invitrogen). The RNA pellet was resuspended in 40 μl of nuclease-free H\textsubscript{2}O and treated with RNase-free DNase (8 units per RNA sample) for 20 min at room temperature to remove any remaining genomic DNA. The RNA concentration and quality were assessed by gel electrophoresis and spectrophotometry (Eppendorf BioPhotometer). Kidney proteins were extracted using 1× RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) with protease inhibitors (complete Mini protease inhibitor cocktail tablets; Roche). The tissues were first ground under liquid N\textsubscript{2} with a precooled mortar and pestle and then incubated on ice for 15 min. Samples were sonicated (2 × 1-s pulses at medium power; micro-ultrasonic cell disruptor by Kontes) and centrifuged at 12,000 × g for 10 min at 4°C, and the supernatant containing soluble proteins was collected. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories) containing soluble proteins was collected. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories) and then incubated on ice for 15 min. Samples were sonicated (2 × 1-s pulses at medium power; micro-ultrasonic cell disruptor by Kontes) and centrifuged at 12,000 × g for 10 min at 4°C, and the supernatant containing soluble proteins was collected. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories) using BSA as a standard. Ultimately, all of the protein samples were diluted to a concentration of ~20 μg/ml and frozen and stored at −80°C until needed.

**Quantification of mRNA Levels Using Real-PCR**

cDNA was synthesized from 1 μg of RNA using random hexamer primers (Boehringer Mannheim) and ReverTra Aid H Minus M-MuLV Reverse Transcriptase (Fermentas). Rainbow trout mRNA levels were measured by real-time PCR on samples of cDNA (1 μl) using a Brilliant SYBR Green QPCR Master Mix Kit (Stratagene) and a Stratagene MX-4000 multiplex quantitative PCR system. ROX (Stratagene) was used as reference dye. The PCR conditions (final reaction volume = 12.5 μl) were as follows: cDNA template = 0.5 μl; forward and reverse primer = 300 nmol/l; 2X Master Mix = 12.5 μl; and ROX = 1:30,000 final dilution. The annealing and extension temperatures over 40 cycles were 56°C (30 s) and 72°C (30 s), respectively. The primers used for real-time PCR (including the reference gene β-actin) were designed using online software (Primer 3; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 1). The specificity of the primers was verified by cloning and sequencing of amplified products. To ensure that residual genomic DNA was not being amplified, control experiments were performed in which reverse transcriptase was omitted during cDNA synthesis. Relative expression of mRNA levels was determined (using β-actin as an endogenous standard) by a modification of the delta-delta Ct (threshold cycle) method (39). Amplification efficiencies were determined from standard curves generated by serial dilution of plasmid DNA.

**Collection of Tissues for Immunocytochemistry and In Situ Hybridization**

Rainbow trout were killed by a blow to the head, and the kidney tissue was dissected, placed in 4% paraformaldehyde, and kept overnight at 4°C. Samples were then placed in 15% sucrose for 2 h at room temperature followed by 30% sucrose at 4°C until sectioning. The tissue was embedded in OCT cryosectioning medium (VWR) and incubated for 20 min. Horizontal sections (10 μm) were obtained using a cryostat (Leica CM 1850) and placed onto glass microscope slides (VWR superfrost plus). Slides were dried at room temperature for ~45 min and then stored at −4°C until required.

**NHE and Na\textsuperscript{+}-K\textsuperscript{+}-ATPase Immunocytochemistry**

Sections were incubated for 2 h at room temperature with the following primary antibodies.

1) First is α\textsubscript{5} (1:100), a mouse monoclonal antibody (University of Iowa Hybridoma Bank) raised against the α\textsubscript{5}-subunit of chicken Na\textsuperscript{+}-K\textsuperscript{+}-ATPase that has been used extensively to localize Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in fish tissues.

2) Second is a custom affinity-purified rabbit polyclonal antibody (Abgent, San Diego, CA) raised against trout NHE3 that was generated using eight-chain multi-antigenic peptides. The synthetic peptide ETKADVDFNKKFRAS corresponded to amino acids 578-593 of the rainbow trout NHE3a protein sequence (GenBank accession ABO32815.1) that is also likely to recognize the rainbow trout NHE3b protein sequence (ENKADVDFNKKFGAD) (20).

