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Cortisol and glucocorticoid receptor 2 regulate acid secretion in medaka (*Oryzias latipes*) larvae

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Abstract

Freshwater fish live in environments where pH levels fluctuate more than those in seawater. During acidic stress, the acid–base balance in these fish is regulated by ionocytes in the gills, which directly contact water and function as an external kidney. In ionocytes, apical acid secretion is largely mediated by H^+-ATPase and the sodium/hydrogen exchanger (NHE). Control of this system was previously proposed to depend on the hormone, cortisol, mostly based on studies of zebrafish, a stenohaline fish, which utilize H^+-ATPase as the main route for apical acid secretion. However, the role of cortisol is poorly understood in euryhaline fish species that preferentially use NHE as the main transporter. In the present study, we explored the role of cortisol in NHE-mediated acid secretion in medaka larvae. mRNA expression levels of transporters related to acid secretion and cortisol-synthesis enzyme were enhanced by acidic FW treatment (pH 4.5, 2 days) in medaka larvae. Moreover, exogenous cortisol treatment (25 mg/L, 2 days) resulted in upregulation of *nhe3* and *rhcg1* expression, as well as acid secretion in 7 dpf medaka larvae. In loss-of-function experiments, microinjection of glucocorticoid receptor (GR)2 morpholino (MO) caused reductions in *nhe3* and *rhcg1* expression and diminished acid secretion, but microinjection of mineralocorticoid receptor (MR) and GR1 MOs did not. Together, these results suggest a conserved action of cortisol and GR2 on fish body fluid acid–base regulation.

Introduction

The pH of environmental freshwater (FW) is readily affected by natural and anthropogenic ambient factors, and high acidity in FW has been shown to cause internal acidosis in fish

(Evans et al. 2005; Hwang et al. 2011). Plasma pCO_2 and bicarbonate are maintained at low levels in fish, and thus cannot provide sufficient buffering capacity to affect plasma pH. Hence, plasma pH must be modulated by secreting acid to the ambient water through the gills (Evans et al. 2005; Perry and Gilmour 2006). While the gills account for over 90% of acid–base regulation in adult fish, this function is mostly performed by the yolk skin in fish embryos, before the development of functioning gills is complete (Evans et al. 2005; Hwang and Perry 2010). At the cellular level, acid secretion from fish gills/skin is primarily accomplished by transport of H^+ across the apical membrane of ionocytes (Hwang et al. 2011).

Apical transport of H^+ is the first step of trans-epithelial acid secretion in the gill ionocytes of FW fish. In zebrafish, a group of ionocytes called H^+-ATPase-rich cells (HRCs) was found to secrete H^+ from the skin of intact larvae. The apical membrane of these HRCs contains both H^+-ATPase and Na^+/H^+ exchanger 3b (NHE3b). In zebrafish, H^+-ATPase is the main transporter in the H^+ secretion pathway (Lin et al. 2006; Horng et al. 2007), and NHE3b is thought to

be a minor player, since exposure to an acidic environment (pH 4.0) stimulates H^+-ATPase expression but suppresses NHE3b (Yan et al. 2007). However, NHE3 (not H^+-ATPase) appears to be the major transporter for apical H^+ secretion in many other species, including FW-acclimated tilapia (*Oreochromis mossambicus*), medaka (*Oryzias latipes*), and Osorezan dace (*Tribolodon hakonensis*) (Furukawa et al. 2011; Hirata et al. 2003; Inokuchi et al. 2009; Hsu et al. 2014; Wu et al. 2010; Lin et al. 2012). Additionally, apical NHE2 and H^+-ATPase were proposed to be the major transporters for acid secretion in the gills of FW rainbow trout; NHE2 is expressed in PNA^+ cells, which appear to perform base secretion, while H^+-ATPase is found in PNA^- cells that mediate acid secretion (Galvez et al. 2002; Ivanis et al. 2008). As such, the utilization of either H^+-ATPase and/or NHE as major apical transporters for acid secretion appears to be species-dependent. Besides apical H^+-ATPase and/or NHE, others acid secretion-related transporters are also involved in the acid secretion function in fish. In zebrafish, the extruded H^+ from H^+-ATPase and/or NHE and environmental HCO_3^- can be utilized by a membrane-bound form of CA15 at HRC and generates H_2O and CO_2. After that, CO_2 enters HRCs and then is catalyzed by cytosolic CA2-like to form H^+ and HCO_3^-. Finally, basolateral AE1b transports the cytosolic HCO_3^- out of the cell (Hwang and Chou 2013). In medaka, homologues of most these transporters have also been identified in acid secretion ionocytes and proposed to execute the same function (Hsu et al. 2014; Liu et al. 2016; Yan and Hwang 2019).

