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Branchial expression and localization of SLC9A2 and SLC9A3 sodium/hydrogen exchangers and their possible role in acid–base regulation in freshwater rainbow trout (*Oncorhynchus mykiss*)

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SUMMARY

Experiments were conducted on adult rainbow trout (*Oncorhynchus mykiss*) to test the hypothesis that SLC9 Na^+/H^+ exchangers (SLC9A2, NHE2; and SLC9A3, NHE3) on the gill epithelium are localized specifically to a subset of mitochondria-rich cells (MRCs) that are unable to bind peanut lectin agglutinin (PNA). This cell type, termed the PNA^- MRC, is a sub-type of MRC believed to function in Na^+ uptake and acid excretion. A technique using biotinylated PNA was used to distinguish between the PNA^- and PNA^+ MRCs on fixed gill sections. In contrast to expectations, both NHE2 (mRNA) and NHE3 (protein) were confined to cells enriched with Na^+/K^+-ATPase and capable of binding PNA. Thus, in trout, NHE2 and NHE3 are localized to PNA^+ MRCs, the cells previously believed to be responsible for Cl^- uptake and base excretion. Levels of mRNA for NHE2, the predominant isoform in the gill, were increased during 72 h of hypercapnic acidosis; NHE3 mRNA and protein levels were unaffected. Because plasma cortisol levels were increased during hypercapnia (from 35.3±9.4 to 100.1±30.9 ng ml^{-1}), the effects of experimentally elevated cortisol levels on NHE expression were investigated. The elevation of plasma cortisol using intraperitoneal implants caused a significant increase in NHE2 mRNA expression without affecting NHE3 mRNA or protein abundance. Thus, we suggest that NHE2 contributes to acid–base regulation during hypercapnia owing to its transcriptional regulation by cortisol. The finding of NHE expression in PNA^+ MRCs is discussed with reference to current models of ionic and acid–base regulation in teleost fish.

Key words: Mitochondria-rich cell, chloride cell, peanut lectin agglutinin, hypercapnia, cortisol, gill, NHE, NHE2, NHE3, sodium/proton exchangers, fish.

INTRODUCTION

Acid–base balance in teleost fish relies, in part, on the dynamic adjustment of acidic and basic equivalent fluxes across the gill (Goss et al., 1998; Claiborne, 1998; Claiborne et al., 2002; Perry et al., 2003b; Evans et al., 2005; Perry and Gilmour, 2006; Marshall and Grosell, 2006). Acid–base balance and ionic regulation are intricately related because of obligatory coupling between Na^+ uptake and acid excretion and Cl^- uptake and base excretion (reviewed by Evans et al., 2005; Perry and Gilmour, 2006; Marshall and Grosell, 2006). However, the specific mechanisms linking NaCl uptake to net branchial acid flux are not yet fully understood. Although Cl^- uptake and bicarbonate (HCO_3^-) efflux are tightly coupled, the specific gene product enabling Cl^-/HCO_3^- exchange and its cellular location have not been identified with certainty.

It is generally accepted that the linkage between branchial Na^+ uptake and acid excretion reflects electroneutral Na^+/H^+ exchange (Maetz, 1973) and/or active H^+ extrusion *via* a vacuolar H^+-ATPase (V-ATPase) pump (Perry et al., 2000) electrically coupled to apical membrane Na^+ channels (Avella and Bornancin, 1989). While there is empirical evidence in support of the V-ATPase–Na^+ channel model (Fenwick et al., 1999; Wilson et al., 2000; Reid et al., 2003; Parks et al., 2007), the apparent absence of epithelial Na^+ channel (ENaC) genes in fish genomes suggests the participation of another conductive cation pathway that has yet to be identified. Based on thermodynamic constraints (Avella and Bornancin, 1989), it was thought that electroneutral Na^+/H^+ exchange, while likely in seawater (SW) fish, would be unlikely to contribute to Na^+ uptake in freshwater (FW) species (Perry, 1997; Marshall, 2002). However, the results of more recent studies have clearly demonstrated that Na^+/H^+ exchangers (NHEs) are expressed in the gills of both SW- and FW-acclimated fish, thus indirectly suggesting their involvement in Na^+ uptake in both media (Claiborne et al., 1999; Claiborne et al., 2008; Edwards et al., 2001; Edwards et al., 2002; Edwards et al., 2005; Choe et al., 2002; Choe et al., 2005; Choe et al., 2007; Hirata et al., 2003; Scott et al., 2005; Tresguerres et al., 2005; Tresguerres et al., 2006b; Catches et al., 2006). Specifically, two paralogs within the SLC9 (NHE) gene family (reviewed by Orlowski and Grinstein, 2004) have been implicated as likely candidates for apical Na^+/H^+ exchange: SLC9A2 (NHE2) and SLC9A3 (NHE3). The localization of NHEs to gill epithelial cells enriched with Na^+/K^+-ATPase (Edwards et al., 2002; Hirata et al., 2003; Choe et al., 2005; Choe et al., 2007; Catches et al., 2006; Tresguerres et al., 2006b) suggests that the mitochondria-rich cell (MRC) is likely to be the principal site of Na^+/H^+ exchange.

By analogy with the A-type intercalated cells of the mammalian collecting duct and in consideration of the previous observations by Goss and co-workers of cell-specific differences in Na^+ uptake and pH_i regulation (Goss et al., 2001; Galvez et al., 2002; Reid et al., 2003; Parks et al., 2007), it was recently proposed that in rainbow trout, the subset of MRCs unable to bind peanut lectin agglutinin (PNA^- MRCs) are the site of Na^+ uptake either by Na^+/H^+ exchange or by conductance through Na^+ channels (Perry et al., 2003a; Perry and Gilmour, 2006). Thus, the present study was undertaken to test the hypothesis that NHEs are specifically expressed in the PNA^- MRC of the rainbow trout gill epithelium and that their expression is transcriptionally increased (potentially by elevated cortisol levels)

during respiratory acidosis, consistent with their potential involvement in Na^+ uptake and acid–base regulation. These ideas were tested by cloning rainbow trout *NHE2* and *NHE3* to provide tools to examine RNA and protein distribution and expression levels using *in situ* hybridization, immunocytochemistry, real-time RT-PCR and Western blotting.

MATERIALS AND METHODS

Experimental animals

Adult rainbow trout (*Oncorhynchus mykiss* Linnaeus) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). Fish (150–300 g) were maintained on a 12 h light:12 h dark photoperiod in circular fibreglass water tanks with flowing, aerated and dechloraminated City of Ottawa tapwater at 13°C. Animals were fed daily with a commercial trout diet and were acclimated for at least 2 weeks before any experiments were performed. Fish were not fed within 48 h of experimentation.

RNA and protein extraction

The fish were killed by a blow to the head and tissues were dissected, flash frozen in liquid N_2 and stored at –80°C until processing. Tissues were processed by grinding on dry ice using a mortar and pestle and then stored at –80°C until needed. Total RNA was extracted from 100 mg aliquots of processed frozen tissue samples using Trizol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. The RNA pellet was resuspended in 40 µl of nuclease-free H_2O and any remaining genomic DNA was removed using RNase-free DNase (8 units per RNA sample; Invitrogen) for 20 min at room temperature. The RNA quality and concentration were assessed by gel electrophoresis and spectrophotometry (Eppendorf Biophotometer, Mississauga, ON, Canada), respectively.

Gill proteins were extracted using 1× RIPA buffer (50 mmol l^{-1} Tris-HCl, pH 8.0, 150 mmol l^{-1} NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate) with protease inhibitors (complete mini protease inhibitor cocktail tablets; Roche, Temecula, CA, USA). The tissues were first ground under liquid N_2 with a pre-cooled mortar and pestle and then incubated on ice for 15 min. Samples were then sonicated two times for 1 s at 50% full power and centrifuged at 12 000 ***g*** for 10 min at 4°C. The supernatant containing soluble protein was transferred to a microcentrifuge tube. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Mississauga, ON, Canada) with bovine serum albumin (BSA) as a standard, and all of the protein samples could be diluted to a concentration of 20 µg $µl^{-1}$. The proteins were frozen and stored at –80°C until needed.

Molecular cloning of trout *NHE2* and *NHE3*

Degenerate primers for *NHE2* and *NHE3* (Table 1) were designed based on known fish and tetrapod sequences. PCR products for *NHE2* and *NHE3* were amplified, cloned and sequenced using standard protocols in our laboratory (Perry et al., 2003a). A search of GenBank protein databases using BLASTX revealed that the cloned *NHE2* cDNA (704 bp) exhibited highest amino acid identity with known fish *NHE2* sequences. A BLASTX search also revealed that the cloned *NHE3* cDNA (1167 bp) sequence shared highest amino acid identity with known fish *NHE3* sequences. Based on these sequences, primers were designed (Table 1) to be used for 3′ and 5′ RACE (rapid amplification of cDNA ends).

