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Strategies for maintaining Na^+ balance in zebrafish (*Danio rerio*) during prolonged exposure to acidic water

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ABSTRACT

The objective of the present study was to characterize the capacity of zebrafish (*Danio rerio*) to regulate whole body Na^+ levels during exposure to acidic (pH 3.8–4.0) water. Exposure to acidic water significantly affected the mRNA levels of 14 claudin and two occludin isoforms, tight junction proteins thought to be involved in regulating paracellular efflux. Despite these changes, Na^+ efflux as well as uptake of polyethylene glycol (PEG), a marker for paracellular pathway, was persistently elevated during the 2-week period of acid exposure, although there was a transient recovery between 12- and 72-h. Pre-exposing fish to acidic water for 2 weeks failed to attenuate the increase in Na^+ efflux associated with acute exposure to acidic water of low [Ca^{2+}]. However, during recovery in water of circumneutral pH following exposure to acidic water, normal rates of Na^+ efflux were restored within 5 h. The rate of Na^+ uptake was significantly elevated between 4 and 7 days of exposure to acidic water; the increase was associated with significant increases in maximal Na^+ uptake capacity ($J_{MAX}Na^{+}$) and affinity constant (K_M). These results demonstrate that in acidic water, zebrafish maintain their whole body Na^+ balance primarily by regulating Na^+ uptake, rather than Na^+ efflux.

1. Introduction

Living in a hypoionic medium, freshwater (FW) teleost fish are faced with the challenge of maintaining their body fluids hyperionic to the environment. Ion homeostasis is defended by actively absorbing salts from the environment and by regulating the obligatory, diffusive loss of ions across the gill. Previous studies have focused largely on the regulation of active ionic uptake (for recent reviews see Evans et al., 2005; Marshall and Grosell, 2006; Hwang and Lee, 2007; Evans, 2008; Hwang, 2009; Hwang and Perry, 2010), owing in part to a limited understanding of the molecular mechanisms controlling the transepithelial efflux of ions. Theoretically, efflux could occur either via transcellular or paracellular pathways. Although the relative contributions of transcellular and paracellular Na^+ efflux to the total Na^+ efflux is still under debate (Wood et al., 2009), it has been theorized that in some environmental conditions such as acidic water, paracellular Na^+ efflux becomes dominant (McDonald et al., 1983 and see references therein).

Tight junctions (TJs), distributed to the apical regions of epithelial and endothelial cells of vertebrates, are the primary regulators of paracellular flux (Tsukita et al., 2001; Schneeberger and Lynch, 2004). Claudins (and to a lesser extent occludins), transmembrane proteins associated with TJs, are considered to be primarily responsible for determining the permeability of a given TJ (Saitou et al., 2000). For example, over-expression of claudin-2 in Madin–Darby Canine Kidney (MDCK) cells increased the permeability to cations (Furuse et al., 2001; Amasheh et al., 2002) whereas over-expression of claudin-4 in MDCK cells reduced cation permeability (Van Itallie et al., 2001). Subsequently, isoforms such as claudins-8 (Yu, 2003), -11 (van Itallie et al., 2003) and -14 (Ben-Yosef et al., 2003) have been shown to increase the "tightness" of membranes to cations whereas claudin-7 (Alexandre et al., 2005) and -15 (van Itallie et al., 2003) have been shown to reduce permeability to cations or increase permeability to anions (for a general review see van Itallie and Anderson, 2006). These charge-selective properties of specific claudin isoforms are responsible for maintaining steep ion concentration gradients across epithelia in various vertebrate transporting tissues, such as distal colon, where claudin 8 defends a steep concentration gradient of Na^+ (Amasheh et al., 2009).

