Ammonia excretion in rainbow trout (Oncorhynchus mykiss): evidence for Rh glycoprotein and H\(^+\)-ATPase involvement

C. Michele Nawata, Carrie C. Y. Hung, Tommy K. N. Tsui, Jonathan M. Wilson, Patricia A. Wright, and Chris M. Wood

Department of Biology, McMaster University, Hamilton; Department of Integrative Biology, University of Guelph, Guelph, Ontario, Canada; and Ecofisiologia CIMAR, Porto, Portugal

Submitted 12 March 2007; accepted in final form 21 August 2007

Nawata CM, Hung CC, Tsui TK, Wilson JM, Wright PA, Wood CM. Ammonia excretion in rainbow trout (Oncorhynchus mykiss): evidence for Rh glycoprotein and H\(^+\)-ATPase involvement. Physiol Genomics 31: 463–474, 2007. First published August 21, 2007; doi:10.1152/physiolgenomics.00061.2007.—Branchial ammonia transport in freshwater teleosts is not well understood. Most studies conclude that NH\(_3\) diffuses out of the gill and becomes protonated to NH\(_4^+\) in an acidified gill boundary layer. Rhesus (Rh) proteins are new members of the ammonia transporter superfamily and rainbow trout possess genes encoding for Rh30-like1 and Rhcg2. We identified seven additional full-length trout Rh cDNA sequences: one Rhag and two each of Rhbg, Rhcg1, and Rhcg2. The mRNA expression of Rhbg, Rhcg1, and Rhcg2 was examined in trout tissues (blood, brain, gill, heart, intestine, kidney, liver, muscle, skin, spleen) exposed to high external ammonia (HEA; 1.5 mmol/l NH\(_3\), pH 7.95, 15°C). Rhbg was expressed in all tissues. Rhcg1 was expressed in brain, gill, liver, and skin, and Rhcg2 was expressed in gill and skin. Brain Rhbg and Rhcg1 were downregulated, blood Rh30-like and Rhag were downregulated, and skin Rhbg and Rhcg2 were upregulated with HEA. After an initial uptake of ammonia into the fish during HEA, excretion was reestablished, coinciding with upregulations of gill Rh mRNA in the pavement cell fraction: Rhcg2 at 12 and 48 h, and Rhbg at 48 h. NHE2 expression remained unchanged, but upregulated H\(^+\)-ATPase (V-type, B-subunit) and downregulated carbonic anhydrase (CA2) expression and activity were noted in the gill and again expression changes occurred in pavement cells, and not in mitochondria-rich cells. Together, these results indicate Rh glycoprotein involvement in ammonia transport and excretion in the rainbow trout while underscoring the significance of gill boundary layer acidification by H\(^+\)-ATPase.

THE CURRENT MODEL OF AMMONIA excretion from the freshwater teleost gill links NH\(_3\) diffusion to CO\(_2\) hydration (62). Expired CO\(_2\) is converted to HCO\(_3^-\) and H\(^+\) by carbonic anhydrase (CA) in the gill mucus, and the resulting acidified gill boundary layer then traps NH\(_3\) to NH\(_4^+\) (68, 69). In addition, this layer could also be acidified by H\(^+\) extruded from an apical vacuolar H\(^+\)-ATPase (29, 30). However, as it is acidified, this boundary layer creates a microenvironment for a favorable transbranchial (blood-to-water) P\(_{\text{NH3}}\) gradient that facilitates ammonia excretion. This is especially advantageous during exposure to high external ammonia (HEA), which reverses the normally positive ammonia gradient. Indeed, abolishing this layer with HEPES buffer during HEA reduces J\(_{\text{Amn}}\) (64).

Address for reprint requests and other correspondence: C. M. Nawata, Dept. of Biology, McMaster Univ., 1280 Main St. W., Hamilton, Ontario, Canada, L8S 4K1 (e-mail: nawatacm@mcmaster.ca).

Despite the dominance of this NH\(_3\) diffusion-trapping theory, many past studies have supported the possibility of apical Na\(^+\)/NH\(_4^+\) exchange (e.g., Refs. 26, 70). It has been argued, however, that freshwater cannot provide the Na\(^+\) gradients necessary to drive this exchange (62). Likewise, basolateral entry of NH\(_4^+\) into the gill via an Na\(^+\)/NH\(_4^+\) exchanger (NHE) is unlikely since the electromotive force for Na\(^+\) is directed into the gill cytosol and NH\(_4^+\) cannot out-compete the much more abundant Na\(^+\) in the plasma for access to the NHEs (62). Also, no evidence exists to support basolateral ammonia transport via NH\(_4^+\) substitution for K\(^+\) on the Na\(^+\)/K\(^+\)-ATPase in the freshwater gill, although there are reports of this occurring in seawater fish (37, 50).

Ammonia transport has been studied for decades in microorganisms, but the molecular identity and function of potential transporters involved were not elucidated until recently. The genes and corresponding proteins were first identified as methylammonium permease (Mep) in yeast (41) and the ammonium transporter (Amt) in plants (48). Based on sequence similarity, mammalian homologs to the Mep/Amt proteins were identified as the Rh blood group-related proteins: Rh30 and the glycoproteins RhAG/Rhag, RhBG/Rhbg, and RhCG/Rhcg (19). The terminologies RhAG, RhBG and RhCG refer to human tissue, whereas Rhag, Rhbg, and Rhcg indicate nonhuman tissue (59). Rh30 and RhAg/Rhag are restricted to erythrocytes (RBCs), whereas RhBG/Rhbg and RhCG/Rhcg are expressed in various other tissues (19, 34, 35). The three glycoproteins conduct ammonia when expressed in heterologous systems, although disagreement exists as to whether NH\(_3\) or NH\(_4^+\) is transported (22). The nonglycosylated Rh30 proteins form part of the Rh complex with RhAG on the RBC membrane, but their function is unknown (60, 61).