Sequence and phylogenetic analysis

Known NHE1, NHE2 and NHE3 amino acid (aa) sequences were used together with the complete 830 aa sequence for NHE2 and the complete 752 aa sequence for NHE3 (NHE3a). Phylogenetic analysis was performed using the neighbour-joining method (TREE-PUZZLE v.5.2, http://www.dkfz.de/tbi_old/tree-puzzle/) with 1000 pseudo-replicates. An additional *NHE3* gene (termed *NHE3b*) was identified by mining the Genomics Research on Atlantic Salmon Project (GRASP) rainbow trout database. Consensus sequence data from three clusters (2843046, 2850627 and 2869330) were assembled into an 862 aa sequence using contig assembly software (DNAMAN; Lynnon Biosoft v.5.2.9, Quebec, Canada). The NHE3b sequence was only used in the phylogenetic analysis; all the other experiments incorporated the NHE3a sequence, referred to as NHE3.

Northern blot analysis

Total RNA (10 µg) was extracted using Trizol (see above) and fractionated by glyoxal/dimethyl sulphoxide (DMSO) denaturing electrophoresis on a 1% agarose gel and transferred to a Duralon nylon membrane (Stratagene, Mississauga, ON, Canada) using 20× standard saline citrate (SSC). Membranes were cross-linked (Fisher UV crosslinker; Ottawa, ON, Canada) twice prior to hybridization.

A probe for *NHE3* was generated from first strand cDNA from rainbow trout mRNA. The 772 bp probe was amplified and subcloned using NHE3-F and NHE3-R primers (Table 1). The probe was then enzymatically cut from extracted plasmids with *Bam*HI and *Xho*I (Invitrogen) for 2 h at 37°C. The probe was labelled using [α^{32}P]dCTP (specific activity 10^9 c.p.m. $µg^{-1}$ DNA) and the Ready-To-Go labelling system (Pharmacia, Piscataway, NJ, USA). Membranes were pre-hybridized at 60°C for 3 h in Church's buffer. Blots were then hybridized overnight in the same solution at 60°C, with approximately 10^9 c.p.m. of denatured probe. The blots were then washed twice using 1× SSC/0.1% SDS solution (20 min, 60°C) and once using 0.25× SSC/0.1% SDS (20 min, 60°C). Finally, blots were exposed to a phosphor screen (Kodak, Rochester, NY, USA) and visualized and quantified using a phosphorimager (Molecular Devices, Sunnyvale, CA, USA) controlled by ImageQuant software.

Quantification of mRNA levels using real-time RT-PCR

cDNA was synthesized from 1 µg of total RNA using random hexamer primers (Boehringer Mannheim, Mannheim, Germany) and RevertAid H minus M-MuLV reverse transcriptase (Fermentas Life Sciences, Burlington, ON, Canada). 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 a reference dye. The PCR conditions (final reaction volume 12.5 µl) were as follows: cDNA template, 1 µl; forward and reverse primer, 300 nmol l^{-1}; 2× master mix, 12.5 µl; 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. All the primers used for real-time PCR (including the reference gene β-actin) were designed using web-based software (primer3; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; Table 1). The specificity of the primers was verified by subcloning and sequencing of the 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 ΔΔ Ct method (Pfaffl, 2001). Amplification efficiencies were determined from standard curves generated by serial dilution of plasmid DNA.

Table 1. Oligonucleotide primers used for cDNA cloning, RACE, real-time PCR and probe construction for *in situ* hybridization and Northern blotting

Primer name	Primer sequence	Uses	Product size	Primer position
DNHE2-F	5′-AAYGAYGSIGTIACIGTIGT-3′	Cloning of partial *NHE2* cDNA	–	730–749
DNHE2-R	5′-GGICKIATIGTIATICCYTG-3′	Cloning of partial *NHE2* cDNA	704	1414–1433
3NHE2F1	5′-TCAGGGAGATAGAACCCCTCT-3′	Outer 3′ RACE		911–931
3NHE2F2	5′-TATGGCCATTGTGACCTGTG –3′	Inner 3′ RACE		993–1012
5NHE2R1	5′-GACCACACCCAGGAAGAAGA-3′	Outer 5′ RACE		1118–1137
5NHE2R2	5′-AGACCAGGGCAAAGGAGATT-3′	Inner 5′ RACE		1314–1333
QNHE2-F	5′-TGTGCCCTGACCATGAAGTA-3′	NHE2 RT-PCR	–	1009–1028
QNHE2-R	5′-CCCAGTTCCACTCGTGTTCT-3′	NHE2 RT-PCR	160	1149–1168
NHE2-F	5′-CGGGCAGAGTCTAGCATTGT-3′	*In situ* hybridization	–	1630–1649
NHE2-R	5′-TGTCGTCACGGTCACCAG-3′	*In situ* hybridization	811	2423–2440
DNHE3-F	5′-CTTCATGTTYCTKGGHATCTCKGC-3′	Cloning of partial *NHE3* cDNA	–	511–534
DNHE3-R	5′-CATGGTCCKSTGGAARATYTC-3′	Cloning of partial *NHE3* cDNA	1167	1657–1677
3NHE3F1	5′-ATCCTCCTCACACTCCTCTTCATCTT-3′	Outer 3′ RACE		1162–1187
3NHE3F2	5′-CAGGTGGTGATGAGCTACGGT-3′	Inner 3′ RACE		1267–1287
5NHE3R1	5′-TTATTCCCTGCAGCATGACA-3′	Outer 5′ RACE		1395–1414
5NHE3R2	5′-GCCGAGGAAGACAAAGATGA-3′	Inner 5′ RACE		1097–1116
NHE3-F	5′-GCTCCCTGGTTGGTATTATC-3′	Northern blot probe	–	833–852
NHE3-R	5′-AACCAGCACAACCACCTCTC-3′	Northern blot probe	772	1585–1604
QNHE3-F	5′-AGAGCAGCCGTGACAGAACT-3′	NHE3 RT-PCR	–	1450–1469
QNHE3-R	5′-AACCAGCACAACCACCTCTC-3′	NHE3 RT-PCR	155	1585–1604
Actin-F	5′-CCAACAGATGTGGATCAGCAA-3′	RT-PCR control	–	1056–1076
Actin-R	5′-GGTGGCAGAGCTGAAGTGGTA-3′	RT-PCR control	138	1173–1193

The expected PCR product sizes and the positions of the PCR primers were deduced on the basis of the GenBank accession numbers EF446605 (*NHE2*) and EF446606 (*NHE3*).

Collection of tissues for immunocytochemistry and *in situ* hybridization

Adult rainbow trout were killed by a blow to the head and the gills were dissected and rinsed with 1× PBS to remove mucus. The gills were placed in 4% paraformaldehyde 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 gills were embedded in OCT cryosectioning medium (VWR, Mississauga, ON, Canada), incubated for 20 min and sectioned horizontally (10 μm section) using a cryostat (Leica CM 1850). Tissue sections were placed on Superfrost Plus slides (VWR) and dried at room temperature for approximately 45 min prior to storage at –4°C until needed.

PNA labelling and Na^+/K^+-ATPase immunocytochemistry

Gill tissue sections, prepared as described above, were incubated for 2 h at room temperature with primary antibodies: α5 (1:100), a mouse monoclonal antibody against the α1 subunit of chicken Na^+/K^+-ATPase (University of Iowa Hybridoma Bank) and 25 mg ml^{-1} PNA conjugated to biotin. The α5 antibody and biotinylated PNA have been used successfully for immunocytochemistry in rainbow trout (Wilson et al., 2000; Galvez et al., 2002). For negative controls, sections were incubated with 1× PBS buffer lacking primary antibodies or biotinylated PNA. Immunofluorescence was detected after incubating the sections with a 1:400 dilution of Alexa Fluor-546 coupled to goat anti-mouse IgG and PNA was detected after incubating sections with streptavidin conjugated to Alexa Fluor-488 (Fisher). After washing (3×10 min in 0.1× PBS), sections were mounted in Vectashield mounting medium (Vector Labs, Burlingame, CA, USA) and cover slipped.