Research on the roles of claudins and occludins in teleosts was triggered by the identification of 56 claudin isoforms in *Fugu rubripes* genome (Loh et al., 2004). Unlike in zebrafish, *Danio rerio*, where the emphasis of research on TJ proteins has been embryo (Siddiqui et al., 2010) or organ development (Hardison et al., 2005; Bagnat et al., 2007), research on TJ proteins in other teleosts has largely focused on their role in osmoregulation. Both the transcriptional and translational levels of claudin isoforms or occludin have been shown to be affected by external salinity in goldfish, *Carassius auratus* (Chasiotis et al., 2009), pufferfish,

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Tetraodon nigroviridis (Bagherie-Lachidan et al., 2008, 2009; Clelland et al., 2010), tilapia *Oreochromis mossambicus* (Tipsmark et al., 2008a) Atlantic salmon, *Salmo salar* (Tipsmark et al., 2008b) and southern flounder, *Paralichthys lethostigma* (Tipsmark et al., 2008c). Further supporting a role of TJ proteins in osmoregulation, a recent *in vitro* study using rainbow trout (*Oncorhynchus mykiss*) gill cells in culture, demonstrated that prolactin, a hormone thought to play a role in the adaptation of euryhaline fish to FW, induced occludin mRNA expression and reduced paracellular permeability (Chasiotis et al., 2010). Similarly, treatment of *T. nigroviridis* gill epithelial cell culture with cortisol, a hormone implicated in the adaptation to seawater (SW), resulted in alterations of transcript levels of some claudin isoforms, though in this case permeability was not measured (Bui et al., 2010). Overall, it seems likely that fish would be capable of regulating their gill epithelial permeability depending on the environment. Thus, the objective of the present study was to characterize the response of zebrafish (*Danio rerio*) to a severe reduction of ambient pH, an environmental condition previously shown to affect branchial permeability. Specifically, exposure to acidic conditions is known to induce a significant increase in Na^+ efflux in variety of FW fishes (McDonald et al., 1983; Freda and McDonald, 1988) which generally has been attributed to a disruption of TJ proteins. In support of this interpretation, Meyer et al. (2010) showed that increasing Na^+ loss in aquatic larval Eastern dwarf tree frog (*Litoria fallax*) exposed to acidic water was associated with a significant reduction in the depth of gill epithelial TJs. Thus, a first objective of the present study was to assess the effect of acidic water on the transcriptional expression of TJ proteins. To do so, we assessed the mRNA expression levels using real time PCR of 18 TJ proteins in adult zebrafish gill over a two-week period of exposure to acidic water (3.8–4.0). A second objective was to directly assess the effects of acidic water on Na^+ efflux and to compare with its effects on non-ionic paracellular permeability (as assessed by measuring the whole body uptake of PEG 400). To more fully understand how zebrafish respond to a disturbance in Na^+ homeostasis caused by acid exposure, whole body Na^+ levels and Na^+ uptake were also measured. Results from this study suggest that in response to acidic water exposure, zebrafish are able to regulate whole body Na^+ balance in the face of prolonged and uncompensated Na^+ loss, primarily by increasing Na^+ uptake. While complex changes in TJ mRNA levels were observed, they apparently have no long term impact on rates of Na^+ efflux.

2. Materials and methods

2.1. Animal care and experimental treatment

Adult zebrafish (*Danio rerio* Hamilton-Buchanan 1822) were purchased from Big Al's Aquarium Services (Ottawa ON, Canada) and kept in the University of Ottawa Aquatic Care Facility where they were maintained in plastic tanks supplied with aerated, dechloraminated City of Ottawa tap water at 28 °C (mean weight = 0.44 ± 0.008 (SEM) g; N = 665). Fish were subjected to a constant 14 h L:10 h D photoperiod and fed daily with No.1 crumble-Zeigler™ (Aquatic Habitats, Apopka, FL, USA) until satiation. To prepare acidic water, H_2SO_4 was added to the City of Ottawa tap water to lower pH to the desired level of 3.8–4.0. Experimental tanks were placed in a temperature-controlled environment to maintain water temperature at 28–28.5 °C. During the two-week period of acid water treatment, approximately 1/3 of the water was changed daily to prevent accumulation of nitrogenous waste and to maintain the desired water pH. Water pH was kept within 3.80–4.10 (mean water pH = 3.93). The experiments were conducted in compliance with guidelines of the Canadian Council of Animal Care (CCAC) and after the approval of the University of Ottawa Animal Care Committee (Protocol BL-226).