Cortisol is a major corticosteroid hormone known to participate in stress response and hydro-mineral balance in fish (Baker 2003; Nelson 2003; Evans et al. 2005; Lin and Hwang 2016; Lin et al. 2011, 2015, 2016). It has been widely reported that circulating cortisol levels are stimulated in fish under acidosis stress (Brown et al. 1986; Gilmour et al. 2011; Kumai et al. 2012; Warren et al. 2004; Wood et al. 1999), and some studies have further addressed the relationship between cortisol and acid secretion mechanisms in FW teleosts. Exogenous cortisol treatment could increase mRNA expression of branchial NHE2 and H^+-ATPase, but it did not affect branchial NHE3 or cytosolic carbonate anhydrase (CA) expression in rainbow trout (Al-Fifi 2006; Ivanis et al. 2008; Gilmour et al. 2011). Furthermore, the activity of branchial H^+-ATPase and CA activity in rainbow trout was reported to be upregulated by exogenous cortisol treatment (Gilmour et al. 2011; Lin and Randall 1993). In zebrafish, exogenous cortisol treatment stimulated the mRNA expression of H^+-ATPase, NHE3, two CA isoforms (CA2 and CA15) and Anion Exchanger 1b (AE1b), as well as upregulating acid secretion from the yolk sac of zebrafish larvae (Lin et al. 2015). Thus, it seems cortisol stimulates acid secretion in several types of fish. However, the known actions

of cortisol on acid regulation are still fragmentary and insufficient to provide a comprehensive model, particularly with regard to species that utilize apical NHE as the main transporter for acid secretion.

Fish lack the ability to synthesize mineralocorticoid (aldosterone), but in vitro studies showed that cortisol treatment could activate transcription of a glucocorticoid-responsive element reporter plasmid via glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (Baker 2003; Bury et al. 2003; Greenwood et al. 2003; Sturm et al. 2005). Thus, cortisol is thought to have both glucocorticoid and mineralocorticoid functions in fish. Nevertheless, cortisol was found to exert its positive effects on the expression of acid-secretion transporters and functional acid secretion through GR and not MR in zebrafish larvae (Lin et al. 2015). Besides this example in zebrafish, the roles of cortisol and its receptors (GR and MR) in regulating acid secretion are still unclear for most fish. Whether cortisol regulates acid secretion through GR in other fish species is an issue that still needs to be clarified.

Serial studies on zebrafish used various molecular physiological approaches to explore the regulation and mechanism of proton secretion during acclimation to an acidic FW (Yan and Hwang 2019). The knowledge obtained in zebrafish, a stenohaline FW species, could not directly be applied to aquaculture species, which are mostly euryhaline or marine species. Medaka, a euryhaline teleost, has several advantages including plenty genetic database and applicability of various molecular physiological approaches (Wu et al. 2010; Lin et al. 2012), as zebrafsh does. Medaka not only is an important species in pet fish industry, but also could be used as an alternative and more-efficient model species for other aquaculture species. In terms of acid secretion, medaka are similar to most other studied species, in that they execute apical acid secretion mainly through NHE3. Furthermore, a specific group of acid-secreting ionocytes, NHE3 cells, has been identified in medaka, and some acid-secretion-related transporters, i.e., NHE3, Rhcg1 and AE1b, are co-expressed in these NHE3 cells (Hsu et al. 2014). As such, medaka is a convenient and suitable model to test the hypothesis that cortisol and its receptors regulate the function of NHE-mediated acid secretion. Herein, we address the following questions: (1) Does medaka exposure to AFW (pH 4.5) stimulate mRNA expression of acid-secretion-related transporters (NHE3, AE1b and Rhcg1) and CYP11B, which encodes an enzyme for the final step of cortisol synthesis? (2) Is cortisol involved in the regulation of acid-secretion-related transporter expression and functional acid secretion in medaka larvae? (3) Do specific receptor(s), such as GR1, GR2 and/or MR, mediate this regulation? By providing answers to these questions, our study enhances understanding of how cortisol controls acid secretion in fish.