3) To identify V-ATPase, a rabbit polyclonal antibody raised against the A subunit of the killifish (*Fundulus heteroclitus*) was generously provided by Dr. Toyoji Kaneko (22).

Sections were incubated for 20 h at 4°C with the NHE3 antibody (1:1,000). For negative controls, sections were incubated with 1× PBS buffer lacking primary antibodies. Immunofluorescence was detected after incubating the sections with a 1:400 dilution of Alexa Fluor 546 coupled to goat anti-mouse IgG and goat anti-rabbit IgG detected after incubating the sections with a 1:400 dilution of Alexa Fluor 546 coupled to goat anti-mouse IgG and goat anti-rabbit IgG. Following the 3× washes in 0.1× PBS buffer lacking primary antibodies. Immunofluorescence was detected after incubating the sections with a 1:400 dilution of Alexa Fluor 546 coupled to goat anti-mouse IgG and goat anti-rabbit IgG (1:100), a mouse monoclonal antibody (University of Iowa Hybridoma Bank) raised against the α\textsubscript{5}-subunit of chicken Na\textsuperscript{+}-K\textsuperscript{+}-ATPase that has been used extensively to localize Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in fish tissues.

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Table 1. Oligonucleotide primers used for real-time PCR and probe construction for in situ hybridization

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Uses</th>
<th>Product Size</th>
<th>Primer Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHE3-F</td>
<td>5'-GCTCCCTGTGGTTGATTTAC-3'</td>
<td>ISH probe</td>
<td>833–852</td>
<td></td>
</tr>
<tr>
<td>NHE3-R</td>
<td>5'-AACAGCGAACACACCCCTC-3'</td>
<td>ISH probe</td>
<td>772</td>
<td></td>
</tr>
<tr>
<td>QNHE3-F</td>
<td>5'-AGAGCGCCGGTCGAGCGAC-3'</td>
<td>NHE3 RT-PCR</td>
<td>1450–1469</td>
<td></td>
</tr>
<tr>
<td>QNHE3-R</td>
<td>5'-AACAGCGAACACCCCTC-3'</td>
<td>NHE3 RT-PCR</td>
<td>155–1604</td>
<td></td>
</tr>
<tr>
<td>Actin-F</td>
<td>5'-GCAAGCAGAAGCTGATCC-3'</td>
<td>RT-PCR control</td>
<td>1107–1127</td>
<td></td>
</tr>
<tr>
<td>Actin-R</td>
<td>5'-CAGTGGCGAAGCTGATCGTA-3'</td>
<td>RT-PCR control</td>
<td>1224–1244</td>
<td></td>
</tr>
</tbody>
</table>

NHE3, Na\textsuperscript{+}/H\textsuperscript{+} exchanging isoform 3; ISH, in situ hybridization. The expected PCR product sizes and the positions of the PCR primers were deduced on the basis of the GenBank accessions EF446605 (NHE2), EF446606 (NHE3), and AF157514 (rainbow trout β-actin).
containing 4,6-diamidino-2-phenylindole to stain nuclei and cover slipped. For negative controls, sections were incubated with 1× PBS buffer lacking primary antibodies and processed as above.

**NHE3 In Situ Hybridization Probes**

Primers were designed to produce a 811-bp digoxigenin (DIG)-labeled ribonucleotide probe for detection of rainbow trout NHE3 by in situ hybridization (Table 1). Gill total RNA (5 μg) was reverse transcribed using oligo (dT) primer (Sigma Genosys) and Stratascript reverse transcriptase (Stratagene). PCR was performed on the resulting cDNA (0.5 μl in a 25-μl reaction). The final concentration of the PCR mix was as follows: 1× PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTP, 2 pmol each of forward and reverse primers, 2.5 units of Taq polymerase (Invitrogen), and 100 ng of trout cDNA. The following cycling parameters were used: 4 min at 94°C followed by 40 cycles of 30 s at 92°C, 30 s at 58°C, and 1 min at 72°C. The final extension of the amplified products was at 72°C for 10 min. A PCR cloning kit was used to clone the PCR products into a pCR II vector (Invitrogen) and used to clone the PCR products into a pCI vector (Invitrogen) for in vitro transcription with SP6 RNA polymerase (Invitrogen), and 100 ng of trout cDNA. The following cycling parameters were used: 4 min at 94°C followed by 40 cycles of 30 s at 92°C, 30 s at 58°C, and 1 min at 72°C. The final extension of the amplified products was at 72°C for 10 min. A PCR cloning kit was used to clone the PCR products into a pCR II vector (Invitrogen) and was transformed into chemically competent DH5α E. coli cells. The desired clone was extracted using a PureLink Quick Plasmid Miniprep Kit (Invitrogen). Purified plasmids were sequenced using M13 forward and reverse primers to confirm identity and determine the orientation of the cloned sequence within the vector. An antisense DIG-labeled RNA probe was synthesized by linearizing 1 μg of plasmid with BanH I (Invitrogen) and followed by in vitro transcription with T7 RNA polymerase (New England BioLabs) containing 4,6-diamidino-2-phenylindole to stain nuclei and cover slipped. For negative controls, sections were incubated with 1× PBS buffer lacking primary antibodies and processed as above.