PNA labelling and NHE3 immunocytochemistry

Custom-made affinity-purified rabbit polyclonal antibodies (Abgent, San Diego, CA, USA) raised against trout NHE2 and trout NHE3a were generated using synthetic multi-antigenic (eight chains) peptides. For trout NHE2, the synthetic peptide VPLHEEKKSSGKPKR corresponded to amino acids 579–593 of the rainbow trout NHE2 full-length amino acid sequence (GenBank accession no. ABO32814). For trout NHE3a, the synthetic peptide ETKADVDFNKKFRAS corresponded to amino acids 579–593 of the rainbow trout NHE3a protein sequence (GenBank accession no. ABO32815). The corresponding region in NHE3b (ENKADVDFNKKFGAD) was 80% identical (12/15 aa). Thus, it is likely that the antibodies recognize both NHE3a and NHE3b. Sections were incubated for 20 h at 4°C with NHE3 antibody (1:1000) and 25 mg ml^{-1} biotinylated PNA. For negative controls, sections were incubated with 1× PBS buffer lacking primary antibodies and PNA. Immunofluorescence was detected after incubating the sections with a 1:400 dilution of Alexa Fluor 546 coupled to goat anti-mouse IgG and streptavidin conjugated to Alexa Fluor 488 (Fisher). Following the 3×10 min wash in 0.1× PBS, sections were mounted in Vectashield mounting medium (Vector Labs) and cover slipped.

Construction of NHE2 *in situ* hybridization probe

Primers were designed to produce an 811 bp digoxigenin (DIG)-labelled riboprobe for rainbow trout NHE2 (Table 1). Gill total RNA (5 μg) was reverse transcribed using oligo-dT primer (Sigma Genosys, Oakville, ON, Canada) and Stratascript reverse transcriptase (Stratagene, Mississauga, ON, Canada). PCR was performed on the resulting cDNA (0.5 μl in a 25 μl reaction) using appropriate primers. An aliquot of the PCR product was run on a 1.25% agarose gel and the rest was ligated into PCR II vector (Invitrogen). 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. Antisense DIG-labelled RNA probes for NHE2 were synthesized

by linearizing 1 μg of plasmid with *Bam*HI (Invitrogen) followed by *in vitro* transcription with T7 RNA polymerase (New England Biolabs, Ipswich, MA, USA) for 1 h at 37°C. Sense DIG-labelled RNA probes were created by linearizing 1 μg of plasmid with *Xho*I (Invitrogen) followed by *in vitro* transcription with SP6 RNA polymerase (Invitrogen) for 1 h at 37°C.

PNA labelling and NHE2 *in situ* hybridization

After desired PNA fluorescence images were taken, the same tissue sections were hydrated in 1× PBST (PBS with 0.1% Tween 20) twice for 15 min each and treated with proteinase K (Gibco BRL, Orand Island, NY, USA) using 20 μg ml^{-1} in PBST for 15 min at room temperature. The slides were rinsed in 1× PBST, twice for 10 min each and then incubated for 10 min at 60°C. DIG-labelled RNA probe (100 ng) was denatured (boiled for 3 min and then cooled on ice) and added to the hybridization buffer [50% deionized formamide, 5× hybridization salts (0.75 mol l^{-1} NaCl, 20 mmol l^{-1} EDTA, 20 mmol l^{-1} Pipes, pH 6.8), 1× Denhardt's solution, 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 50% PBS for 10 min at room temperature. For DIG detection, the sections were incubated first with 0.1 mol l^{-1} PBS containing 1% goat serum, 2 mg ml^{-1} BSA and 0.3% Triton X at room temperature for 1 h. This was followed by incubation in anti-DIG conjugated to alkaline phosphatase (Roche Molecular Biochemicals) diluted 1:1000 in 0.1 mol l^{-1} PBS containing 1% goat serum, 2 mg ml^{-1} BSA and 0.3% Triton X overnight at 4°C. The next day, the slides were washed twice in 0.1 mol l^{-1} PBS (15 min each at room temperature). The slides were then washed twice (5 min each) in coloration buffer (100 mmol l^{-1} Tris pH 9.5, 50 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} NaCl, 0.1% Tween-20). One nitroblue tetrazolium and 5-bromocresyl-3-indolyl phosphate (NBT/BCIP) 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 (at room temperature in a humid chamber) until satisfactory coloration was achieved. The slides were then washed twice with 0.1 mol l^{-1} PBS (15 min each). The sections were covered with mounting media (60% glycerol) and cover slipped.

Microscopy and image acquisition

Both bright field and fluorescence images from tissue sections were visualized using a Zeiss Axiophot epifluorescence microscope. All images were captured using an Olympus DP70 digital microscope camera and image-Pro Plus v.6.0.0 (Media Cybernetics Inc., Bethesda, MD, USA).

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% PBST–milk for 1 h at room temperature. After blocking, the membranes were probed with an NHE2 or NHE3 antibody (1:200) for 2 h at 37°C. To demonstrate specificity of the NHE2 and NHE3 antibodies, a second blot was incubated simultaneously with the appropriate primary antibody that had previously been incubated over night at 4°C in the presence of 100× excess synthetic peptide. In addition, another blot was incubated with pre-immune serum only (1:100). All membranes were incubated in goat anti-rabbit IgG, horseradish peroxidase (1:5000, Amersham Life Sciences, Little Chalfont, Bucks, UK) for 1 h at room temperature. After washing (3×5 min in TBST), the proteins were visualized using Western Lightning Chemiluminescence reagent plus kit (PerkinElmer, Woodbridge, ON, Canada). The protein size marker used was obtained from Fermentas Life Sciences.

To estimate changes in protein level between control and hypercapnia tissue samples (see below), experimental and control protein samples were separated by SDS-PAGE and transferred onto the same 0.45 mm nitrocellulose membranes. The blots were blocked in 5% PBST–milk for 1 h at room temperature and the membrane was probed with NHE3 antibody (1:200) for 2 h at 37°C. To compensate for variation in the amount of protein loaded, the same membrane was stripped using Re-Blot Plus mild stripping solution (Chemicon, Temecula, CA, USA). The membrane was then probed with a β-actin antibody (1:500, Sigma) for 1 h at 37°C, incubated in anti-mouse IgG, horseradish peroxidase (1:5000) for 1 h at room temperature and washed 3 times for 5 min in TBST. The proteins were visualized as described above. The size and the opacity of the NHE3 bands, relative to the size and opacity of the β-actin bands, were calculated using ImageJ analysis software (http://rsb.info.nih.gov/ij/).

Exposure of fish to hypercapnia

Adult fish were placed into black plastic boxes supplied with flowing and aerated water and were allowed to acclimate for 24 h. Fish were exposed to external hypercapnia for 24 h with an intended final water P_{CO_2} of 7.5 mm Hg. To achieve hypercapnia, a water equilibrium column was gassed with mixtures of CO_2 and air (Sierra C100L Smart-trak mass flow controllers; SRB Controls, Markham, ON, Canada). Water P_{CO_2} was monitored by using a CO_2 electrode connected to a blood gas meter (Cameron Instruments, Port Aransas, TX, USA). Differences from the intended water P_{CO_2} were corrected by adjusting the gas and water flow through the equilibration column. For investigating changes in protein level, fish were killed and tissues were collected after 24 h of exposure to hypercapnia (N=6) or normocapnia (controls; N=6). To assess the changes in NHE2 and 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

Fish (N=6) were lightly anaesthetized by immersion in a solution of benzocaine (0.5 g l^{-1} for ~30 s or until they did not respond to touch), weighed and given an intra-peritoneal implant (Perry and Reid, 1994) of cortisol (0.11 mg g^{-1} body weight; hydrocortisone 21-hemisuccinate sodium salt; Sigma-Aldrich, Inc.) dissolved in cocoa butter (22 mg of cortisol per 1 ml of cocoa butter). This protocol has been shown to reliably increase plasma cortisol levels to approximately 150 ng ml^{-1} in rainbow trout (Dibattista et al., 2005). After 24, 48 and 72 h, six fish were killed and gill tissues were dissected and frozen for extraction of RNA for real-time RT-PCR. Protein samples extracted from the gill samples were degraded. Thus, the same protocol was repeated on a separate group of fish (N=6) treated with cortisol for 72 h to obtain gill tissue for analysis of NHE3 protein by Western blotting.

Statistical analyses

The effect of exposure to hypercapnia on gill NHE2 and NHE3 mRNA expression as determined by real-time PCR was analysed using Student's one-sample t-tests. The effects of hypercapnia on NHE3 protein were analysed by Student's unpaired t-tests and one-way ANOVA (P<0.05).