Materials and methods

Experimental animals

Japanese medaka (*Oryzias latipes*) were kept in local tap water at 28.5 °C under a 14:10-h light–dark photoperiod at the Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan. Experimental protocols were approved by the Academia Sinica Institutional Animal Care and Utilization Committee (approval no.: 17-12-1163).

Acclimation experiments

Local tap water (freshwater; FW, pH 7.0) and acidic FW (AFW, pH 4.5) were prepared for experiments to determine the effects of an acidic medium. The acidic medium was made by adding H_2SO_4 to FW, while the concentrations of other ions in AFW were the same as those in FW. Fertilized medaka eggs were incubated in FW until 5 days post fertilization (dpf), and then transferred to either FW or AFW for 2 days. Media were changed twice per day. The pH values of all experimental media were verified using a pH meter (MP225; Mettler-Toledo, Schwer-zenbach, Switzerland) and ion concentrations were determined with an atomic absorption spectrometer (U-2000; Hitachi, Tokyo, Japan).

Cortisol incubation experiments

For cortisol incubation experiments, the dosages were taken from previous studies (Kiilerich et al. 2007; Lin et al. 2011; Cruz et al. 2013a; Trayer et al. 2013). A cortisol (hydrocortisone, Sigma-Aldrich, St Louis, MO, USA) stock solution was prepared in dimethyl sulfoxide (DMSO), and the stock was diluted to the final working solution (25 mg/L) in local tap water. Medaka larvae (5 dpf) were incubated in the cortisol-containing media for 2 days and were sampled at 7 dpf for analysis. The incubation media were changed with new cortisol solution each day to maintain constant levels of cortisol. During incubation, neither significant mortalities nor abnormal behaviors were observed. Although the dosages of cortisol in the present study are higher than those used in one previous study (Kumai et al. 2012), similar dosages have been applied for experiments on cultured gills and fish larvae in many previous studies (Lin et al. 1999, 2011, 2015, 2016; Kiilerichet al. 2007; Cruz et al. 2013a).

Cortisol measurements

Seven dpf medaka larvae with FW, AFW, and exogenous cortisol treatment were anesthetized by MS-222 and then washed several times with 1X phosphate-buffered saline (PBS). Fifteen larvae were pooled into 1 vial as one sample. For the cortisol extraction, we referred to the method of Trayer et al. (2013). In brief, the samples were homogenized in 500 μL of ice-cold PBS. Next, diethyl ether (Merck, Darmstadt, Germany) was added to the homogenates and then the mixture was vortexed for 1 min. After that, the mixture was centrifuged at $2000 \times g$ at 4 °C for 10 min. The diethyl ether phase was collected and dried with the nitrogen flux at room temperature. Finally, the dried samples were re-suspended in Standard/Sample Diluent (R1) of General Cortisol ELISA Kit (RK00639, ABclonal, MA, USA), and cortisol was quantified according to the manufacturer's instructions of ELISA kit.

RNA extraction

After anesthetization with 0.03% MS222, appropriate numbers of medaka larvae were sampled and homogenized in 1 mL Trizol reagent (Invitrogen, Carlsbad, CA, USA), mixed with 0.2 mL chloroform, and then thoroughly shaken. Supernatants were obtained by centrifugation at 4 °C and $12{,}000 \times g$ for 30 min. The samples were then mixed with an equal volume of isopropanol. Pellets were precipitated by centrifugation at 4 °C and $12{,}000 \times g$ for 30 min, washed with 70% alcohol, and then stored at −20 °C until use.

Reverse-transcription polymerase chain reaction (RT-PCR) analysis

For complementary (c)DNA synthesis, 1–5 μg total RNA was reverse-transcribed in a final volume of 20 μL containing 0.5 mM dNTPs, 2.5 μM oligo $(dT)_{20}$, 250 ng random primers, 5 mM dithiothreitol, 40 units RNase inhibitor, and 200 units Superscript RT (Invitrogen) for 1 h at 50 °C, followed by incubation at 70 °C for 15 min.