Rh proteins have been identified in different fish species (20, 21, 47), and functionally, it has been shown that pufferfish Takifugu rubripes Rh glycoproteins, like their mammalian counterparts, mediate the transport of the ammonium analog, methylammonium, when expressed in Xenopus oocytes (47). Recently we identified three Rh cDNA sequences in the unexposed and HEA-exposed mangrove killifish Kryptolebias marmoratus (21). HEA exposure resulted in upregulated Rh mRNA expression in the gill, liver, muscle, and skin. Two rainbow trout Rh genes (Rh30-like1 and Rhcg2) were identified in a previous study (20), but their tissue expression and potential involvement in ammonia transport have not been investigated.

With this background in mind, the objectives of this study were to identify and sequence Rh orthologs in the rainbow trout, to examine their expression pattern in different trout tissues under control conditions and when exposed to HEA,
and to quantify the HEA-exposed gill expression of Rh orthologs as well as other proteins thought to be involved in the ammonia transport process.

MATERIALS AND METHODS

Animals

Rainbow trout [Oncorhynchus mykiss, Walbaum; 178–260 g], obtained from Humber Springs Trout Hatchery, Ontario, Canada were used for all experiments. The fish were held in dechlorinated Hamilton tap water (moderately hard: [Na+] = 0.6 mequiv/l, [Cl−] = 0.8 mequiv/l, [Ca2+] = 0.8 mequiv/l, [Mg2+] = 0.3 mequiv/l, [K+] = 0.05 mequiv/l; titration alkalinity 2.1 mequiv/l; pH ~8.0; hardness ~140 mg/l as CaCO3 equivalents; temperature 12–16°C), and food was withheld for at least 1 wk prior to experimentation to stabilize the endogenous fraction of nitrogenous waste excretion (15). All fish were transferred to individual, darkened acrylic flux boxes supplied with aerated, continuously flowing dechlorinated tap water at 15 ± 0.5°C and allowed to recover overnight prior to experimentation. All procedures used were approved by the McMaster University Animal Research Ethics Board and are in accordance with the Guidelines of the Canadian Council on Animal Care.

HEA Experiment

Six fish were exposed to a nominal total ammonia (TAM) concentration of 1.5 mmol/l NH3·HCO3 (pH 7.95 ± 0.05, 15.0 ± 0.5°C) for 12 or 48 h. Fluxes were conducted during which time the water flow was stopped and the box volume was set to 4 l. For the 48-h flux, a period of flow-through flushing was performed every 12 h to prevent toxic build-up of ammonia. Control fish (six each) were treated identically for 12 and 48 h without the addition of ammonia.

Water samples (10 ml) were taken at 12-h intervals, frozen at −20°C and later assayed in triplicate for TAM using a modified salicylate-hypochlorite method (58). After the flux periods, the fish were anesthetized with MS222 (~0.1 g/l, Sigma, St. Louis, MO), and caudal blood was drawn into a heparinized syringe. Samples were centrifuged to separate plasma from RBCs (2 min, 20,000 × g), immediately frozen in liquid nitrogen, stored at −70°C, and later analyzed enzymatically for plasma TAM (Raichem; Hemagen Diagnostics, San Diego, CA). Net flux rates of total ammonia (JAMM, μmol·kg−1·h−1) were calculated as: JAMM = (TAMf − TAMi) × V/(t × M), where i and f refer to initial and final concentration (μmol/l), V is water volume (l) in the box, t is the time elapsed (h), and M is the fish mass (kg). A negative JAMM indicates a net excretion of ammonia to the water. A positive JAMM indicates a net uptake of ammonia into the fish.

Sequencing and Characterization of Rainbow Trout Rh cDNAs

Tissue sampling. Gill and blood samples for cloning purposes were removed from fish that were exposed to 1.5 mmol/l NH3·HCO3 for 24 h. Control samples for all subsequent analyses were collected from fish that were held in individual flux boxes with flowing dechlorinated tap water for 24 h. Blood samples were collected (as described above) immediately after anesthetization and prior to tissue collection. Preliminary studies indicated Rhbg expression on RBCs, so all analyses subsequent to cloning were performed on saline-perfused tissues to minimize the contribution of RBC mRNA to tissue levels. Samples of brain, eye, gill, heart, intestine, kidney, liver, muscle, skin, and spleen were removed after the fish were anesthetized, placed on ice, and perfused free of blood with chilled, heparinized (50 IU/ml lithium heparin, Sigma) Cortland saline (66). Perfusions were performed by cannulation of the bulbus arteriosus with a 23-gauge butterfly needle attached to a peristaltic pump, and the ventricle was severed immediately after initiation of perfusion to allow drainage.

Tissue samples were immediately frozen in liquid nitrogen and stored at −70°C until analysis. RNA extraction and reverse-transcriptase PCR amplification. Total RNA was extracted from blood and tissues using TRIzol (Invitrogen, Burlington, ON, Canada), quantified spectrophotometrically, and electrophoresed on 1% agarose gels stained with ethidium bromide to verify integrity. First strand cDNA was synthesized from 1 μg total RNA using an oligo(dT)17 primer and Superscript II reverse transcriptase (Invitrogen). Samples were stored at −70°C.

