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Cortisol regulates Na^+ uptake in zebrafish, *Danio rerio*, larvae via the glucocorticoid receptor

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ABSTRACT

Unlike other freshwater fish previously examined, zebrafish are capable of increasing their rate of Na^+ uptake during chronic exposure to acidic water (pH 4). In the present study, the potential role of cortisol in the induction of Na^+ uptake during acid-exposure was investigated. When zebrafish larvae (4 days post-fertilization) were treated with waterborne cortisol, the rate of Na^+ uptake was significantly increased; this effect was blocked by co-incubating larvae with RU-486, an antagonist selective for the glucocorticoid receptor (GR). A similar induction in Na^+ uptake, which was also blocked by RU-486, was observed when larvae were treated with dexamethasone, a selective GR agonist. Conversely, treating larvae with aldosterone, a selective agonist for the mineralocorticoid receptor (MR) had no effect on Na^+ uptake. Acid-exposure increased whole body cortisol levels and translational knockdown of GR using antisense morpholinos prevented the full induction of Na^+ uptake during exposure to acidic water, further confirming the role of cortisol and GR in Na^+ uptake stimulation. Using immunohistochemistry, GR was localized to ionocytes known to be responsible for Na^+ uptake (HR-cells). Knockdown of Rhcg1, an apical membrane ammonia channel or Na^+/H^+ exchanger 3b (NHE3b), proteins known to play an important role in facilitating Na^+ uptake in acidic water, prevented the stimulatory effects of cortisol treatment on Na^+ uptake, suggesting that cortisol regulates Na^+ uptake by stimulating an Rhcg1–NHE3b "functional metabolon".

1. Introduction

Cortisol, through its glucocorticoid and mineralocorticoid actions, plays an important role in regulating a wide range of physiological processes in fish including metabolism, immune responses, growth, reproduction and osmoregulation (Mommsen et al., 1999; Wendelaar Bonga, 1997). Although initially recognized as a key osmoregulatory hormone promoting acclimation to seawater (SW) in euryhaline teleosts (Hwang and Wu, 1993; see reviews by McCormick, 2001; McCormick and Bradshaw, 2006), there is increasing acknowledgement of its involvement in freshwater (FW) fish (Evans et al., 2005). For example, cortisol treatment was shown to stimulate Na^+, Cl^- and Ca^{2+} uptake (Laurent and Perry, 1990; Lin et al., 2011; Perry et al., 1992) while increasing the functional surface area of chloride cells (Bindon et al., 1994a,b) (more recently referred to as ionocytes), specialized subtypes of branchial epithelial cells responsible for ion absorption in FW fish (Hwang and Lee, 2007; Hwang, 2009; Hwang and Perry, 2010; Hwang et al., 2011; Perry, 1997). Moreover, exogenous cortisol stimulates, either at the enzyme activity or protein/mRNA expression levels, the molecular transporters thought to be mediating ionic uptake, including H^+-ATPase (Lin and Randall, 1993), Na^+/H^+ exchanger 2 (Ivanis et al., 2008b), epithelial Ca^{2+} channel (Lin et al., 2011; Shahsavarani and Perry, 2006) and plasma membrane Ca^{2+}-ATPase (Flik and Perry, 1989). In addition, cortisol can influence gill cell permeability in several species (Chasiotis et al., 2010; Kelly and Chasiotis, 2011; Wood et al., 2002a) by regulating the expression of several tight junction associated proteins (Bui et al., 2010; Chasiotis et al., 2010; Tipsmark et al., 2009), and potentially affect the rate of passive loss of ions to the environment.

Although there is clear evidence for a role of cortisol in modulating osmoregulation by FW fish, the signaling pathways through which these effects are exerted remain more controversial. Because aldosterone is lacking in fish (Colombo et al., 1972), it was traditionally assumed that cortisol acts through a single receptor which confers both "glucocorticoid" and "mineralocorticoid" functions in teleosts. With the discovery in rainbow trout of a mineralocorticoid receptor (MR) like gene (Colombe et al., 2000), as well as alternative hormones (e.g. 11-deoxycorticosteroid) that could potentially activate MR (Kiilerich et al., 2011; Sturm et al., 2005), a significantly more complicated mechanism is emerging for corticosteroid signaling in teleosts (for recent reviews see Bury and

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Sturm, 2007; Prunet et al., 2006). Sloman et al. (2001) demonstrated that treating rainbow trout with the presumed MR antagonist, spironolactone, prevented branchial chloride cell proliferation following their exposure to soft water, suggesting a role for the MR in osmoregulation. Similarly, Scott et al. (2005) demonstrated that spironolactone injections prevented the increase in gill mRNA expression levels of the α1a subunit of Na^+–K^+-ATPase in killifish, *Fundulus heteroclitus*, following FW transfer. However, GR also has been implicated in osmoregulation in FW fish. For example, in FW Atlantic salmon, *Salmo salar*, cortisol treatment is associated with increased mRNA expression of the α1a subunit of Na^+–K^+-ATPase, which is partially inhibited by RU-486 (Kiilerich et al., 2007; McCormick et al., 2008). Lin et al. (2011) demonstrated that knockdown of GR, but not MR, significantly reduced the whole body Ca^{2+} content of zebrafish larvae following transfer to a low Ca^{2+} environment. Using a pharmacological approach, Kelly and Chasiotis (2011) demonstrated a role for GR in influencing the expression levels of tight junction protein (occludin) and paracellular permeability in cultured trout gill cells. Clearly, the mechanism(s) by which cortisol affects various aspects of osmoregulation in FW fish is complex and likely dependent on environmental conditions and species.