Quantitative real-time PCR (qPCR)

A Light Cycler real-time PCR system (Roche, Penzberg, Germany) was used to perform qPCR in a final reaction volume of 10 μL, containing 5 μL 2× SYBR Green I Master Mix (Roche Applied System), 300 nM of the primer pairs, and 20–30 ng cDNA. A standard curve was used to verify gene expression was detected in the linear range, and *rpl7* was used as an internal control. The primer set for *cyp11b* qPCR was GAATGGAGATCCGACCGTCT (forward) and GGGGAAGAAACCTCCTCACA (reverse). The primer sets for *nhe3*, *rhcg1*, *ae1b* and *rpl7* qPCR were taken from previous studies (Lin et al. 2012; Hsu et al. 2014).

Morpholino oligonucleotide (MO) knockdown and rescue

The medaka MR MO (5′-ATTACGACATTTAGAGCT GTCAGGT-3′), GR1 MO, (5′-TGAGCTTCAGTTCCCCTT GATCCAT-3′) and GR2 MO (5′-ATCCTTGACATGGCT GGTTGCTGAT-3′) were designed as previously described (Trayer et al. 2013). A standard control MO (5′-CCTCTT ACCTCAGTTACAATTTATA-3′) was used as the control. The optimal concentration of each MO (MR, 4.6 ng/embryo; GR1, 3 ng/embryos; GR2, 1.5 ng/embryo) was that which caused maximal knockdown efficiency and minimal toxicity (Trayer et al. 2013). MOs were injected into embryos at the 1–2 cell stage using an IM-300 microinjector system (Narishige Scientific Instrument Laboratory, Tokyo, Japan). MO-injected embryos were sampled at 7 dpf for subsequent analyses.

Whole-mount immunocytochemistry

Seven dpf medaka larvae were fixed with 4% paraformaldehyde solution overnight. After being washed with PBS, the samples were incubated with 3% BSA for 2 h to block non-specific binding. Samples were then incubated overnight at 4 °C with α5 monoclonal antibody against avian NKA (Developmental Studies Hybridoma Bank, University of Iowa, Ames, IA, USA). After being washed with PBS for 20 min, samples were further incubated in Alexa Fluor 488 goat anti-mouse IgG antibodies (Molecular Probes; diluted 1:200 with PBS) for 2 h at room temperature. To count the ionocyte densities in medaka larvae, we the protocols from previous studies (Lin et al. 2012; Hsu et al. 2014). Images were acquired with an Axioplan 2 microscope (Zeiss, Oberkochen, Germany).

Scanning ion-selective electrode technique (SIET)

SIET was used to detect H^+ flux at the surface of medaka larvae. The method was performed as described previously (Wu et al. 2010). In brief, micropipettes with tip diameters of 3–4 µm were pulled by a Sutter P-97 Flaming Brown pipette puller (Sutter Instruments, San Rafael, CA, USA). Micropipettes were then baked at 120 °C overnight and coated with dimethyl chlorosilane (Sigma-Aldrich) for 30 min. To make an ion-selective microelectrode (probe), micropipettes were backfilled with a 1-cm column of electrolytes and frontloaded with a 20- to 30-µm column of liquid ion-exchange cocktail (Sigma-Aldrich). The following ionophore cocktail and electrolytes were used: H^+ ionophore I cocktail B (40 mM KH_2PO_4, 15 mM K_2HPO_4, pH 7). To calibrate the ion-selective probe, the Nernstian property of each microelectrode was measured by placing the microelectrode in a series of standard solutions (pH 6, 7, and 8 for

the H^+ probe). Linear regression was performed on plots of probe voltage output against $[H^+]$ values, yielding a Nernstian slope of 58.6 ± 0.8 ($n = 10$) for H^+.

Measurement of surface H^+ gradients in the skin

SIET was performed at room temperature (26–28 °C) in a small plastic recording chamber filled with 1 mL normal recording medium containing 0.5 mM NaCl, 0.2 mM $CaSO_4$, 0.2 mM $MgSO_4$, 0.16 mM KH_2PO_4, 0.16 mM K_2HPO_4, 300 µM MOPS, and 0.3 mg/L ethyl 3-aminobenzoate methanesulfonate (Tricaine, Sigma-Aldrich). The pH of the recording media was adjusted to 7.0 by addition of NaOH or HCl. Before measurement, an anesthetized larva was positioned in the center of the chamber with its lateral side in contact with the base of the chamber. After 3 min to allow for signal stabilization, the ion-selective probe was moved to the target position (10–20 µm away from the yolk sac membrane), and ionic activities were recorded for 10 s; the probe was then immediately moved away (~ 1 cm) to record the background for another 10 s. The average voltage (mV) from the 10 s serial recording was used to calculate the ionic concentration at the target and background sites. To calculate ionic gradients, the background concentration was subtracted from the concentration at the target site. $\Delta[H^+]$ therefore represents the measured H^+ gradient between the target (at the surface of larval skin) and background. Lin et al. (2006) previously showed that the measured ionic gradients vary with distance between the probe tip and the larval skin (Lin et al. 2006); therefore, the probe location was kept as consistent as possible between samples. The noise of the system was usually less than 10 µV and was neglected when calculating ionic gradients (the voltage difference for larvae was usually 1–10 mV).