2.2. Experimental protocols

To investigate the effect of exposure of zebrafish to acidic conditions on Na^+ homeostasis, the following eight series of experiments were performed:

Series 1 Effect of acid exposure on whole body Na^+ content.
For this series, the same fish used in series 3 (unidirectional Na^+ efflux) were used to calculate the whole body Na^+ content. Following the measurement of unidirectional Na^+ efflux as described in series 3, fish were pulverized in liquid nitrogen. Half of the pulverized samples was placed into a pre-weighed vial, weighed, and digested with 7% perchloric acid overnight at 65 °C. Once digestion was complete, samples were supplemented with deionized water to a total volume of 20 ml and Na^+ concentration was measured by flame emission spectrophotometry (model AA260, Varian, Palo Alto, CA, USA) to calculate the whole body Na^+ content. This information was used to calculate the internal Na^+ specific activity for series 3.

Series 2 mRNA expression profiles of claudin and occludin isoforms.
Tissues (brain, liver, gut, heart, kidney, eye, gill and muscle) were dissected from adult zebrafish and snap frozen in liquid N_2 to assess the tissue distribution patterns of 18 TJ proteins (16 claudins and 2 occludins), which included all claudin/occludin isoforms listed in ZFIN when this study was conceived. We followed the nomenclature published on ZFIN (http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg). Concurrently, fish were subjected to a 2-week exposure to acidic or regular aquarium water (controls). After 1, 3, 6, 12, 24, 48, 72, 168 and 336 h of exposure, fish were killed with an overdose of ethyl 3-aminobenzoate methanesulfonate (MS-222; N = 6 at each time point) and their gills were dissected and snap-frozen in liquid N_2 for later RNA extraction for real time PCR.

2.2.1. RNA extraction and tissue distribution of TJ proteins

Total RNA was extracted from the tissue using Trizol reagent (Invitrogen) following the manufacturer's instructions. After quantifying the extracted RNA using a spectrophotometer (model ND-1000, NanoDrop, Wilmington, DE, USA) with NanoDrop ND-1000 software (version 3.3.0), cDNA was synthesized by treating the 2 μg of extracted RNA with DNase (Invitrogen) and RevertAid TM M-MNuLV reverse transcriptase (Fermentas, Burlington, ON, Canada) according to the manufacturer's instructions. To establish the tissue distribution patterns of TJ proteins, RT-PCR was performed with the primers listed in Table 1. Primers were designed using Primer3 web-based software (http://frodo.wi.mit.edu/primer3/) and all amplicons were sequenced to verify the products. Conditions of PCR were as follows: initial denaturation at 94 °C for 30 s followed by 40 cycles of 94 °C for 30 s, 58 °C for 60 s, and 72 °C for 45 s, with final extension for 5 min at 72 °C. PCR products were run on 2% agarose gels and images were acquired using GelDoc equipped with Quantity-One 1-D analyzer software (BioRad, Mississauga, ON, Canada).

2.2.2. Real time RT-PCR

Fish were killed and cDNA was synthesized from gill tissue as described earlier. Real time PCR was performed using a MX3000P qPCR system with analysis completed on MXPro 4.0 (Stratagene). Each reaction consisted of 4.06-5.06 μl of sterile water, 6.3 μl of Brilliant II SYBR Green Master Mix (Stratagene), 0.25–0.75 μl of both forward and reverse primers (Table 1) and 0.19 μl of diluted reference dye. PCR conditions were identical to those described earlier. cDNA templates were added to the final volume of 12.5 μl. 18S ribosomal