RESULTS

Molecular cloning of trout *NHE2* and *NHE3*

Using homology cloning techniques, two cDNAs were sequenced, translated and assembled into open reading frames encoding a deduced NHE2 protein of 830 amino acids (aa) and a deduced NHE3a protein of 752 aa; a deduced NHE3b protein of 862 aa was mined from the GRASP database (Fig. 1A). A BLAST search of the GenBank protein databases indicated that the rainbow trout putative NHE orthologues (NHE2, NHE3a and NHE3b) shared sequence identity with other vertebrate NHE amino acid sequences. For example, NHE3a (GenBank accession no. EF446606) shared approximately 66–68% aa identity with other fish NHEs and

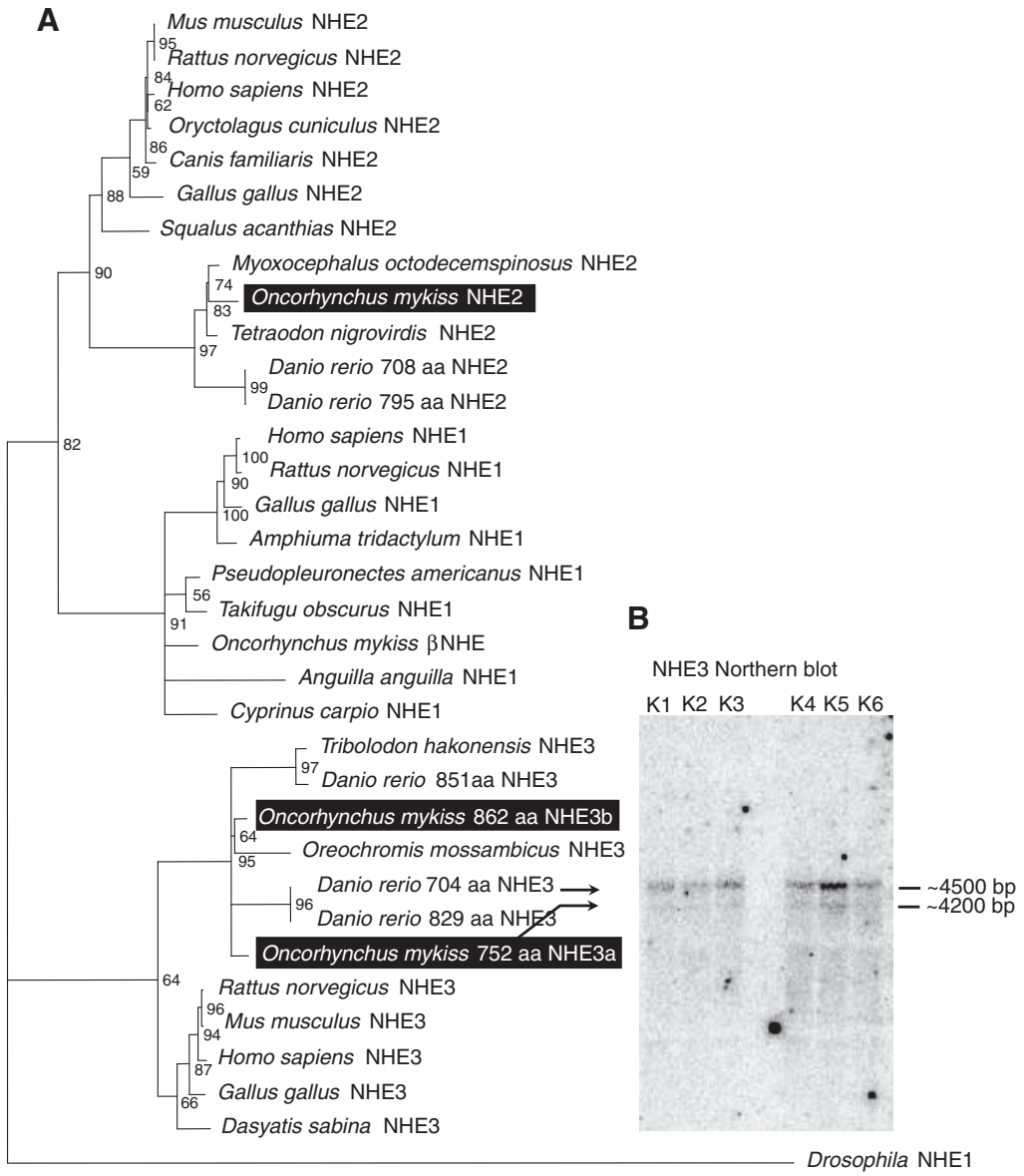

Fig. 1. (A) Phylogenetic relationship of selected NHE1–3 proteins from different species and orthologous rainbow trout NHE2, NHE3a and NHE3b amino acid sequences. The grouping of each rainbow trout orthologue with its corresponding form is an indication that the rainbow trout clone is most closely related to that NHE isoform. (B) The existence of the two rainbow trout NHE3 transcripts is indicated in a representative Northern blot. K1–6, kidney samples 1–6. The tree in A was constructed using the neighbour-joining method, and numbers indicate bootstrap values for 1000 replicates. GenBank accession numbers are as follows.

NHE1: *Oncorhynchus mykiss* (βNHE) Q01345, *Pseudopleuronectes americanus* AAO32340, *Amphiuma tridactylum* AAD33928, *Takifugu obscurus* BAE75800.1, *Rattus norvegicus* NP_036784.1, *Anguilla anguilla* CAB45085, *Cyprinus carpio* CAB45232, *Homo sapiens* NP_003038, *Drosophila melanogaster* AAF51559, *Gallus gallus* ABB82239.

NHE2: *Canis familiaris* XP_531775.2, *Gallus gallus* XP_416918.2, *Mus musculus* NP_001028461 XP_129721, *Oryctolagus cuniculus* P50482, 708 amino acid (aa) *Danio rerio* XP_001336127.1, 795 aa *Danio rerio* XP_691364, *Homo sapiens* NP_003039, *Myoxocephalus octodecemspinosus* AAD46576, *Rattus norvegicus* P48763, partial 692 aa *Oncorhynchus mykiss* ABO32814, *Squalus acanthias* ABC54565, *Tetraodon nigroviridis* CAG05798.

NHE3: 704 aa *Danio rerio* CAM47009.1, 829 aa *Danio rerio* XP_696670, 851 aa *Danio rerio* XP_696728, *Gallus gallus* XP_418895.2, *Mus musculus* NP_001074529.1, *Dasyatis sabina* AAT45738.2, *Homo sapiens* NP_004165.1, *Rattus norvegicus* NP_036786.1, NHE3a; 752 aa *Oncorhynchus mykiss* ABO32815.1 and NHE3b; 862 aa *Oncorhynchus mykiss* assembled sequence, *Oreochromis mossambicus* BAF80347, *Tribolodon hakonensis* AB055466.

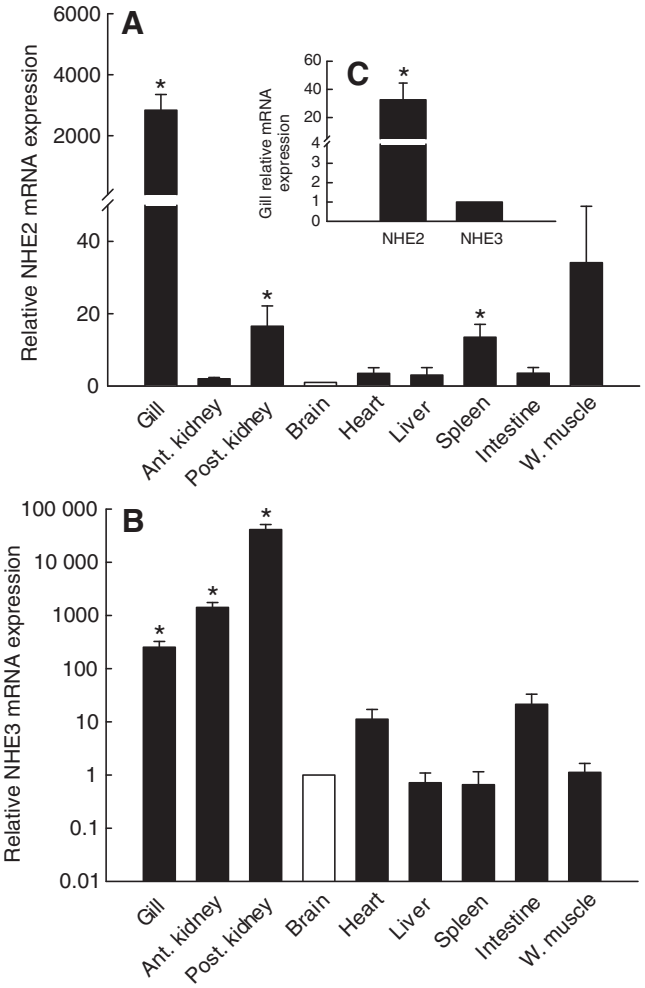

Fig. 2. Tissue distribution analysis of rainbow trout NHE2 (A) and NHE3 (B) mRNA as determined by real-time PCR and expressed relative to a reference tissue (brain) set to a relative value of 1. The inset (C) represents the relative mRNA levels of NHE2 and NHE3 in the gill. Values shown are means + 1 s.e.m.; asterisks indicate significant differences (P<0.05) from the reference tissue or gill NHE3 (inset). Ant., anterior; Post., posterior; W. muscle, white muscle.

