Comparative Biochemistry and Physiology, Part C 171 (2015) 1–7

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

A novel variant of aquaporin 3 is expressed in killifish (*Fundulus heteroclitus*) intestine

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ARTICLE INFO

Article history:
Received 7 December 2014
Received in revised form 1 March 2015
Accepted 3 March 2015
Available online 9 March 2015

Keywords:
Fundulus heteroclitus
Aquaglyceroporin
Arsenite uptake
Intestine
Salinity

ABSTRACT

Killifish (*Fundulus heteroclitus*) are euryhaline teleosts that are widely used in environmental and toxicological studies, and they are tolerant to arsenic, in part due to very low assimilation of arsenic from the environment. The mechanism of arsenic uptake by the intestine, a major route of arsenic uptake in humans is unknown. Thus, the goal of this study was to determine if aquaglyceroporins (AQPs), which transport water and other small molecules including arsenite across cell membranes, are expressed in the killifish intestine, and whether AQP expression is affected by osmotic stress. Through RT-PCR and sequence analysis of PCR amplicons, we demonstrated that the intestine expresses kfAQP3a and kfAQP3b, two previously identified variants, and also identified a novel variant of killifish AQP3 (kfAQP3c) in the intestine. The variants likely represent alternate splice forms. A BLAST search of the *F. heteroclitus* reference genome revealed that the AQP3 gene resides on a single locus, while an alignment of the AQP3 sequence among 384 individuals from eight population ranging from Rhode Island to North Carolina revealed that its coding sequence was remarkably conserved with no fixed polymorphism residing in the region that distinguishes these variants. We further demonstrate that the novel variant transports arsenite into HEK293T cells. Whereas kfAQP3a, which does not transport arsenite, was expressed in both freshwater (FW) and saltwater (SW) acclimated fish, kfAQP3b, an arsenic transporter, was expressed only in FW acclimated fish, and kfAQP3c was expressed only in SW acclimated fish. Thus, we have identified a novel, putative splice variant of kfAQP3, kfAQP3c, which transports arsenic and is expressed only in SW acclimated fish.

1. Introduction

Arsenic is a naturally occurring metalloid that is the number one substance of concern in the priority list of hazardous substances by the Agency for Toxic Substances and Disease Registry (ATSDR, 2011). Globally, at least 500 million people are exposed to arsenic in their drinking water. In addition, recent studies have indicated that rice-based food products are a major source of arsenic exposure (Jackson et al., 2012). Chronic exposure to arsenic leads to a number of diseases including cancer of the skin, bladder, kidney and liver; diabetes; cardiovascular and lung diseases (Abernathy et al., 2003; Hughes et al., 2011). In teleosts, exposure to sublethal concentrations of arsenic has been linked to reduced growth, attenuated stress response, and increased oxidative stress (Eisler, 1988; Bears et al., 2006; Ventura-Lima et al., 2009; Erickson et al., 2010).

The Atlantic killifish (*Fundulus heteroclitus*) is an estuarine fish that is widely distributed throughout the coast of the eastern United States. Because killifish readily acclimate to sudden and extreme changes in salinity, this organism has been extensively used to study osmoregulation in teleosts (Wood and Marshall, 1994; Burnett et al., 2007). Interestingly, although high levels of arsenic interfere with osmoregulation in killifish (Shaw et al., 2007a), studies have shown that killifish are highly tolerant to waterborne arsenic exposure. Notably, killifish are able to survive exposure to very high concentrations of arsenic (Shaw et al., 2007b), and accumulation of arsenic from water is dramatically lower compared to accumulation of other metals (Dutton and Fisher, 2011b, 2014). Although arsenic accumulation is greater in fish acclimated to

Abbreviations: AQP, aquaglyceroporin; FW, freshwater; SW, seawater; kf, killifish; As, arsenic; qRT-PCR, quantitative real time polymerase chain reaction.

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http://dx.doi.org/10.1016/j.cbpc.2015.03.001
1532-0456/

seawater (SW) compared to accumulation by fish acclimated to freshwater (FW) (Dutton and Fisher, 2011b) the reason for this is unknown, as is the reason why overall accumulation of arsenic is low in killifish.