Identification of Rh orthologs. Partial Rh30-like, Rhag, Rhbg, and Rhcg1 sequences were obtained from cDNA of HEA-exposed blood or gill samples using primers (Table 1) designed from the trout Rh30-like1 sequence and the pufferfish Rhag, Rhbg, and Rhcg2 sequences (GenBank: AY207445, AY618933, AY116074, AY116076, respectively). PCR were carried out in a PTC-200 MJ Research thermocycler with Taq DNA polymerase (Invitrogen) at 94°C (2 min) followed by 45 cycles of 94°C (30 s), 52°C (30 s), and 72°C (1 min). Products were electrophoresed on ethidium bromide-stained 1% agarose gels, and bands of appropriate size were excised and extracted (GeneRax kit, Qiagen, Mississauga, ON, Canada). Purified gel products were ligated to a pGEM-T Easy vector (Promega; Fisher Scientific, Nepean, ON, Canada), transformed into heat-shock competent Escherichia coli (XL-Blue; Stratagene, Mississauga, ON, Canada), and then grown on ampicillin LB agar plates. Colonies containing the ligated product were inoculated into liquid media and grown overnight. Plasmids were harvested using GeneJet Plasmid Miniprep Kit (Fermentas Canada, Burlington, ON, Canada), and sequencing was performed on an ABI 3100 Gene Analyzer at the MOBIX Lab, McMaster University (Hamilton, ON, Canada). Primers designed specific to the Rh orthologs (Table 1) were used to obtain full-length cDNA sequences by 5′– and 3′-rapid amplification of cDNA ends (Smart RACE cDNA amplification kit; BD Bioscience Clontech, Mississauga, ON, Canada). Several clones of the 3′- and 5′-ends were sequenced in both directions, and full-length transcripts were determined by majority-rule consensus with BioEdit (17). Sequences have been deposited into GenBank (Table 2).

Phylogenetic, amino acid sequence identity, hydropathy, and glycosylation analysis. Phylogenetic analyses were conducted using ClustalW (57) and MEGA 3.1 software (27) by the neighbor-joining method with support for each node using 500 bootstrap replicates. Amino acid sequence analyses were performed using BioEdit (17), hydropathy profiles were determined using EMBOSS (52) and SPLiT 4.0 (23), and N-glycosylation sites were predicted using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

Tissue distribution (PCR). Following the methods described above, total RNA and cDNA were prepared from the RBCs and saline-perfused tissues collected from the initial HEA experiment. Distribution of Rh orthologs in control and HEA-exposed (48 h) tissues was determined using ortholog-specific primers (Table 1) with the PCR protocol described earlier. Products were electrophoresed on ethidium bromide-stained 1.5% agarose gels.

Tissue expression (quantitative real-time PCR). Rh expression in tissues exposed to HEA for 12 and 48 h was compared with control tissues by quantitative real-time PCR (qPCR) using the cDNA prepared above and ortholog-specific primers (Table 1). Primer specificity was confirmed in preliminary experiments, and qPCR analyses were performed on an MX3000P QPCR System (Stratagene, Cedar Creek, TX). Reactions (20 μl) containing 4 μl of DNaseI-treated (Invitrogen) cDNA, 4 pmol of each primer, 10 μl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), and 0.8 μl of ROX (1:10 dilution) were performed at 50°C (2 min), 95°C (2 min), followed by 40 cycles of 95°C (15 s) and 60°C (30 s). Melt-curve analysis confirmed production of a unique product, and gel electrophoresis verified presence of a single band. A nonreversed-transcribed sample controlled for possible genomic DNA contamination. Elongation factor-1α (EF-1α, GenBank AF498320) for gill and β-actin (GenBank AF157514) for other tissues had the most stable expression.
Enzyme/Transporter Expression in the Gill
measured relative to
from both cell fractions, and the mRNA expression of Rh was
density gradient. Pavement cells were harvested from the 1.03–1.05
treated with RBC lysis buffer and layered onto a discontinuous Percoll
branchial baskets were removed from control and HEA-exposed fish,
cells using a technique modified from Galvez et al. (16). Whole
gill epithelial
ATPase (V-type, B subunit; GenBank AF14002), carbonic anhy-
Table 2. Primers used for cloning, PCR, and qPCR analysis

