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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Submitted 31 March 2008; accepted in final form 16 July 2008

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 HCO$_3^-$ reabsorption (36, 43, 46, 47). Clearly, the accumulation of HCO$_3^-$ within the plasma as a strategy to regulate hypercapnic acidosis can only succeed if the accumulating HCO$_3^-$ filtered at the kidney is reabsorbed (reviewed by Perry and Gilmour (35)). In mammals, the renal reabsorption of HCO$_3^-$ involves acidification of the filtrate predominantly at the proximal tubule (40). Acidification of the filtrate is accomplished by H$^+$/Na$^+$ exchange (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 HCO$_3^-$ reabsorption in the mammalian proximal tubule have been identified in rainbow trout. Trout Na-HCO$_3^-$ 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-HCO$_3^-$ cotransporter 1, V-ATPase, tCAc, and tCA IV occurs in trout exposed to hypercapnia, presumably to increase HCO$_3^-$ reabsorption (16, 32–34).

One key component required for HCO$_3^-$ 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 HCO$_3^-$ 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...
HCO₃⁻ 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 dechloraminated 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 H₂O 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 N₂ with a precooled mortar and pestle and stored at −80°C until needed.

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

cDNA was synthesized from 1 μg of RNA using random hexamer primers (Boehringer Mannheim) and RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas). Rainbow trout mRNAs 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; 2× 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 (WVR) and incubated for 20 min. Horizontal sections (10 μm) were obtained using a cryostat (Leica CM 1850) and placed onto glass microscope slides (WVR superfrost plus). Slides were dried at room temperature for ~45 min and then stored at −4°C until required.

**NHE and Na⁺/K⁺-ATPase Immunocytochemistry**

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

1) First is α₁ (1:100), a mouse monoclonal antibody (University of Iowa Hybridoma Bank) raised against the α₁-subunit of chicken Na⁺/K⁺-ATPase that has been used extensively to localize Na⁺/K⁺-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 amino acids 578-593 of the NHE3b protein sequence (ENKADVFKNNFKGAD) (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 conjugated to Alexa Fluor 488 (Fisher, Ottawa, Ontario, Canada). Following the 3 × 10 min washes in 0.1× PBS, sections were mounted with Vectashield mounting medium (Vector Laboratories)

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'-GCTCCCTGTTGTTATATTAC-3'</td>
<td>ISH probe</td>
<td>833–852</td>
<td></td>
</tr>
<tr>
<td>NHE3-R</td>
<td>5'-AACACGACACAACCCCTTC-3'</td>
<td>ISH probe</td>
<td>772</td>
<td></td>
</tr>
<tr>
<td>QNHE3-F</td>
<td>5'-AGACGAGCAGCTGACAGAAT-3'</td>
<td>NHE3 RT-PCR</td>
<td>1450–1469</td>
<td></td>
</tr>
<tr>
<td>QNHE3-R</td>
<td>5'-AACACGACACAACCCCTTC-3'</td>
<td>NHE3 RT-PCR</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>Actin-F</td>
<td>5'-GCTTGGCTTACAGTGAATGCT-3'</td>
<td>RT-PCR control</td>
<td>1107–1127</td>
<td></td>
</tr>
<tr>
<td>Actin-R</td>
<td>5'-GCTTGGCTTACAGTGAATGCT-3'</td>
<td>RT-PCR control</td>
<td>138</td>
<td></td>
</tr>
</tbody>
</table>

NHE3, Na⁺/H⁺ exchanger 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 constitution 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 was transformed into chemically competent DH5 α 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 kb plasmid with BanHI (Invitrogen) followed by in vitro transcription with T7 RNA polymerase (New England Biolaboratories) for 1 h at 37°C. Sense DIG-labeled (Roche) RNA probes were created by linearizing 1 μg of plasmid with XhoI (Invitrogen), followed by in vitro transcription with SP6 RNA polymerase (Invitrogen) for 1 h at 37°C.

NHE3 In Situ Hybridization

The slides were incubated for 10 min at 60°C. The DIG-labeled RNA probe (100 ng) was denatured (boiled for 3 min and then rapidly chilled on ice) and added to the hybridization buffer [50% deionized formamide, 5% hybridization salts (0.75 M NaCl, 20 mM EDTA, 20 mM PIPES, pH 6.8), 1× Denhardt’s, 0.2% SDS, 5% dextran sulphate (Sigma)]. Hybridization was performed for 20 h at 63°C in a humid chamber in a hybridization oven. The next day, the sections were washed twice in 2× SSC (15 min each at 60°C), twice in 0.2× SSC (15 min each at 60°C), and once in 0.1× SSC for 10 min at room temperature. For DIG detection, the sections were incubated first with 0.1 M PBS containing 1% goat serum, 2 mg/ml BSA, and 0.3% Triton X at room temperature for 1 h. This was followed by incubation in anti-DIG conjugated with alkaline phosphatase (Roche Molecular Biochemicals) diluted 1:1,000 in 0.1% Tween 20, 0.1% Tween 20. One nitroblue tetrazolium and 5-bromocresyl-3-indolyl phosphate tablet (Sigma) was dissolved in 10 ml water, and this solution was layered over the sections. The chromogenic reaction was allowed to proceed in the dark until satisfactory coloration was achieved (at room temperature in a humid chamber). The slides were then washed twice with 0.1 M PBS (15 min each). The sections were covered with mounting media (60% glycerol) and cover slipped.