Arsenic uptake into cells is achieved by two major routes. Transport of organic and inorganic arsenate (As^{5+}) occurs by sodium/phosphate cotransporter (Huang and Lee, 1996; Ballatori, 2002). Transport of organic and inorganic arsenite (As^{3+}) into cells is primarily mediated by aquaglyceroporins (AQPs), which also transport water, urea and glycerol (Lee et al., 2006; Bhattacharjee et al., 2008). AQP3, AQP7, AQP9, and AQP10 have been identified in teleosts (Cerdà and Finn, 2010) and have been shown to play an important role in cellular arsenic uptake in zebrafish (Hamdi et al., 2009). The expression of kfAQP3 in the gill is salinity dependent reflecting its role in maintaining osmotic balance (Whitehead et al., 2012; Shaw et al., 2014). In a previous study we identified a novel AQP3 expressed in killifish gill (kfAQP3a), which is expressed in FW acclimated fish and does not transport arsenic (Jung et al., 2012a). In fact, kfAQP3a is the only known AQP3 that does not transport arsenic. Because kfAQP3a was the only aquaporin identified in killifish gill, this observation supported the conclusion that the gill is not a route of arsenite assimilation in the killifish (Jung et al., 2012a).

In the present study we conducted experiments to identify AQP transporters in killifish intestine since it is well known that arsenic uptake by the intestine is a major pathway for arsenic assimilation in several species including humans (WHO, 2001; Calatayud et al., 2011; Dutton and Fisher, 2011a; Erickson et al., 2011). We hypothesized that arsenite uptake in killifish intestine is mediated in part by AQP3, and that transfer from FW to SW may affect AQP3 expression. To these ends we used quantitative real-time PCR (qRT-PCR) and Western blot to identify APQs in killifish intestine, and a functional assay to examine arsenite transport. We found two variants of killifish AQP3 that we previously identified (kfAQP3a, kfAQP3b) as well as a novel variant, kfAQP3c, which we demonstrate is likely a splice variant and an arsenic transporter. We further demonstrate that the expression of the kfAQP3 arsenic transporters 3b and 3c, is salinity dependent with kfAQP3b expressed only in FW fish and kfAQP3c only expressed in SW fish.

2. Materials and methods

2.1. Fish care

Fish were caught in Northeast Creek, Salisbury Cove, ME and transferred to Mount Desert Island Biological Laboratory (MDIBL) or the Geisel School of Medicine at Dartmouth (GSM). Fish were either kept in 100% seawater (SW, natural seawater at MDIBL and artificial seawater (Aquarium Systems Inc., Mentor, OH) at GSM) or kept in 10% seawater for 2 weeks, then moved to freshwater (FW, 100 μM $NaHCO_3$, 50 μM $CaSO_4$, 50 μM $MgSO_4$, 20 μM KCl) (ASTM, 1985). Results from fish studied at MDIBL and GSM were identical, thus, the data were pooled. Fish were acclimated in each salinity condition for at least 1 month before experimental use. Fish were fed commercial flake food (Tetracichlid, Tetra, Blacksburg, VA) that had minimal arsenic concentration, as reported previously (Shaw et al., 2010). Fish were kept under natural light (MDIBL) or 15:9 (light: dark) cycle (GSM). All fish care and experimental protocols were in accordance with the Institutional Animal Care and Use Guidelines of GSM (stan.ba.1) and MDIBL (13-01).