<table>
<thead>
<tr>
<th>Name</th>
<th>Application</th>
<th>Sequence (5'-3')</th>
<th>Name</th>
<th>Application</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh30-like</td>
<td>cloning-forward</td>
<td>cccagccatgctgaagaagtgcaag</td>
<td>Rhag</td>
<td>qPCR-forward</td>
<td>cgccgggcaagtatggttg</td>
</tr>
<tr>
<td>Rh30-like</td>
<td>cloning-reverse</td>
<td>cagctgacctcaagggcagaaaat</td>
<td>Rhag</td>
<td>qPCR-reverse</td>
<td>atggccgaaggtctgatgagt</td>
</tr>
<tr>
<td>Rhag</td>
<td>cloning-forward</td>
<td>ccacaggtgtcagcgagag</td>
<td>Rhbg</td>
<td>qPCR-forward</td>
<td>gcaacacgctttaactgccg</td>
</tr>
<tr>
<td>Rhbg</td>
<td>cloning-reverse</td>
<td>ccacaggtgtcagcgagag</td>
<td>Rhbg</td>
<td>qPCR-reverse</td>
<td>gcaacacgctttaactgccg</td>
</tr>
<tr>
<td>Rhbg1</td>
<td>qPCR-forward</td>
<td>caatctccactctcataagcg</td>
<td>Rhbg1</td>
<td>qPCR-forward</td>
<td>caatctccactctcataagcg</td>
</tr>
<tr>
<td>Rhbg2</td>
<td>qPCR-forward</td>
<td>cctctcggcttcctcataagcg</td>
<td>Rhbg2</td>
<td>qPCR-forward</td>
<td>cctctcggcttcctcataagcg</td>
</tr>
<tr>
<td>Rhbg</td>
<td>3' RACE</td>
<td>ggagctctacgggtagcgagag</td>
<td>CA</td>
<td>qPCR-forward</td>
<td>gccgtctccattgacactac</td>
</tr>
<tr>
<td>Rhbg</td>
<td>5' RACE</td>
<td>gacacctcagagcttctcttgcct</td>
<td>CA</td>
<td>qPCR-forward</td>
<td>gccgtctccattgacactac</td>
</tr>
<tr>
<td>Rhag</td>
<td>3' RACE</td>
<td>gcacaggtgaagggagctgagg</td>
<td>HATP</td>
<td>qPCR-forward</td>
<td>tlaccccttctggagagag</td>
</tr>
<tr>
<td>Rhag</td>
<td>5' RACE</td>
<td>gacacctcagagcttctcttgcct</td>
<td>HATP</td>
<td>qPCR-forward</td>
<td>tlaccccttctggagagag</td>
</tr>
<tr>
<td>Rhbg</td>
<td>3' RACE</td>
<td>ggctcagtgacgtagagacggagaag</td>
<td>NHE</td>
<td>qPCR-forward</td>
<td>tatggcccatgtgacactgc</td>
</tr>
<tr>
<td>Rhbg</td>
<td>5' RACE</td>
<td>ccccttccggcttctcataagcg</td>
<td>NHE</td>
<td>qPCR-forward</td>
<td>tatggcccatgtgacactgc</td>
</tr>
<tr>
<td>Rhcg1</td>
<td>3' RACE</td>
<td>cggtgctaggcaaggtgagcccagtc</td>
<td>NKA1a</td>
<td>qPCR-forward</td>
<td>gagccatcgagagacagagag</td>
</tr>
<tr>
<td>Rhcg1</td>
<td>5' RACE</td>
<td>ccctgctggcattcgtctcagc</td>
<td>NKA1a</td>
<td>qPCR-forward</td>
<td>gagccatcgagagacagagag</td>
</tr>
<tr>
<td>Rhbg</td>
<td>PCR-forward</td>
<td>gcacaggtgaagggagctgagg</td>
<td>NKA1b</td>
<td>qPCR-reverse</td>
<td>gctacccagctcctacccag</td>
</tr>
<tr>
<td>Rhbg</td>
<td>PCR-reverse</td>
<td>gcacaggtgaagggagctgagg</td>
<td>NKA1b</td>
<td>qPCR-reverse</td>
<td>gctacccagctcctacccag</td>
</tr>
<tr>
<td>Rhcg1</td>
<td>PCR-forward</td>
<td>ccacaggtgaagggagctgagg</td>
<td>NKA1c</td>
<td>qPCR-forward</td>
<td>ggcctgctgagagacagagag</td>
</tr>
<tr>
<td>Rhcg1</td>
<td>PCR-reverse</td>
<td>ccctgctggcattcgtctcagc</td>
<td>NKA1c</td>
<td>qPCR-forward</td>
<td>ggcctgctgagagacagagag</td>
</tr>
<tr>
<td>Rhcg2</td>
<td>PCR-forward</td>
<td>gcacaggtgaagggagctgagg</td>
<td>NKA3</td>
<td>qPCR-forward</td>
<td>gccaatctcctcctcagcaag</td>
</tr>
<tr>
<td>Rhcg2</td>
<td>PCR-reverse</td>
<td>gcacaggtgaagggagctgagg</td>
<td>NKA3</td>
<td>qPCR-forward</td>
<td>gccaatctcctcctcagcaag</td>
</tr>
<tr>
<td>Rh30-like2</td>
<td>qPCR-forward</td>
<td>gccggtgtcagctgagcgagg</td>
<td>b-actin</td>
<td>qPCR-forward</td>
<td>aggctccttcggagagagag</td>
</tr>
<tr>
<td>Rh30-like3</td>
<td>qPCR-forward</td>
<td>gccggtgtcagctgagcgagg</td>
<td>b-actin</td>
<td>qPCR-forward</td>
<td>aggctccttcggagagagag</td>
</tr>
<tr>
<td>Rh30-like2</td>
<td>qPCR-forward</td>
<td>gccggtgtcagctgagcgagg</td>
<td>EF-1a</td>
<td>qPCR-forward</td>
<td>gaaataccagctcctacccag</td>
</tr>
<tr>
<td>Rh30-like3</td>
<td>qPCR-forward</td>
<td>gccggtgtcagctgagcgagg</td>
<td>EF-1a</td>
<td>qPCR-forward</td>
<td>gaaataccagctcctacccag</td>
</tr>
<tr>
<td>Rh30-like3</td>
<td>qPCR-forward</td>
<td>gccggtgtcagctgagcgagg</td>
<td>EF-1a</td>
<td>qPCR-forward</td>
<td>gaaataccagctcctacccag</td>
</tr>
</tbody>
</table>

qPCR, quantitative real-time PCR.

across samples and were used as endogenous standards to calculate relative mRNA expression by the standard curve method. Standard curves were generated by serial dilution of a random mixture of control samples. To discriminate between erythroid and nonerythroid Rhbg mRNA expression, unperfused samples of brain, kidney, and liver were analyzed and compared with samples that had been perfused free of blood, by the same methods as those described earlier.

Pavement cell and mitochondria-rich cell expression. Pavement and mitochondria-rich (MR) cells were isolated from gill epithelial cells using a technique modified from Galvez et al. (16). Whole branchial baskets were removed from control and HEA-exposed fish, and gill filaments were trypsin-digested, passed through 96-μm filters, treated with RBC lysis buffer and layered onto a discontinuous Percoll density gradient. Pavement cells were harvested from the 1.03–1.05 g/ml interface, and MR cells were harvested from the 1.05–1.09 g/ml interface. Total RNA and cDNA were processed as described above from both cell fractions, and the mRNA expression of Rh was measured relative to β-actin.