Recently, it was demonstrated that zebrafish are able to increase their rate of Na^+ uptake when exposed to acidic water (pH 4), a response partially attributed to activation of a functional metabolon linking Na^+ uptake via NHE3b and ammonia excretion via Rhcg1, an apically distributed ammonia-conducting channel (Kumai et al., 2011; Kumai and Perry, 2011). Given that cortisol is known to (1) increase in zebrafish larvae at 3–4 days post fertilization (dpf) in response to external stressors such as handling and salinity challenge (Alderman and Bernier, 2009; Alsop and Vijayan, 2008), (2) stimulate ion uptake in FW fish (see above), (3) induce NHE3 expression or increase the surface activity of NHE3 in mammalian cells (Bobulescu et al., 2005; Donowitz et al., 2009; Kinsella et al., 1984) and rainbow trout kidney (Ivanis et al., 2008a), and (4) activate compensatory mechanisms facilitating acid-excretion in trout during metabolic acidosis (Gilmour et al., 2011), it was hypothesized that cortisol is partially responsible for inducing the rise in Na^+ uptake in acid-exposed zebrafish larvae.

Thus, the principal objective of the present study was to test the hypothesis that cortisol is responsible for increasing Na^+ uptake by zebrafish larvae, in particular under acidic conditions by stimulating NHE3b and if so, to determine whether the effect is mediated by GR or MR (or both). The results clearly demonstrated that increasing levels of cortisol stimulate Na^+ uptake in zebrafish larvae via the specific interaction of cortisol with GR. The results suggests a potentially widespread role of cortisol under a variety of stress-inducing environmental conditions, including low pH, in regulating Na^+ uptake by zebrafish.

2. Materials and methods

2.1. Experimental animals and husbandry

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. Fish were subjected to a constant 14 h L:10 h D photoperiod and fed daily until satiation with No. 1 crumble-Zeigler™ (Aquatic Habitats, Apopka, FL, USA). Embryos were collected following the standard method (Westerfield, 2000). Collected embryos were reared in 50 ml petri dishes supplemented with either dechloraminated City of Ottawa tap water (pH 7.3–7.5) or acidified water (water pH was lowered to 3.9–4.0 by adding H_2SO_4 to the Ottawa tap water) supplemented with 0.05% ethylene blue. The petri dishes were kept in incubators set at 28.5 °C. Dead embryos were removed and water was changed daily. As all experiments were performed on 4 dpf fish, they were not fed for the duration of the experiment. 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). Unless stated otherwise, all chemicals used for the experiments were purchased from Sigma.

2.2. Effects of cortisol on Na^+ uptake and whole body Na^+ content

To determine whether Na^+ uptake is affected by cortisol, hatched 2 dpf larvae were transferred and maintained in zebrafish medium containing 500 nM cortisol (hydrocortisone) for 2 days. A comparable, physiologically relevant dose of cortisol was successfully used to induce physiological responses in previous studies on *Xenopus laevis* tadpole (500 nM; Yao et al., 2008) and cell culture derived from rainbow trout gill (150–1500 nM; Chasiotis et al., 2010). Stock solutions were prepared by dissolving cortisol in dimethyl sulfoxide (DMSO); the concentration of DMSO never exceeded 0.1% in the final working solution. In all series of experiments, controls were performed in which DMSO, alone, was dissolved in water. To measure the rate of Na^+ uptake, 0.25 μCi ^{22}Na in the form of NaCl (Perkin Elmer, Woodbridge, ON, Canada) was added to each tube to a final activity of 0.15 μCi/ml. Water samples (50 μl) were collected at 5 min and 2 h after the addition of radioisotope. At the end of the 2 h flux period, larvae were killed with overdose of ethyl 3-aminobenzoate methanesulfonate (MS-222) and briefly washed in isotope-free water containing high levels of Na^+ (>200 mM) to remove any residual radioisotope attached to the surface of the fish. The remaining radioactive water in the tube was stored separately for later measurement of the total [Na^+]. For the processing of samples and calculation of influx rate, see "analytical methods and calculation" section below.

To measure the whole body Na^+ content, larvae were treated with waterborne cortisol as described above. When they reached 4 dpf, 10 larvae were pooled to yield one (1) sample for whole body ion content measurement. Larvae were killed with MS-222 overdose, briefly washed in ion-free water and digested in 1 N HNO_3 solution at 65 °C. The digest was then supplemented with ion-free water and the Na^+ concentration was measured using a flame emission spectrophotometer (model AA240, Varian). The ion content was expressed as nmol/larvae.

2.3. Effect of pharmacological manipulation of GR and MR on Na^+ uptake

To determine whether the effects of cortisol were being mediated by GR or MR (or both), 2 dpf larvae were administered the following treatments for 2 days; (1) 500 nM dexamethasone (a GR-selective agonist), (2) 500 nM dexamethasone combined with 1 μM RU-486 (a GR-selective antagonist), (3) 500 nM cortisol, (4) 500 nM cortisol combined with 1 μM RU-486, and (5) 500 nM aldosterone (Steraloids Inc., Newport, RI, USA). All chemicals were dissolved in DMSO and the rate of Na^+ uptake was measured at the end of the treatment using 4 dpf larvae.

To determine whether cortisol signaling was required to induce Na^+ uptake during exposure to acidic water, 2 dpf larvae were treated with either 1 μM RU-486 or 10 μM eplerenone (a selective MR antagonist; see Discussion for details) for 24 h in the control zebrafish medium. After the initial exposure to the drugs, larvae were transferred to acidified zebrafish medium supplemented with the same concentration of antagonists and kept there for another