2.2. kfAQP3 sequence verification of amplicons

Sequence analysis of the amplicons derived by RT-PCR of AQP3 identified a variant of kfAQP3 (kfAQP3a) in killifish gill that differed from our transcriptome database (kfAQP3b) in the three C-terminal amino acids (Jung et al., 2012a; Shaw et al., 2014). To examine the expression of these AQP3 variants in killifish intestine, intestines were isolated from nine fish acclimated to FW and nine fish acclimated to SW and stored in RNA later (Ambion, Austin, TX) at −20 °C. Total RNA was isolated using Qiagen RNeasy Minikit (Valencia, CA) according to the manufacturer's protocol. The quality and quantity of the isolated RNA were measured using the Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE). Only samples that exhibited 18S and 28S peaks without significant degradation were further processed for cDNA synthesis. cDNA was synthesized using Ambion Reverse Transcription System (San Luis Obispo, CA) from 1 μg RNA. PCR analysis was performed with primers that span nucleotides that code for the three C-terminal amino acids of kfAQP3 (5′–3′: GGTTTCCATGTGGAAGGAGA, 3′–5′: TGCCCTCATGACTAGCCTTT). Specifically, the primers were designed to include a 150 base pair (bp) target sequence that spans the 38 terminal amino acids, the stop codon, and 33 bp 3′ to the stop codon. The conditions for PCR reaction were as follows: segment 1–1 cycle for 2 min at 95 °C, segment 2–35 cycles (45 s at 95 °C, 30 s at 58 °C, and 1 min at 72 °C), and segment 3–10 min at 72 °C. PCR products were run on 2.0% agarose gel to confirm the product of a single amplicon. Products were then cloned into PCR 4.0-TOPO vector (Invitrogen, Carlsbad, CA), and transformed into DH5α-T1 cells according to the manufacturer's protocol (Invitrogen). Transformation products were incubated overnight at 37 °C on LB plates containing 50 μg/mL ampicillin. At least twenty colonies from each plate (i.e., each individual fish from each condition) were picked, cultured overnight in LB medium containing 50 μg/mL ampicillin. Plasmids were isolated with QIAprep Miniprep kit (Qiagen), and sequenced using ABI Big Dye technology with the ABI Model 3100 Genetic Analyzer at the GSM Molecular Biology Core using M13 reverse primer. Chromatograms of each sequencing result were visually inspected and checked with MacVector software with Phred for base calling (MacVector Inc., Cary, NC), and any sequence data with high background noise was excluded from further analysis.

2.3. Arsenic transport assay

Arsenic transport by kfAQP3 was measured in HEK293T cells transfected with kfAQP3a, kfAQP3b, or kfAQP3c. Vectors containing full-length kfAQP3a, 3b, and 3c were constructed as described previously. Briefly, full length AQP3a was cloned into TopoTA pCR2.1 vector (Invitrogen, from the gill of killifish acclimated to SW and sequence was verified by Sanger sequencing (Jung et al., 2012a). Next, the 3′-terminus of the cloned kfAQP3a was mutated using the forward primer (5′-CTCCAAATCTCACCAGCC-3′) and reverse primer (5′-CCTT TCTGCGCCTCTTTTTTAGCAGTTAGCCTCTTTGCCGTTGG-3′) to match kfAQP3b, and forward primer (5′-CCAACGGCAAAGAGGGTAATTGCT AAAAAAGAGGCGCAGAA-3′) and reverse primer (5′-TTCTGCGCCTCT TTTTTAGCAATTACCCTCTTTGCCGTTGG-3′) to match kfAQP3c, respectively. Mutated clones were sequenced for verification by Sanger sequencing. Once verified, full length cDNAs were cut from the vectors and inserted into pcDNA3.1(−) expression vectors (Invitrogen) by standard molecular cloning techniques.

HEK293T cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 μg/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine in a 5% CO_2–95% air incubator at 37 °C, and transfected with 1.0 μg of empty vector, kfAQP3a, or kfAQP3c in pcDNA3.1(−) using Effectene (Qiagen) as described previously ($n = 3$ per treatment) (Jung et al., 2012a). Efficiency of transfection was validated by checking the expression of AQP3 mRNA with qRT-PCR using primers and conditions described in Section 2.6. For each experimental treatment, 200,000 cells were plated in each well of a 6-well plate. At 48 h after transfection, cells were cultured in serum-free media containing 2 μg/L (2ppb) sodium arsenite. After 1 h, cells were washed twice with PBS, detached from the plates, transferred into preweighed centrifuge tubes, and pelleted by centrifugation (600 ×g for 10 min). Tubes were weighed again to calculate the mass of the cells. The concentration of arsenic and treatment time was chosen after preliminary experiments identified the conditions where arsenic uptake was linear and was dependent on the expression of kfAQP3b or kfAQP3c. Cells were resuspended in 100 μL of optima grade HNO_3