Enzyme/Transporter Expression in the Gill

Following the qPCR protocol outlined earlier, expression of H+\textsuperscript{+}ATPase (V-type, B subunit; GenBank AF14002), carbonic anhy-

Table 2. Accession number and size of each Rh cDNA identified

<table>
<thead>
<tr>
<th>Ortholog</th>
<th>GenBank Accession Number</th>
<th>5'UTR</th>
<th>Coding Region</th>
<th>3'UTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh30-like2</td>
<td>EF062577</td>
<td>83</td>
<td>1263</td>
<td>265</td>
</tr>
<tr>
<td>Rh30-like3</td>
<td>EF062578</td>
<td>83</td>
<td>1263</td>
<td>551</td>
</tr>
<tr>
<td>Rhag</td>
<td>EF067352</td>
<td>140</td>
<td>1305</td>
<td>358</td>
</tr>
<tr>
<td>Rhbg-a</td>
<td>EF051113</td>
<td>188</td>
<td>1386</td>
<td>1080</td>
</tr>
<tr>
<td>Rhbg-b</td>
<td>EF051114</td>
<td>188</td>
<td>1386</td>
<td>607</td>
</tr>
<tr>
<td>Rhcg1-a</td>
<td>DQ431254</td>
<td>73</td>
<td>1463</td>
<td>562</td>
</tr>
<tr>
<td>Rhcg1-b</td>
<td>EF051115</td>
<td>73</td>
<td>1463</td>
<td>215</td>
</tr>
</tbody>
</table>

UTR, untranslated region.

drase (cytoplasmic, CA2; GenBank ASY514870), four isoforms of Na+/K+\textsuperscript{+}\textsuperscript{+}ATPase (α1a, α1b, α1c, α3; GenBank: ASY31939, ASY319390, ASY319387, ASY319388), and NHE2 (GenBank EF446605) was quantified in control and HEA-exposed whole gill samples relative to EF-1α, and in control and HEA-exposed pavement and MR cells relative to β-actin. Expression of Na+/K+\textsuperscript{+}\textsuperscript{+}ATPase isoforms α1a and α1b were quantified in the gill cell fractions, but as the results were similar, only the results of α1a are reported here.

Enzyme Activity in the Gill

Enzyme assays were performed in triplicate on homogenates of gill tissues that were perfused free of blood, extracted, and frozen from the previous HEA experiment. Protein assays were performed using Bradford Reagent (Sigma) and BSA standards.

ATPase activity. Protocols for Na+/K+\textsuperscript{+}\textsuperscript{+}ATPase (44) and H+\textsuperscript{+}ATPase (32) assays were modified and adapted to measure activity levels in control and HEA-exposed fish gills. Activities are reported as control activity (no inhibitor) minus inhibitor-treated activity [ouabain for Na+/K+\textsuperscript{+}\textsuperscript{+}ATPase and sodium azide with N-ethylmaleimide (NEM) for V-type H+\textsuperscript{+}ATPase], measured at 340 nm in a kinetic microplate reader (SpectraMAX Plus; Molecular Devices, Menlo Park, CA) at 15-s intervals for 30 min. We obtained the same results for H+\textsuperscript{+}ATPase activity using 10 μM bafilomycin as an inhibitor but are only reporting results obtained using NEM.

CA. CA activity was determined using the enzymatic ΔpH method (18). Briefly, samples were assayed in a pH 7.4 buffer (225 mmol mannitol, 75 mmol sucrose, 10 mmol Tris) held at 4°C, and the reaction was initiated with 200 μl of CO₂-saturated water. The reaction velocity was measured, using hydrogen and reference electrodes (Kwik-Tip; World Precision Instruments, Sarasota, FL) attached to an Accumet XL15 pH meter (Fisher Scientific), over a 0.15 pH change, and the activity is reported as the observed rate minus the uncatalyzed rate.

Physiol Genomics • VOL 31 • www.physiolgenomics.org
Statistical Analysis

All data are presented as means ± SE (n, number of fish). Values at each time point were compared with control values using one-way analysis of variance with Fisher’s least significant difference post hoc test. Student’s t-test was used for comparisons of \( T_{Amn} \) and \( J_{Amn} \) values and for comparisons between unperfused and perfused tissues. Significance was set at \( \alpha = 0.05 \).

RESULTS

HEA Experiment

Exposure to HEA initially resulted in net uptake of ammonia from the water into the fish during the first 12 h. Thereafter, net excretion of ammonia occurred and by 48 h, \( J_{Amn} \) exceeded the 12- and 48-h control rates, which remained unchanged (Fig. 1). Plasma \( T_{Amn} \) levels in HEA-exposed fish were significantly higher than the control value (Fig. 2), but they remained lower than water levels throughout the course of the experiment. There is however, a noticeable upward trend toward 48 h approaching a toxic level (36), but no signs of stress were visible in these fish.

Identification of Rh Orthologs in Trout

Seven Rh ortholog cDNA sequences were identified in this study. Phylogenetic analysis (Fig. 3) showed that two sequences with different coding regions (CDS), isolated from HEA-exposed RBCs, fell into a cluster of fish sequences described as Rh30-like (designated Rh30-like2 and Rh30-like3). Another sequence, also isolated from RBCs, fell into the Rhag cluster (designated Rhag). Two sequences with identical CDS but different 3’-untranslated regions (3’-UTR) were cloned from HEA-exposed gill, and these fell into the Rhbg cluster (designated Rhbg-a and Rhbg-b). A further two sequences that differ only in the 3’-UTRs were identified in the HEA-exposed gill, and these fell into subgroups 1 in the Rhcg cluster (designated Rhcg1-a and Rhcg1-b). Amino acid sequence analysis revealed that the Rh30-like2 sequence is 98.8% identical to the previously identified Rh30-like1 sequence, and all Rh30-like sequences are 44.5–96.4%, similar to their counterparts in other fish. Rhag is 78.5–83.7%, Rhbg is 76.1–80.6%, and Rhcg1 is 75.8–82.7% identical to their respective homologs in other fish (Fig. 4). The CDS of the identified sequences vary in length from 1050 to 1463 nucleotides (Table 2), and hydrophobicity analyses of all sequences...
revealed 12 predicted transmembrane domains (Fig. 5). Rhag, Rhbg, and Rhcg1 have predicted N-glycosylation sites in the extracellular domain at Asp45, Asp48, and Asp60, respectively. No potential glycosylation sites were identified in the Rh30-like sequences.