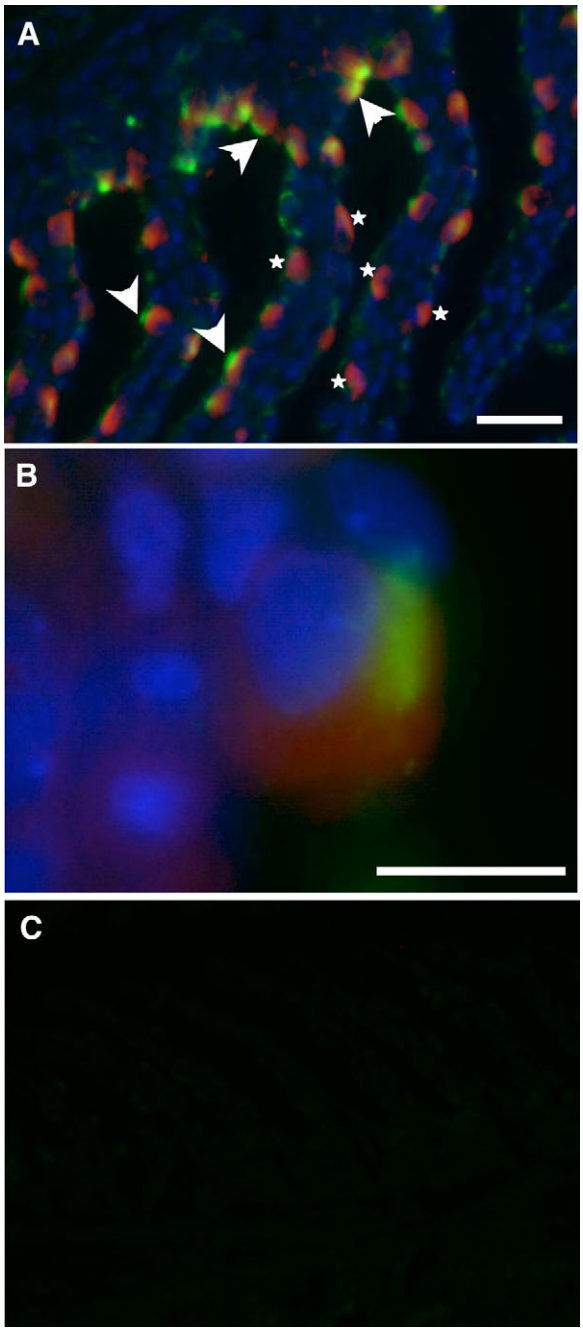

Fig. 3. (A) Identification of PNA$^+$ and PNA$^-$ negative mitochondria-rich cells (MRCs) in gill sections from adult rainbow trout. PNA$^+$ MRCs are labelled with arrows; stars indicate PNA$^-$ MRCs. MRCs were identified on the basis of immunoreactivity against Na^+/K^+-ATPase (red); green indicates apical PNA and blue indicates nuclei (DAPI). (B) Higher magnification image of a PNA (green)-positive MRC. Negative controls included (C) omission of primary antibody against Na^+/K^+-ATPase and of biotinylated PNA. The image in C was captured using the same exposure and microscope settings as in A. Scale bar in A, 20 μm; and in B, 5 μm.

approximately 60–62% identity with mammalian NHEs. NHE2 (GenBank accession no. EF446605) shared 62–76% aa identity with other fish NHE2s and roughly 50% identity with mammalian NHE2s. Fig. 1A illustrates the phylogenetic analysis of the complete trout NHE2 and NHE3a and NHE3b sequences with other vertebrate NHEs. Included in the phylogenetic analysis is trout βNHE, which is most closely related to NHE1. The tree clearly shows the grouping of rainbow trout NHE2, NHE3a and NHE3b with corresponding putative orthologues in the different species analysed. Confirming the presence of two NHE3 transcripts (NHE3a and NHE3b), the Northern blot (Fig. 1B) on kidney RNA shows two bands, a putative NHE3a band at ~4200 bp and a putative NHE3b band at ~4500 bp. Even though the estimated band sizes were larger than predicted based on the NHE3a and NHE3b coding sequences (probably due to the untranslated regions), the estimated difference between the two bands was ~300 bp and agrees with the difference between NHE3a and NHE3b sequences of 330 bp. Because NHE3a and NHE3b shared only 83% nucleotide identity over common regions, these isoforms are likely to be products of different genes rather than splice variants.

Tissue distribution of NHE2 and NHE3 mRNA

The relative levels of NHE2 and NHE3 mRNA across different tissues were examined using real-time PCR (Fig. 2), revealing that

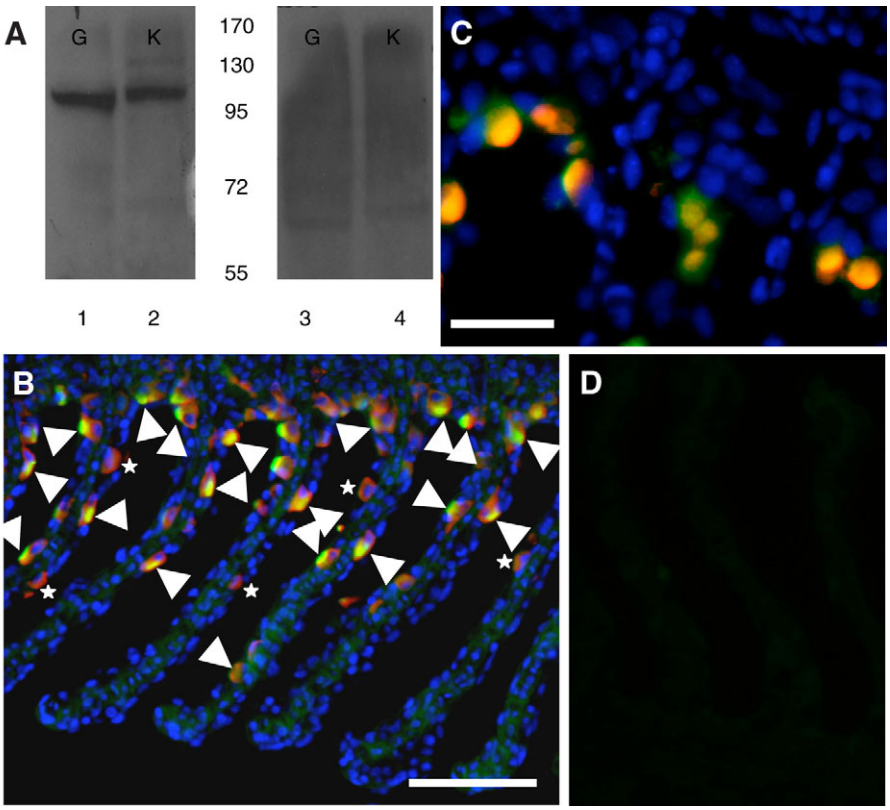

Fig. 4. Identification of NHE3-expressing cells as PNA^+ MRCs in gill sections from adult rainbow trout. (A) NHE3 protein was detected in gill (G) and kidney (K) protein extracts using Western blots (N=6) to reveal a 98 kDa immunoreactive band (lanes 1 and 2) that was absent after the NHE3 antibody was incubated with excess peptide against which the antibody was raised (lanes 3 and 4). Size markers are given in kDa. (B) Co-localization of NHE3 (green) and Na^+/K^+-ATPase (red) in MRCs (nuclei are stained blue); NHE3-expressing MRCs are labelled with arrows and NHE3 negative MRCs are indicated by stars. (C) Localization of NHE3 to PNA^+ MRCs; red indicates NHE3, green indicates PNA and nuclei are stained blue. (D) The NHE3 immunofluorescence disappeared with omission of primary NHE3 antibody. Scale bar in A, 40 μm; and in C, 10 μm.

the distribution of NHE mRNA was isoform specific. The highest amount of NHE2 mRNA (Fig. 2A) was detected in the gills followed by lower levels in posterior kidney and spleen. On the other hand, the level of NHE3 mRNA (Fig. 2B) was highest in the posterior kidney, followed by anterior kidney and the gill. On a relative scale, NHE2 was the predominant isoform in the gill (Fig. 2C) and NHE3 was the predominant isoform in the kidney (G.I. and S.F.P., unpublished data). Although there are limitations associated with comparing the levels of expression of two genes using real-time PCR, the differences measured in these studies are almost certainly too large (>40-fold) to be explained by methodological constraints. It is unclear whether these large differences in mRNA expression correspond to similar differences in protein expression.