**Microscopy and Image Acquisition**

Bright-field and fluorescence images were acquired using a Zeiss Axioskop epifluorescence microscope equipped with an Olympus DP70 digital camera. Images were processed using image-Pro Plus version 6.0.0 (Media Cybernetics).

**Western Blots and Antibody Specificity**

Proteins (50–100 μg per lane) were separated by SDS-PAGE on 10% Tris-tricine polyacrylamide gels and then transferred onto 0.45-μm nitrocellulose membranes (Bio-Rad Laboratories) using a wet transfer unit. The membranes were blocked in 5% PBS-Tween-20 for 1 h at room temperature. After blocking the membranes were probed with an NHE3 antibody (1:200) for 2 h at 37°C. The specificity of the NHE3 antibody was established previously (20). All membranes were incubated in goat anti-rabbit IgG and horseradish peroxidase (1:5,000, Amersham Life Sciences) for 1 h at room temperature. After washing (3 times, 5 min in Tris-buffered saline-Tween), the proteins were visualized using Western Lightning Chemiluminescence Reagent Plus Kit (PerkinElmer). The protein size marker was obtained from Fermentas Life Sciences. To compensate for deviation in the amount of protein loaded, the same membrane was stripped using Re-Blot Plus mild stripping solution (CHEMICON). The membrane was then probed with a β-actin antibody (hypercapnia experiment; 1:500, Sigma) or α-tubulin antibody (cortisol experiment; 1:1,000, Santa Cruz Biotechnology) for 1 h at 37°C, incubated in anti-mouse IgG, horseradish peroxidase (1:5,000; hypercapnia), or anti-rabbit IgG, horseradish peroxidase (1:10,000, cortisol) for 1 h at room temperature, and washed three times for 5 min in Tris-buffered saline-Tween. The proteins were visualized as described above. The size and the opacity of NHE3 bands, relative to the size and the intensity of β-actin or α-tubulin bands, were calculated using Image J analysis software (http://rsb.info.nih.gov/ij/). The switch from actin to tubulin as a reference protein was to reduce costs.

**Exposure of Fish to Hypercapnia**

Adult fish were placed into black plastic boxes supplied with flowing and aerated water and were allowed to recover for 24 h. After acclimation, fish were exposed to external hypercapnia for 24 h with intended final water PCO2 of 7.5 Torr. To achieve hypercapnia, a water equilibrium column was gassed with mixtures of CO2 and air (Sierra C100L Smart-trak mass flow controllers; SRB Controls). Water PCO2 was monitored by using a CO2 electrode connected to a blood gas meter (Cameron Instruments). Differences from the intended water PCO2 were corrected by adjusting the gas and the water flows through the equilibration column. For investigating changes in protein levels, fish were killed, and kidney tissues were collected after 24 h of exposure to hypercapnia (N = 6) or normocapnia (controls; N = 6); blood samples were withdrawn by caudal puncture to obtain plasma for cortisol determinations. To assess the changes in NHE3 mRNA levels using real-time PCR, tissues were collected after 3, 12, and 24 h (N = 6 at each time point) of exposure to hypercapnia. Control fish were also killed at 3, 12, and 24 h (N = 6 at each time point) of exposure to normocapnia.