**Tissue Distribution and Expression of Rh**

PCR analysis of the various trout tissues revealed the presence of Rhbg in all control and HEA-exposed tissues examined, including the blood (Fig. 6). Since Rhbg was expressed in
RBCs, it was important to ensure that expression in tissues nominally cleared of blood was not due to residual RBCs. When mRNA copy number is taken into consideration, Rhbg was always higher in the unperfused tissues due to contribution from the blood. The contribution of Rhbg mRNA from the blood, however, was small compared with that of β-actin, and therefore the removal of blood in perfused tissues produced a more profound reduction in the relative amount of β-actin. On average, perfusion of brain, kidney, and liver reduced the Rhbg mRNA copy number by 1.7-fold, but β-actin copy number was reduced by sixfold. While this large reduction in β-actin expression represents a substantial elimination of contaminating blood, the smaller reduction in Rhbg indicates that Rhbg expression was originating from the tissues. As a result, Rhbg mRNA expression was always higher in the perfused tissues when normalized to β-actin (Fig. 7). Interestingly, Rhbg expression decreased significantly at 48 h of HEA in the perfused brain (Fig. 7), but increased by almost 200-fold at 48 h HEA in the skin (Fig. 9). Of particular note also is the increased Rhbg expression in the pavement cell fraction that is devoid of contaminating RBCs after lysis treatment (see below and Fig. 11A).

Rhcg1 was clearly detected in the gill and skin by PCR in both control and HEA conditions, and there was some evidence of expression in the brain and liver during HEA as well (Fig. 6). Analysis by qPCR confirmed that Rhcg1 mRNA was
expressed in control and HEA conditions in brain, gill, liver, and skin, but the only significant change noted was a decrease in expression at 48 h of HEA in the brain (Fig. 10), parallel to that seen with Rhbg in the brain at this time (cf. Fig. 7).

Although PCR analysis revealed Rhcg2 expression in the gill only (Fig. 6), qPCR analysis also detected Rhcg2 in the skin, and its expression increased about ninefold with 48 h HEA (Fig. 9). In the HEA-exposed gill, Rhcg2 expression increased by 6.2-fold at 12 h and remained elevated by 4.2-fold at 48 h (Fig. 8).

Gill cell fractionation was performed to ascertain the cellular location of these changes in expression. In the pavement cell fraction, there was no change in Rhcg1 expression, but there was a 4.3-fold increase in Rhbg expression at 48 h HEA and an 8.1 and 10.8-fold increase in Rhcg2 expression at 12 and 48 h of HEA, respectively (Fig. 11A). No significant changes in Rh mRNA expression were noted in the MR cell fraction, though Rhcg1 expression tended to fall (Fig. 11B).

With the Rh30-like1 and Rh30-like2 sequences being nearly identical, only the expression of Rh30-like2 and Rh30-like3 was analyzed. The expression of these as well as Rha decreased significantly to less than half the control value in RBCs exposed to 12 and 48 h of HEA (Fig. 12).

**Enzyme/Transporter Expression and Activity in the Gill**

The expression of H⁺-ATPase in the HEA-exposed gill increased by 2-fold at 12 and 48 h (Fig. 13A) and H⁺-ATPase enzyme activity increased significantly by 1.4-fold at 48 h HEA (Fig. 13D). CA2 expression decreased significantly at 48 h to half of the control value (Fig. 13B), and this corresponded to a 1.7-fold decrease in CA enzyme activity at 48 h HEA (Fig. 13E). Expression of four α-isosforms of Na⁺/K⁺-ATPase in the gill did not change significantly with HEA exposure (Fig. 13C), and Na⁺/K⁺-ATPase enzyme activity also remained unchanged (Fig. 13F). Similarly, NHE2 expression did not change significantly with control, 12 and 48 h HEA values of 1.00 ± 0.21, 1.82 ± 0.41, and 0.72 ± 0.09, respectively (n = 6).

In the pavement cells, there was no change in NHE2 expression, but there was a 8.3-fold decrease in CA2 expression at 48 h, a 3-fold increase in H⁺-ATPase expression at 12 h, and a 5.3-fold increase in Na⁺/K⁺-ATPase expression at 48 h HEA (Fig. 14A). In MR cells, Na⁺/K⁺-ATPase was significantly downregulated by 5.6-fold at 48 h HEA while CA2, H⁺-ATPase, and NHE2 expression remained unchanged (Fig. 14B).

**DISCUSSION**

Marini et al. (39) first reported that RhAG and RhCG rescued the growth of Mep-deficient yeast and that RhAG mediated efflux of methylammonium. Since then, the role of Rh proteins as potential ammonia transporters has become an active area of investigation. Previous work by Huang and Peng (20) identified an Rh30-like1 and Rhcg2 gene in the rainbow trout...
trout, but the physiological significance of these genes has not been explored. In the present study, we identified additional cDNA sequences encoding for Rh30-like, Rhag, Rhbg, and Rhcg1 and examined their potential involvement in ammonia transport. Rh expression was studied exclusively at the mRNA expression level in the present investigation. An important next step in future studies will be to quantify expression at the protein level, once suitable antibodies have been developed for use in rainbow trout.

All sequences identified in this study have 12 predicted transmembrane domains, a highly conserved characteristic observed in the Rh protein family (19). The Rh30-like1 and Rh30-like2 sequences are identical, with the exception of a single amino acid deletion and four amino acid substitutions in Rh30-like1 (Fig. 4). Rh30-like2 and Rh30-like3 differ only at the 3'-end due to a 73-base pair (bp) deletion in Rh30-like2. We also identified a partial Rh30-like4 sequence with a more varied CDS as well as two variants of Rhag lacking the 12 predicted transmembrane spanning domains typical of the other Rh family members.