Localization of NHE2 and NHE3 to PNA^+ MRCs

Identification of PNA^+ and PNA^- MRCs on gill tissue sections (Fig. 3) was used to localize NHE2 and NHE3 to specific MRC subtypes. Fig. 3A clearly shows that a subset of the MRCs (red) co-localized with apical PNA (arrows) and others did not (stars). It is evident that the majority of the PNA^+ MRCs are located near the base of the lamellae whereas most of the PNA^- MRCs are found on the lamellae but rarely near the base (Fig. 3A). Fig. 3B is a higher magnification of one PNA^+ MRC, supporting the basolateral localization of Na^+/K^+-ATPase (red) and apical localization of PNA (green). Immunofluorescence was eliminated after omission of primary antibody and PNA (Fig. 3C) clearly indicating that non-specific binding of the secondary antibody and/or streptavidin to gill sections was not a confounding issue.

The NHE3 antiserum yielded an immunoreactive band at 98.6 kDa that was not observed after the antibody was incubated with excess peptide against which it was raised (Fig. 4A). The size of the band was slightly larger than expected based on either the predicted NHE3a (84.4 kDa) or NHE3b (95.8 kDa) sequences. Using the homologous polyclonal antibodies against trout NHE3, it was possible to co-localize NHE3 to some, but not all MRCs (Fig. 4B). Clearly, some of the MRCs (red) co-localized with apical NHE3 (arrows) and others did not (stars). Moreover, as observed for the PNA^+ cells (Fig. 3A), the NHE3-positive MRCs typically were localized to the basal portions of the lamellae. Indeed, after incubating the gill tissue with the NHE3 antibody and PNA (Fig. 4C) it is apparent that NHE3 (red) co-localized with PNA (green), confirming the localization of NHE3 to PNA^+ MRCs. Immunofluorescence was eliminated after omission of the NHE3 primary antibody (Fig. 4D).

Despite trying a variety of antigen retrieval techniques, we were unable to demonstrate specific immunofluorescence on gill tissue sections using the homologous NHE2 antibody. Furthermore, the results of Western blotting experiments suggested that the trout NHE2 antibody was not specific. Thus, it was necessary to develop other methods to identify the cell type(s) expressing NHE2. A technique was successfully developed to perform *in situ* hybridization using DIG-labelled antisense NHE2 probes on the same gill sections previously subjected to immunocytochemistry to identify PNA^+ and PNA^- MRCs. Thus it was possible to identify the MRC type expressing NHE2 mRNA. Fig. 5 clearly demonstrates that NHE2 mRNA is expressed in PNA^+ MRCs. In this particular instance it can be observed that numerous cells showing distinct NHE2 mRNA expression (Fig. 5B; black arrows) are PNA^+ MRCs (Fig. 5A; white arrows). In this particular photo (Fig. 5), 2 of 11 PNA^+ cells did not exhibit prominent NHE2 mRNA staining. Although no PNA^- cells were present in this photo, we never observed such cells to exhibit obvious NHE2 MRNA staining. Negative control experiments incorporating either a DIG-labelled sense NHE2 probe (Fig. 5C) or omission of probe (Fig. 5D) on gill sections produced no obvious cellular staining.

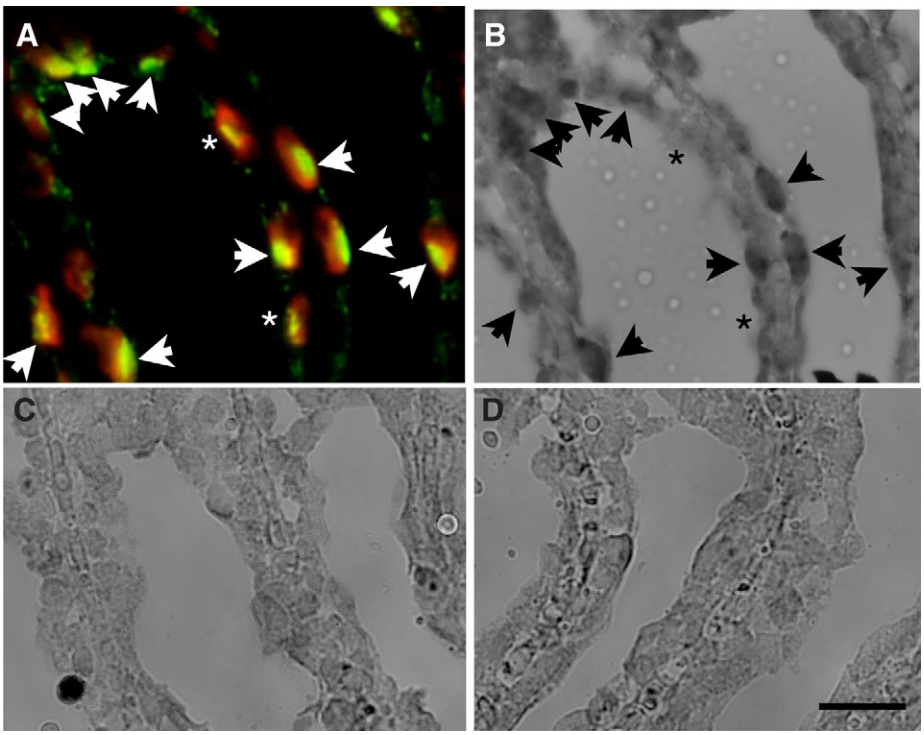

Fig. 5. Detection of NHE2-expressing cells in rainbow trout gill by combined immunofluorescence and *in situ* hybridization. (A) PNA^+ MRCs (labelled with arrows) were detected using a primary antibody against Na^+/K^+-ATPase (red) and biotinylated PNA (green). (B) *In situ* hybridization on the same section demonstrated that the PNA^+ MRCs were enriched with NHE2 mRNA (labelled with arrows). Two PNA^+ cells (labelled with asterisks) did not exhibit strong mRNA staining. No staining was observed when a sense probe was used (C) or when probe was omitted (D). Scale bar, 20 μm.

Effects of hypercapnia on gill NHE2 and NHE3 levels

Changes in gill NHE2 and NHE3 mRNA levels during hypercapnia (nominal P_{CO_2}=7.5 mmHg), as assessed by real-time PCR, appeared to be isoform specific (Fig. 6). Gill NHE2 mRNA levels were significantly increased after 3, 12 and 24 h exposure to hypercapnia (Fig. 6A), whereas NHE3 mRNA levels were statistically unaffected (Fig. 6B).

Because the trout NHE2 antibody was unreliable, it was not possible to test whether NHE2 protein levels were altered during hypercapnia. Consistent with the mRNA data, gill NHE3 protein levels were unchanged after 24 h of hypercapnia exposure (Fig. 7B). Plasma cortisol levels were increased significantly after 24 h of hypercapnia from 35.3±9.4 to 100.1±30.0 ng ml^{-1}.

Effects of elevated plasma cortisol levels on gill NHE2 mRNA expression

Fish subjected to intra-peritoneal cortisol implants exhibited an increase in gill NHE2 mRNA level after 48 and 72 h; NHE3 mRNA and protein were unaffected (Fig. 8).