**Cortisol Implants and Measurements**

Fish (N = 6) were lightly anesthetized with benzocaine (0.5 g/l for ~30 s or until they did not respond to touch), weighed, and given an intraperitoneal implant of cortisol (0.11 mg/g body wt; hydrocortisone
21-hemisuccinate sodium salt; Sigma-Aldrich) dissolved in coco but-
ter (22 mg of cortisol per 1 ml of coco butter) or coco butter alone
(shams). After 1, 2, or 3 days, fish were anesthetized with benzocaine,
and kidney tissues were dissected, frozen, and then processed for
real-time PCR. After 3 days, kidney tissue was processed for Western
blotting, and blood was collected into heparinized syringes from the
caudal vessels for subsequent determination of plasma cortisol levels.
Cortisol levels were determined on 25-μl samples using a commercial
radioimmunoassay (ICN).

Statistical Analyses

The effect of exposure to hypercapnia or cortisol implants on kidney
NHE3 mRNA expression, as determined by real-time PCR, was analyzed
using one-sample Student’s t-tests. Differences in band intensities on
Western blots were assessed using unpaired Student’s t-tests.

RESULTS

Relative Abundance of NHE2 and NHE3 mRNA
in the Kidney

The abundance of NHE2 and NHE3 mRNA levels in the
kidney tissues was examined using real-time PCR (N = 6).
Statistical analysis of the data demonstrated that, on a relative
basis, NHE3 mRNA was ∼15,000× more abundant in kidney
than NHE2 (P = 0.002; one-tailed Student’s t-test). The reference
gene used was β-actin, and data were compared relative to NHE2
mRNA, which was given a relative value of 1. Thus all ensuring
studies focused exclusively on the NHE3 isoform.

Localization of NHE3 and V-ATPase to the proximal nephron.
In situ hybridization clearly demonstrated that some renal
tubules contained NHE3 mRNA (Fig. 1). Tissues processed as
negative controls, either using a DIG-labeled sense probe (Fig.
1C) or by omission of probe (Fig. 1D), exhibited background
staining only. Using a homologous NHE3 polyclonal anti-
body, NHE3 protein was also localized to the apical mem-
brane of a subpopulation of renal tubules (Fig. 2A) express-
ing basolateral Na+/K+-ATPase. Some tubules that were
particularly enriched with Na+/K+-ATPase exhibited no
NHE3 immunoreactivity (Fig. 2A). Subsequent treatment
with periodic acid Schiff (to identify brush borders) of the
same tissue sections processed for immunofluorescence
identified the proximal tubule as the site of apical NHE3
(Fig. 2B). Immunofluorescence was eliminated by omission
of primary antibodies (Fig. 2A, inset), indicating that non-
specific binding of the secondary antibody to kidney tissue sec-
tions was negligible. Similar staining pattern was observed using
a rabbit antibody raised against killifish (Fundulus heteroclitus) A
subunit of V-ATPase (22), in which V-ATPase protein was
localized to apical membrane of proximal tubules (Fig. 3, A and
B). Immunofluorescence was absent after omission of primary
antibodies (Fig. 3A, inset).

Effects of hypercapnia and cortisol on kidney NHE3 levels.
Kidney NHE3 mRNA levels were significantly increased after
3, 12, and 24 h of exposure to hypercapnia (Fig. 4). Consistent
with the increase in mRNA expression, kidney NHE3 protein
levels increased significantly after hypercapnia exposure (Fig. 5). In
a parallel experiment (20), plasma cortisol concentrations were
significantly elevated after 24 h of hypercapnia from 35.3 ±
9.4 to 100.1 ± 30.9 ng/ml, suggesting that cortisol could be
regulating renal NHE3 at the transcriptional level. In support of
this idea, elevating plasma cortisol levels using intraperitoneal
cortisol implants was associated with increased NHE3 mRNA
expression after 48 and 72 h (Fig. 6A), as well as increased
protein abundance at 72 h (Fig. 6B). At 72 h, plasma cortisol
levels were 27.7 ± 7.6 ng/ml in shams (N = 6) and 167.9 ±
34.6 ng/ml in cortisol-treated fish (N = 6).