These multiple Rh30-like and Rhag sequences are likely the result of polyploidization events that occurred in the lineage leading to modern ray-finned fishes (65). Salmonids have further undergone a recent (25–100 million years ago) tetraploidization event (1), resulting in duplicated genes. Indeed, multiple Wilms’ tumor suppressor (5) and glutamine synthetase (46) genes in trout are likely the result of both ancient and recent duplication events. It is possible, therefore, that additional (yet unidentified) Rh genes will be characterized in the future.

The cDNA sequences of Rhbg-a and Rhbg-b are identical with the exception of a 474-bp deletion in the 3'-UTR of Rhbg-b. Rhcgl-a and Rhcgl-b are also identical, except for a 299-bp deletion in the 3'-UTR of Rhcgl-b. Presumably the two Rhbg sequences are RNA splice variants from the same gene, and this is likely the case for the two Rhcgl sequences. Although we cannot speculate about the function of the Rhbg and Rhcgl variants, it is reasonable to conclude that they are differentially expressed given that 3'-UTRs are important for posttranscriptional regulation (43). Furthermore, preliminary results using primers designed specifically to Rhbg-a suggest that the Rhbg expression changes that we report here in the brain and skin (Figs. 7 and 9) likely reflect changes in Rhbg-b.

Both Rhbg and Rhcgl are very similar (>75%) to their counterparts in other fish species, but there is more variability between the Rh30-like sequences (Fig. 4). The Rh30-like sequences fall into a cluster of homologs related to the Rh30 proteins (Fig. 3) that were first described in humans (3). There is ~60% amino acid similarity between trout Rhbg/Rhcg and human RhBG/RhCG, but trout Rh30-like sequences are <31% similar to human Rh30 (not shown). This agrees well with Huang and Peng (20), who showed that Rh30 is more functionally divergent across species than the other Rh genes. Rh30 proteins do not directly transport ammonia, but their membrane expression depends on the presence of RhAG (60). In the present study, the mRNA expression of Rh30-like and Rhag followed the same pattern of downregulation in HEA-exposed RBCs (Fig. 12), suggesting that these proteins could also be coexpressed together on the trout RBC membrane. Whether Rhag is additionally expressed in pillar cells of the trout gill, as they are in the pufferfish (47), remains to be determined.

Exposure to 1.5 mmol/l NH₄HCO₃ for 48 h resulted in an initial net uptake of ammonia into the fish with subsequent...
recovery of excretion (Fig. 1). This is consistent with earlier investigations that reported the same phenomenon in both freshwater and seawater fish exposed to high (up to 1 mmol/l) external concentrations of NH4Cl or (NH4)2SO4 (6, 7, 8, 63). These studies hypothesized that an active NH4+/H+ extrusion mechanism is induced to counteract the inward diffusion of NH3, but active NH4+/H+ exchange has not yet been demonstrated in the freshwater teleost gill, leaving NH3 diffusion as the main route of excretion (54, 62).

The lipid solubility of NH3 is, however, only moderate at best (13, 67), and a protein channel would greatly facilitate NH3 passage even under routine conditions. This is particularly advantageous when potentially toxic concentrations of ammonia need to be transported out quickly. In HEA conditions, fish need to counter the influx of ammonia as well as eliminate endogenously produced ammonia. This became apparent at 48 h of HEA when plasma TAmm approached water TAmm (Fig. 2). Recovery of excretion after 12 h, and the increased JAm at 48 h (Fig. 1) was accompanied by the simultaneous upregulation of Rhcg2 at 12 and 48 h (Fig. 8). This suggests that Rhcg2 is upregulated in response to HEA, resulting in enhanced ammonia excretion.

Although Rhcg2 was upregulated during HEA, all three Rh mRNAs (Rhbg, Rhcg1, and Rhcg2) were expressed in the whole gill and gill cell fractions during control and HEA conditions (Figs. 8 and 11). Taken together, this suggests that while both cell types may be involved in routine ammonia transport via Rh glycoproteins during control and HEA conditions, pavement cells respond to HEA with an upregulation of Rhcg2, restoring excretion and elevating rates above basal levels. Rhbg may be involved in the maintenance or further regulation of this process. These ideas still need to be validated with trout-specific Rh protein studies, which will reveal whether changes in Rh protein levels or subcellular distribution occur as they do in rat renal cells during metabolic acidosis (55).

In the gill of the pufferfish, localization of Rh mRNAs and proteins suggests that at least under basal conditions, Rhcg1 is expressed apically and is confined to the MR cells while Rhcg2 and Rhbg are restricted to pavement cells in the apical and basolateral regions, respectively (47). In the gills of the rainbow trout, on the other hand, our findings show that the trout Rh mRNAs are expressed in both the MR and pavement cells under control conditions but respond differentially under HEA: Rhbg and Rhcg2 expression increases in pavement cells only, while Rhcg1 expression does not change in pavement cells but tends to fall in MR cells. Whether this discrepancy in findings is due to species differences and whether or not the same polarized localization of Rh glycoproteins occurs in the trout gill await further investigation.

Previous studies demonstrated that expired water passing over the gills of freshwater fish is acidified by up to 1.5 pH units, depending on initial water pH and buffer capacity (30, 49, 69). This acidified gill boundary layer traps excreted NH3 to NH4+ (51, 64), but whether the acidification is due to H+...
arising from CO2 hydration or is a result of H+ extruded by H+-ATPase is unknown (62). In our study, an increase in H+-ATPase expression and activity (Fig. 13, A and D) accompanied the Rhcg2 upregulation and recovery of ammonia excretion during HEA, suggesting that H+-ATPase is responsible for the acidification. Moreover, this upregulation of H+-ATPase occurred in the pavement cells (Fig. 14), indicating a possible functional coupling with Rhcg2, which has also been hypothesized to occur in the intercalated cells of the rat nephron (11).