DISCUSSION

In contrast to the extensive literature on NHE proteins in mammals (for reviews, see Counillon and Pouyssegur, 2000; Hayashi et al., 2002; Orlowski and Grinstein, 2004), considerably less is known about the physiological roles of the various NHE paralogues in fish and indeed several members of the *NHE* gene family have yet to be identified or characterized. An obvious exception is the unique βNHE of rainbow trout red blood cells (Borgese et al., 1992), which was extensively characterized using pharmacological and molecular techniques (Nikinmaa and Boutilier, 1995; Malapert et al., 1997). In the present study, we report the identification of two additional rainbow trout NHE isoforms, NHE2 and NHE3 (Fig. 1). The physiological role of the two NHE isoforms may be tissue specific; relatively high levels of NHE2 mRNA were detected in the gills, kidney and intestine (Fig. 2A), all of which include acid-/base-excreting epithelia (Marshall and Grosell, 2006). The kidney and gill exhibited abundant expression of NHE3 mRNA when compared with other tissues (Fig. 2B). Based on analysis of mRNA levels, the predominant NHE isoform in the gill, however, was NHE2 (Fig. 2C) suggesting that NHE2 may be the major NHE protein expressed in the gill.

Sequence analysis and Northern blot results indicated that there are at least two different NHE3 transcripts expressed in the rainbow trout (Fig. 1). Similar results were obtained by Yan and colleagues who reported two *NHE3* genes, *NHE3a* and *NHE3b*, in zebrafish (*Danio rerio*) of which only *NHE3b* was detectable in the gill (Yan et al., 2007). A search of GenBank revealed three *NHE3* genes in zebrafish ranging between 2112 and 2553 bp. Because the antibody we developed for NHE3 is likely to recognize both of the trout isoforms, we cannot determine whether there is preferential expression of one form as in the zebrafish. Unfortunately, the primers used for real-time PCR and subsequent experiments were designed and reactions performed before we realized there were multiple NHE3 transcripts. Differences between *NHE3a* and *NHE3b* in nucleotide sequence at the primer sites were slight (only 3 out of 20 nucleotides differed in the forward and reverse primers). Thus, it is possible that both *NHE3a* and *NHE3b* were being amplified during real-time PCR. Despite the occurrence of genome duplication, it is unclear whether there are multiple *NHE2* genes expressed in trout. Thus, it is difficult to know whether the tools (PCR primers, *in situ* hybridization probe) that were developed to examine NHE2 mRNA expression were indeed specific for a single *NHE2* gene. However, on the basis of sequence alignment and comparison, we are confident that the PCR primers and *in situ* probe were not detecting other *NHE* paralogues (specifically *NHE3* and *NHE1*).

The presence of NHE in the gills of rainbow trout is not particularly surprising because the occurrence of branchial NHE2 or NHE3 was previously documented in several species within the agnathans (Edwards et al., 2001; Choe et al., 2002; Tresguerres et al., 2005), elasmobranchs (Edwards et al., 2002; Choe et al., 2002;

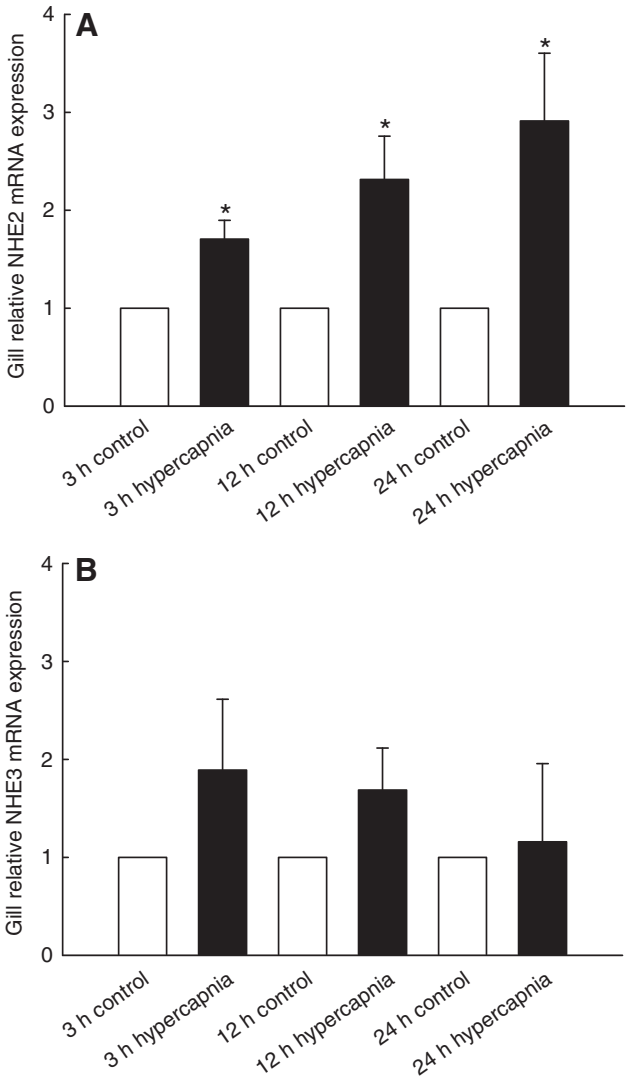

Fig. 6. The effects of hypercapnia (water P_{CO_2}=7.5 mmHg) on trout gill NHE2 (A) and NHE3 (B) relative mRNA levels. Gill NHE2 mRNA levels were significantly increased at 3, 12 and 24 h of hypercapnia (P values of 0.014, 0.031 and 0.040, respectively). Gill NHE3 mRNA levels were unchanged throughout the period of hypercapnia exposure.

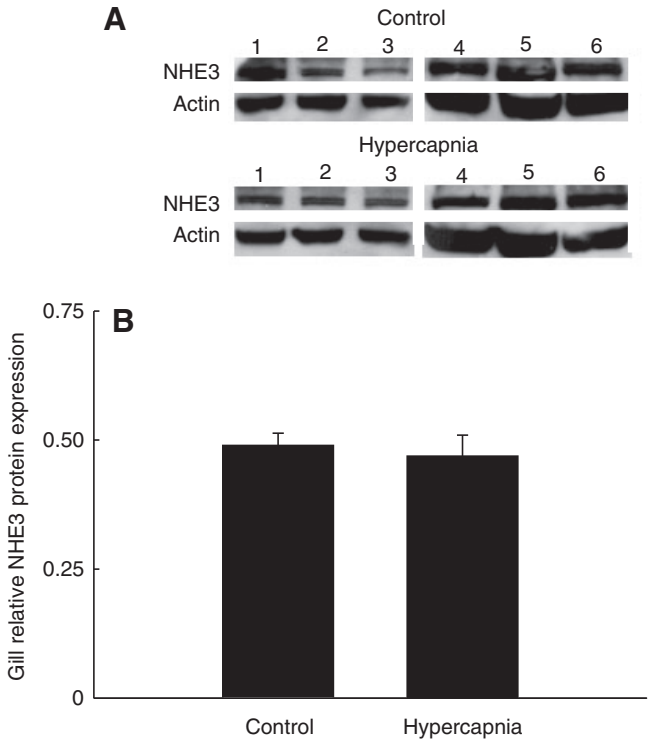

Fig. 7. (A) The effects of exposure to hypercapnia for 24 h (water P_{CO_2}=7.5 mmHg) on trout gill NHE3 protein levels as revealed by Western blot analysis (N=6; each lane represents a different fish). Densitometric analysis of Western blots (B), normalized to β-actin intensities, revealed unaltered levels of NHE3 protein after 24 h of hypercapnia.

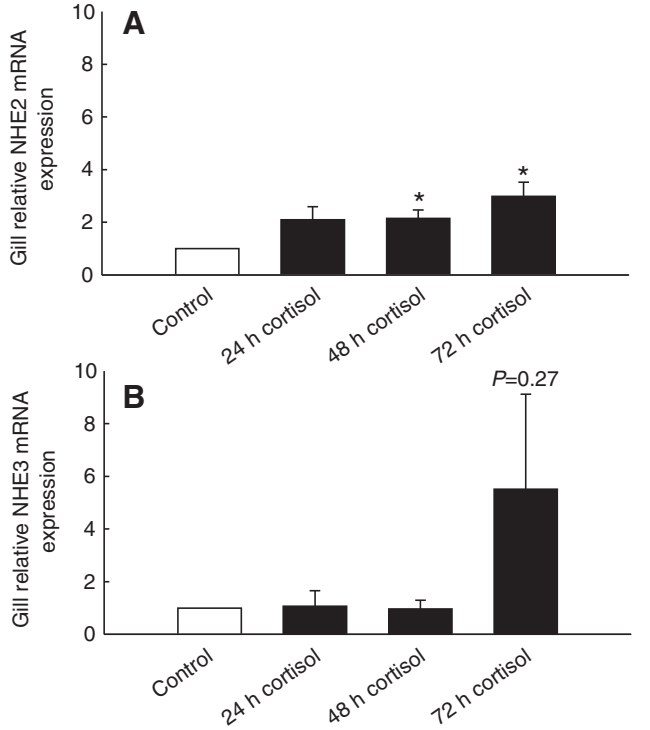

Fig. 8. The effects of elevated plasma cortisol levels on (A) gill relative NHE2 and (B) relative NHE3 mRNA expression after 24 (N=6), 48 (N=6) and 72 h (N=6) of administration of exogenous cortisol. NHE2 mRNA levels were significantly elevated at 48 (P=0.017) and 72 h (P=0.014) postinjection; NHE3 mRNA levels were unchanged.