Fig. 1. Identification of Na+/H+ exchanger iso-
form 3 (NHE3) mRNA-enriched renal tubules in
rainbow trout (Oncorhynchus mykiss) kidney by in
situ hybridization. As indicated by the black arrow-
heads in A and B, digoxigenin (DIG)-labeled anti-
sense NHE3 probe (purple staining) was detected
in some renal tubules. Using labeled sense strand
NHE3 probe (C) or excluding probe altogether
(D) eliminated specific staining; the black staining
indicates pigmented cells. Bar, 40 μm.
Although the fish gill is the major site of acidic equivalent exchanges between the internal and external environments, the kidney also plays an essential role in systemic acid-base balance by regulating the extent of HCO₃⁻/H⁺ reabsorption (16, 36, 43, 46, 47). For example, during metabolic compensation of respiratory acidosis, plasma HCO₃⁻/H⁺ levels can become markedly elevated (e.g., 50 mmol/l in European eel Anguilla anguilla) (30), owing to increased net branchial acid excretion (5). To sustain elevated levels of plasma HCO₃⁻, the additional HCO₃⁻ being filtered at the kidney must be reabsorbed. The reabsorption of HCO₃⁻ requires equimolar secretion of H⁺ into the filtrate, and thus, while the net output of acid by the kidney may be minor compared with the gill, H⁺ secretion is increased markedly. For example, to sustain plasma levels of HCO₃⁻ at 20 mmol/l (a typical value for rainbow trout experiencing metabolic compensation of hypercapnic acidosis) (17) would require additional renal H⁺ secretion of ~150 μmol·kg⁻¹·h⁻¹ [this estimate assumes a glomerular filtration rate of 7 ml·kg⁻¹·h⁻¹ or 1.77 × urine flow rate (36, 43)]. This estimate of renal acid secretion is similar to measured rates of branchial net acid excretion during compensation of hypercapnia (17). Moreover, unlike the transient increase in branchial acid excretion that is required to elevate plasma HCO₃⁻ concentration, the increased rates of renal acid secretion must be maintained even after metabolic compensation is complete.

The mechanisms underlying renal acid secretion and HCO₃⁻ reabsorption in fish have not been fully elucidated. Indeed, current schemes for tubular acid secretion in fish (34, 35, 38) are largely extrapolated from mammalian models. In these models, intracellular H⁺ is generated via the hydration of CO₂ by tCAc (11) within the proximal convoluted tubule, the predominant site of HCO₃⁻ reabsorption. Secretion of H⁺ across the apical membrane is achieved via V-ATPase (42) and NHE3 (1, 48). It was recently demonstrated that, in trout kidney, the secretion of H⁺ and associated HCO₃⁻ reabsorption is facilitated by externally oriented apical (luminal) membrane-bound CA IV (16). As in mammalian models (41, 44), the facilitation of HCO₃⁻ reabsorption by CA IV stems from its Fig. 3. Localization of vacuolar-type H⁺-ATPase (V-ATPase) protein to apical regions of proximal renal tubules in rainbow trout (Oncorhynchus mykiss) kidney using immunocytochemistry. A: apical V-ATPase (stained green) was localized to cells containing basolateral Na⁺-K⁺-ATPase (stained red); nuclei are stained blue (4,6-diamidino-2-phenylindole). B: periodic acid Schiff (PAS) staining of the same section demonstrated that V-ATPase was specifically localized to the brush border of proximal tubule cells. The V-ATPase and Na⁺-K⁺-ATPase immunofluorescence were absent with omission of primary antibodies (inset in A) when image was acquired under the same exposure as in A. Bar, 50 μm.
role in catalyzing the dehydration of filtered $\text{HCO}_3^-/\text{H}_2\text{CO}_3$ within the lumen of the proximal tubule. The $\text{CO}_2$ thus formed diffuses into the tubule cells where it is hydrated to $\text{HCO}_3^-/\text{H}_2\text{CO}_3$ and $\text{H}^+$, a reaction catalyzed by tCAc; the $\text{HCO}_3^-/\text{H}_2\text{CO}_3$ is moved across the basolateral membrane via a $\text{Na}^+\text{HCO}_3^-/\text{H}_2\text{CO}_3$ cotransporter (34).

Overall, $\text{HCO}_3^-$ reabsorption is favored because $\text{H}^+$ is recycled via NHE3 and V-ATPase. $\text{H}^+$ not used for $\text{HCO}_3^-$ reabsorption are excreted in the urine as titratable acidity or combine with $\text{NH}_3$ to form $\text{NH}_4^+$; together, titratable acidity and $\text{NH}_4^+$ contribute to net renal acid excretion.