The CA-catalyzed hydration of CO2 normally fuels the H+-ATPase with H+ in the trout gill (31), so we expected to see an increase in CA expression and/or activity in conjunction with the upregulated H+-ATPase in HEA conditions. Instead, CA expression and activity were significantly decreased at 48 h (Fig. 13, B and E), a response that again appeared specific to the pavement cells (Fig. 14), in agreement with one study that reported inhibition of CA activity by HEA in trout (2). Downregulation or lowered CA activity suggests that CO2 is no longer the main source of H+ for the H+-ATPase pump. Under ammonia-loaded conditions, NH4+ may be the major supplier of H+. This correlates with the present model of the ammonium transporter (AmtB) of E. coli, which is structurally and functionally similar to Rh proteins (9, 38, 72). AmtB is a trimeric membrane-spanning complex (10, 24, 71) functioning as an NH3 channel. The NH4+ is deprotonated before entering the channel so that it can pass through as NH3 and reprotonate after exiting (24, 25). Under HEA, a continuous and abundant supply of H+ stripped off from NH4+ entering the Rh channel may be fuelling the H+-ATPase (Fig. 15), making elevated CA activity unnecessary.

Alternative routes of ammonia transport in the gill are via the NHE and Na+/K+-ATPase. So far, only the giant mudskipper, Periophthalmodon schlosseri, is documented to actively excrete NH4+ by a basolateral Na+/K+-ATPase and an apical NHE during HEA (50). The lack of evidence for ammonia transport via Na+/K+-ATPase in the freshwater fish gill may be related to higher Na+/K+-ATPase activity levels in seawater fish (62); however, not all seawater species display high enzyme activity (42). In our study, HEA did not result in changes in Na+/K+-ATPase at the transcript (Fig. 13C) or activity level (Fig. 13F) in whole gill samples. Analysis of mRNA expression in the gill cell fractions, however, revealed upregulation in the pavement cells and downregulation in the MR cells (Fig. 14). These opposing cell-specific differences may explain the absence of change in expression and activity in the whole gill. Therefore, we cannot rule out the potential for basolateral NH4+ transport via this route until more cell-specific studies are performed. No changes in NHE2 mRNA expression were seen in whole gill or the gill cell fractions (Fig. 14), so a role for this transporter during HEA is not apparent. NH4+ transport through the gill remains a possibility, but a mechanism has yet to be characterized.

Results from this study revealed a wide tissue expression of Rh mRNAs with a noted downregulation of Rhbg and Rhcg1 occurring in the brain with HEA exposure (Figs. 7 and 10). Since trout brain cells have only a limited capacity to detoxify ammonia (45), we could hypothesize that this downregulation of Rh was a protective response to prevent ammonia overloading into the cells. Downregulation of Rhbg did not occur in the mangrove killifish brain in response to similar HEA conditions (21), and this may reflect species differences: the mangrove killifish is very ammonia tolerant (14) compared with the trout (45). Interestingly, Rh glycoproteins were not detected in the pufferfish brain (47).
During HEA, Rhbg and Rhcg2 were upregulated in the skin (Fig. 9). This was surprising as the skin under normal conditions contributes very little to total ammonia excretion in freshwater teleosts compared with seawater species (67). Up-regulation of Rhcg1 and Rhcg2 was also observed in the mangrove killfish skin exposed to HEA or air (21). This species, however, is known to volatilize ammonia via the skin during air exposure (33). Whether the trout skin also assumes an excretory role during HEA is not clear, but Rh proteins appear to play some role in this tissue during HEA.

An important controversy regarding the function of Rh proteins is whether or not they transport CO2. Chlamydomonas reinhardtii is a green alga with Amh and Rh (Rh1) genes. When Rh1 is inhibited, ammonium uptake is unaffected but the growth increase that normally occurs in high CO2 is impaired. Elevating the CO2 content of the growth medium increases Rh1 expression (28, 56). Also, RBCs lacking RhAG (Rhnull) have reduced CO2 permeability (12), and RhCG-expressing oocytes have increased CO2 permeability (4). Contrary to this, however, Ripoche et al. (53) reported no change in CO2 permeability in Rhnull ghost cell preparations. Future studies should include investigations into the potential CO2 transport via Rh proteins.

In summary, we identified seven full-length Rh cDNA sequences in the rainbow trout and evaluated the gene expression of these as well as Rhcg2 at the transcript level in the rainbow trout challenged with HEA. Rh mRNAs were expressed in many tissues, and changes in expression upon HEA exposure were noted in some. In the gill, the main site of ammonia excretion, Rhcg2 expression as well as $H^+$/ATPase expression and activity were upregulated during HEA and this coincided with the net recovery of ammonia excretion. CA expression and activity were significantly downregulated at 48 h of HEA, suggesting that CA is no longer fuelling the $H^+$/ATPase pump at this point. Furthermore, pavement cells and not MR cells appear to be instrumental in the regulation of ammonia transport during HEA since clear expression changes in Rh and enzymes were occurring in this cell type only. The contribution of Rh glycoproteins and $H^+$/ATPase to enhanced ammonia excretion across the gill during HEA has been highlighted, adding to our current knowledge and understanding of branchial ammonia transport in the freshwater rainbow trout.

ACKNOWLEDGMENTS

We thank Richard Smith, Paul Craig, and Grant McClelland for logistic support and three anonymous reviewers for constructive criticisms.

GRANTS

Funded by the Natural Sciences and Engineering Research Council of Canada Discovery Grants and Canada Foundation for Innovation/Ontario Innovation Trust equipment awards to C. M. Wood and P. A. Wright. C. M. Wood is supported by the Canada Research Chair Program.

REFERENCES


Physiol Genomics • VOL 31 • www.physiolgenomics.org