Choe et al., 2005; Choe et al., 2007; Tresguerres et al., 2005) and teleosts (Claiborne et al., 1999; Edwards et al., 1999; Edwards et al., 2005; Wilson et al., 2000; Hirata et al., 2003; Scott et al., 2005; Catches et al., 2006). These studies not only included examples from FW- and SW-acclimated fish but also demonstrated that transferring euryhaline species to lowered salinity could induce an increase in mRNA levels of NHE2 [*Fundulus heteroclitus* (Scott et al., 2005)] or NHE3 [*Dasyatis sabina* (Choe et al., 2005)]. The finding of NHEs in FW fish is particularly significant because it provides indirect evidence that electroneutral Na^+/H^+ exchange may function even when the concentration gradient for Na^+ across the apical membrane apparently is unfavourable. Perhaps even more surprising is the localization of an NHE3 isoform to the apical membrane of MRCs in Osorezan dace (*Tribolodon hakonensis*) inhabiting highly acidic (pH 3.5) water (Hirata et al., 2003). In this case, an apical NHE would be required to function against both Na^+ and H^+ concentration gradients (see below).

A subset of MRCs expresses NHEs in rainbow trout

Aside from the study of Wilson and colleagues (Wilson et al., 2000) that provided evidence for NHE localization to a sub-population of MRCs and pavement cells (PCs) in tilapia (*Oreochromis mossambicus*), all previous studies (with one exception; see below) demonstrated that NHE2 and NHE3 are co-localized with Na^+/K^+-ATPase-enriched MRCs. The novel finding of the present study was the demonstration that in trout, both NHE2 and NHE3 are confined to a specific sub-type of MRC, the PNA^+ MRC (Galvez et al., 2002). Unlike the PNA^- MRCs, which tended to be situated along the more distal segments of the lamellae, the PNA^+ cells were typically found on the lamellar surfaces more proximal to the filament as well as within the interlamellar regions. The localization of NHEs to PNA^+ MRCs obviously does not support our initial hypothesis, based on the model of Perry and Gilmour (Perry and Gilmour, 2006), that positioned NHEs on the apical membrane of PNA^- MRCs. Because the NHE2 antibody was ineffective it was not possible to determine the sub-cellular distribution of NHE2. The results of immunocytochemistry utilizing the NHE3 antibody clearly showed that NHE3 was confined to the MRC apical membrane or sub-apical regions. Although not attempted in this study, previous experiments have demonstrated that the cells expressing apical NHEs generally do not contain high levels of V-ATPase (Choe et al., 2005; Choe et al., 2007; Catches et al., 2006). Two apparent exceptions are the Pacific hagfish (*Eptatretus stoutii*), in which NHE2, V-ATPase and Na^+/K^+-ATPase are localized to the same gill epithelial cells (Tresguerres et al., 2005), and zebrafish (Yan et al., 2007), in which NHE3b is confined to cells enriched with apical membrane V-ATPase and not Na^+/K^+-ATPase.

Are the PNA^+ MRCs acid- or base-secreting cells?

The PNA^+ MRC of trout is characterized by a tubular network arising from extensive infolding of the basolateral plasma membrane. The PNA^- MRC has no such elaboration of the basolateral membrane and levels of Na^+/K^+-ATPase, while high relative to PCs, are lower than in the PNA^+ cells (Galvez et al., 2002). Thus, the PNA^+ MRC is presumed to be analogous to the so-called 'chloride cell' of FW fish (Perry, 1997). These PNA^+ MRCs or chloride cells are believed to be the site of apical membrane Cl^-/HCO_3^- exchange and hence are thought to function as net base-secreting cells (Perry et al., 2003b; Perry and Gilmour, 2006; Tresguerres et al., 2006a). Indeed, there is extensive indirect evidence to suggest that net base excretion in trout (Goss and Perry, 1993; Perry and Goss, 1994; Goss et al., 1994b) and other species (Goss et al., 1992a; Goss et al., 1994a) is regulated by the number of PNA^+ MRCs (chloride cells) exposed to the water. Thus, the increase in net acid excretion during acidosis required for pH regulation is thought to arise, in part, from reduced rates of Cl^-/HCO_3^- exchange, reflecting the physical covering of chloride cells (now presumed to be PNA^+ MRCs) by adjacent PCs (see reviews by Goss et al., 1992b; Goss et al., 1995; Goss et al., 1998; Laurent and Perry, 1995; Perry and Gilmour, 2006). The unexpected finding of this study that NHE3 is also expressed on the apical membrane of PNA^+ MRCs clearly complicates the current model of a net base-secreting PNA^+ MRC because, if correct, physical covering of these cells during acidosis would presumably cause equivalent reductions in H^+ and HCO_3^- efflux (via Na^+/H^+ and Cl^-/HCO_3^- exchange) and thus have no impact on net acid excretion. We believe that the present finding of NHE3 expression on the apical membrane of PNA^+ MRCs warrants a re-assessment of the role of this cell type in acid–base regulation and acknowledgement that the PNA^+ MRC may in fact be an acid-secreting cell in rainbow trout. Indeed, the significant increases in mRNA levels during hypercapnia for the predominant NHE isoform in the gill (NHE2) supports the idea that net acid excretion by the PNA^+ MRC increases during acidosis. Obviously, these observations must be weighed against the substantial (albeit largely indirect) data that implicate the PNA^- MRC as the acid-secreting cell type in the trout gill (Galvez et al., 2002; Reid et al., 2003; Laurent et al., 1994; Sullivan et al., 1995). Finally, if truly an acid-secreting cell, one must also re-evaluate the utility of the physical covering of the PNA^+ MRCs that occurs during acidosis. One possibility is that the morphological rearrangement of the gill during acidosis enables the creation of chemical microenvironments in the vicinity of the apical plasma membrane that favour acid efflux.

Previous studies examining the effects of acidosis on NHEs in other species have produced conflicting results. For example, while expression of NHE3 in Atlantic hagfish [*Myxine glutinosa* (Edwards et al., 2001)] and NHE2 in *Fundulus heterolclitus* (Edwards et al., 2005) and *Squalus acanthias* (Tresguerres et al., 2005) increased during acidotic conditions, there were no detectable changes caused by acidosis in NHE2 expression in the sculpin [*Myoxocephalus octodecimspinosus* (Catches et al., 2006)] or NHE3 expression in the Atlantic stingray [*Dasyatis Sabina* (Choe et al., 2005)].

How is a favourable gradient for electroneutral Na^+/H^+ exchange established?

Clearly, if Na^+/H^+ exchange is to operate in FW, mechanisms must exist to establish a favourable chemical gradient within the microenvironment in which the NHE functions. As discussed by Hirata and colleagues, it is possible that activity of the basolateral Na^+/K^+-ATPase in close proximity to the apical membrane creates pockets of low cytoplasmic Na^+ to establish an inwardly directed gradient for Na^+ diffusion (Hirata et al., 2003). Although our previous model (e.g. Perry and Gilmour, 2006) placed V-ATPase on the basolateral membrane of PNA^+ cells, its presence there would probably reduce or abolish an otherwise favourable gradient for H^+ and would not be conducive to net acid secretion. Thus, the presence of basolateral V-ATPase in the NHE-containing PNA^+ cells of trout must be reconsidered.

Regulation of NHE by cortisol

Concomitant with the increased expression of NHE2, plasma cortisol levels increased significantly during hypercapnia. Because previous research on mammals has shown that NHEs in kidney are transcriptionally regulated by glucocorticoids (Hayashi et al., 2002), we sought to determine whether cortisol might be playing a role in regulating NHE2 levels in trout gill. The data showing a significant increase in branchial NHE2 mRNA after cortisol treatment are consistent with the notion that cortisol mobilization during hypercapnic acidosis contributes to pH regulation by transcriptional activation of NHE2 in PNA^+ MRCs. For such a scheme to be beneficial to net H^+ excretion, NHE2 would need to be expressed on the apical membrane; clearly, future research should be directed at determining the sub-cellular distribution of NHE2 in rainbow trout gill.

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