An important contribution of the present study was the first demonstration of NHE3 in proximal tubule of any fish species and its colocalization with V-ATPase, thereby substantiating two putative components of the models for piscine renal acid secretion and $\text{HCO}_3^-$ reabsorption (34). Although Perry and Fryer (33) previously demonstrated the presence of apical membrane V-ATPase expression in trout proximal tubules, they did not comment on the coexpression of NHE3. Furthermore, Hirata et al. (19), while reporting an increase in renal NHE3 mRNA in Osorezan dace ($\text{Tribolodon hakodensis}$) exposed to water of low pH, did not attempt to identify the sites of expression within the kidney. Similar to the Osorezan dace experiencing metabolic acidosis when exposed to acidic water...
(19), the results of the present study demonstrated that renal NHE3 (mRNA and protein) expression is also increased by respiratory acidosis. The increased levels of NHE3 protein during hypercapnia presumably reflect the need to secrete greater quantities of H\(^+\) into the filtrate to reabsorb the greater quantities of HCO\(_3^-\) being filtered at the glomerulus. Evidence for increased activity of renal Na\(^+\)/H\(^+\) exchange during hypercapnia was provided by Georgalis et al. (16) by demonstrating that inhibition of renal CA (a key component of the Na-HCO\(_3^-\) reabsorption process) using acetazolamide caused a much larger increase in urinary Na\(^+\) levels during hypercapnia compared with normocapnia. Although not assessed in this study, previous research has shown V-ATPase, tCAc, and tCA IV mRNA in the trout kidney also increase during hypercapnic acidosis (16, 34). Thus the net consequence of systemic respiratory acidosis in rainbow trout is the induction of the cellular machinery required to enhance renal HCO\(_3^-\) reabsorption.

We propose that the elevated levels of cortisol (from 35.3 ± 9.4 to 100.1 ± 30.9 ng/ml) during hypercapnia are responsible, at least in part, for the transcriptional activation of renal NHE3. Only a few studies have examined the impact of hypercapnia on plasma cortisol levels in fish, and these have yielded variable results. Although elevated levels of cortisol have been reported in hypercapnic white sturgeon (Acipenser transmontanus) (9) and Atlantic salmon smolts (Salmo salar) (15), the levels in the European eel (Anguilla anguilla) (30) remained unaltered during exposure to high ambient PCO\(_2\). It is likely that species differences account for the inconsistent data. While indirect, the evidence for the involvement of cortisol in the increased expression of NHE3 is compelling. First, the elevation of plasma cortisol levels in hypercapnic trout occurred concurrently with increasing NHE3 mRNA and protein expression. Second, the administration of exogenous cortisol resulted in an increase in NHE3 mRNA and protein levels. This conclusion is also supported by previous studies on mammals demonstrating that glucocorticoids transcriptionally activate renal NHE3 expression (18, 21) and promote renal tubular acidification (4). It is important to point out that an elevation of plasma cortisol levels is not a prerequisite for increased renal acid excretion. Indeed, Wood et al. (47) demonstrated significant increases in renal acid excretion during respiratory acidosis associated with hyperoxia, with no accompanying change in plasma cortisol levels. Further studies should be directed at teasing apart the relative participation of glucocorticoids, other hormones including endothelin (26) and angiotensin II (27), and the direct effects of acidosis (18).

**Perspectives**

The kidney must play a central role in metabolic compensation of respiratory acid-base disorders. With the current and previous studies, it is becoming increasingly evident that the modes of renal acid-base compensation are conserved from fish to mammals. Indeed, it would appear that the basic machinery required for HCO\(_3^-\) reabsorption at the proximal tubule evolved in the actinopterygians nearly 300 million years ago. The need to reabsorb HCO\(_3^-\) arose with the evolution of a glomerular (filtering) kidney. Certain fish species possess agglomerular kidneys, and in these species one might predict a reduced role for the kidney in acid-base regulation. In fact, because fish with agglomerular kidneys are not faced with the problem of reabsorbing filtered HCO\(_3^-\) during metabolic compensation of respiratory acidosis, one would expect more efficient acid-base compensation in these species. Clearly, there is a need to assess the capacities of fish with glomerular and agglomerular kidneys to regulate respiratory acidosis.

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