Periodic Acid Schiff Staining of Proximal Tubules Following Immunocytochemistry

After the appropriate immunocytochemistry was completed on kidney sections and the images were captured, a periodic acid Schiff staining protocol was performed, according to the directions of the manufacturer (Sigma-Aldrich). Briefly, the sections were hydrated (2 × 10 min) in 1× PBS, and then periodic acid solution was applied for 5 min. Sections were then rinsed with water (2 × 10 min), and then Schiff’s reagent was applied for 15 min. After the sections were washed for 5 min in running tap water, hematoxylin solution was applied as counterstain for 90 s. The images were acquired from the same area from which they were taken previously for immunocytochemistry.

Microscopy and Image Acquisition

Bright-field and fluorescence images were acquired using a Zeiss Axiosphot epifluorescence microscope equipped with an Olympus DP70 digital camera. Images were processed using image-Pro Plus version 6.0 (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 used for confirmation of the band. 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 butter (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 antibody, NHE3 protein was also localized to the apical membrane of a subpopulation of renal tubules (Fig. 2A) expressing 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 nonspecific binding of the secondary antibody to kidney tissue sections was negligible. Similar staining pattern was observed using a rabbit antibody raised against killifish (Fundulus heteroclitus) 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).
DISCUSSION

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 HCO$_3^-$/H$_2$CO$_2$ reabsorption (16, 36, 43, 46, 47). For example, during metabolic compensation of respiratory acidosis, plasma HCO$_3^-$/H$_2$CO$_2$ 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 HCO$_3^-$, the additional HCO$_3^-$ being filtered at the kidney must be reabsorbed. The reabsorption of HCO$_3^-$ 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 HCO$_3^-$ at 20 mmol/l (a typical value for rainbow trout experiencing metabolic compensation of hypercapnic acidosis) (17) would require additional renal H$^+$ secretion of $\sim$150 $\mu$mol$\cdot$kg$^{-1}$$\cdot$h$^{-1}$ [this estimate assumes a glomerular filtration rate of 7 ml$\cdot$kg$^{-1}$$\cdot$h$^{-1}$ or 1.77 $\times$ 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 HCO$_3^-$ concentration, the increased rates of renal acid secretion must be maintained even after metabolic compensation is complete.

The mechanisms underlying renal acid secretion and HCO$_3^-$ 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 CO$_2$ by tCaC (11) within the proximal convoluted tubule, the predominant site of HCO$_3^-$ 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 HCO$_3^-$ reabsorption is facilitated by externally oriented apical (luminal) membrane-bound CA IV (16). As in mammalian models (41, 44), the facilitation of HCO$_3^-$ reabsorption by CA IV stems from its...
role in catalyzing the dehydration of filtered $\text{HCO}_3^-$ within the lumen of the proximal tubule. The $\text{CO}_2$ thus formed diffuses into the tubule cells where it is hydrated to $\text{HCO}_3^-$ and $\text{H}^+$, a reaction catalyzed by tCAc; the $\text{HCO}_3^-$ is moved across the basolateral membrane via a $\text{Na}^+\text{-HCO}_3^-$ cotransporter (34).

Fig. 4. Effects of 3, 12, and 24 h of exposure to hypercapnia (water $\text{PCO}_2 = 7.5$ Torr) on rainbow trout ($\text{Oncorhynchus mykiss}$) kidney NHE3 mRNA levels, as determined by real-time PCR. Kidney NHE3 mRNA levels were significantly increased at all periods of hypercapnia exposure (*$P < 0.05$; one-tailed Student’s $t$-test).

Fig. 5. Effects of 24 h of hypercapnia exposure (water $\text{PCO}_2 = 7.5$ Torr) on trout kidney NHE3 protein levels, as determined by Western blotting. A: Western blots using trout NHE3 antibody indicated an increase in kidney NHE3 protein levels (relative to $\beta$-actin) after exposure to 24-h hypercapnia. B: quantitative analysis of blots from normocapnic and hypercapnic tissues demonstrated a significant increase (*$P < 0.05$; Student’s $t$-test) of relative NHE3 protein levels ($\text{NHE3/} \beta$-actin) after 24-h hypercapnia exposure.

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 $\text{H}^+$ into the filtrate to reabsorb the greater quantities of $\text{HCO}_3^-$ 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 aglomerular kidneys, and in these species one might predict a reduced role for the kidney in acid-base regulation. In fact, because fish with aglomerular 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 aglomerular kidneys to regulate respiratory acidosis.

**ACKNOWLEDGMENTS**

We appreciate the helpful comments of Dr. Katie Gilmour and the technical assistance of Branka Vulesevic. The V-ATPase antibody was kindly provided by Dr. Toyoji Kaneko.

**GRANTS**

This study was supported by Natural Sciences and Engineering Research Council of Canada Discovery and Research Tools and Instruments grants to S. F. Perry.

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