Statistical analysis

Normal distributions were confirmed by the Anderson Darling Normality Test ($p < 0.05$). Data are presented as mean $\pm$ SD and were analyzed by Student's t test.

Results

Effects of acidic (AFW) treatment on gene expression, whole body cortisol levels, and proton secretion in 7 dpf medaka larvae

To explore the effects of AFW (pH 4.5) treatment on genes related to acid secretion and on proton secretion in medaka larvae, 5 dpf medaka larvae were exposed to FW (pH 7.0) or AFW for 2 d. Exposure to AFW resulted in significantly elevated expression of *nhe3*, *rhcg1*, *ae1b* and *cyp11b* and

cortisol level, as compared to the expression levels in fish exposed to FW (Fig. 1A, B). However, proton secretion was not changed in medaka larvae with AFW treatment (Fig. 1C).

Effects of exogenous cortisol treatment on whole body cortisol levels, ionocyte cell density, mRNA expression of genes related to acid secretion, and proton secretion in 7 dpf medaka larvae

Expression of *cyp11b* and whole body cortisol levels was dramatically upregulated in medaka larvae in the AFW condition (Fig. 1A, B). Therefore, we wanted to further investigate whether exogenous cortisol treatment causes changes in ionocyte cell density, expression of relevant genes, or proton secretion. To accomplish this goal, 5 dpf medaka larvae were treated with cortisol for 2 d and then sampled for analysis. The exogenous cortisol treatment was able to dominantly increase cortisol levels in medaka larvae (Fig. 2A). We
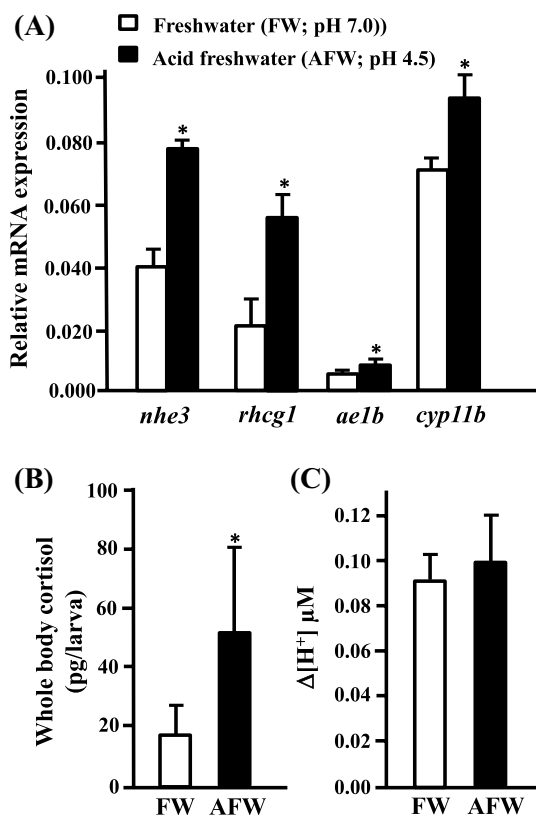

Fig. 1 AFW treatment increases expression of acid-secretion-related genes in medaka larvae. Effects of FW (pH 7.0) and acidic FW (AFW, pH 4.5) on **A** mRNA expression of indicated genes, **B** whole body cortisol levels and **C** proton secretion in 7-d post-fertilization (dpf) medaka larvae. The expression levels of *nhe3b, rhcg1, ae1b* and *cyp11b* mRNA were analyzed by qPCR, and the values were normalized to *rpl7*. SIET was used to measure the H$^+$ activity on the skin. Values are mean ± SD ($n = 6$–12). Student's t test, $*p < 0.05$
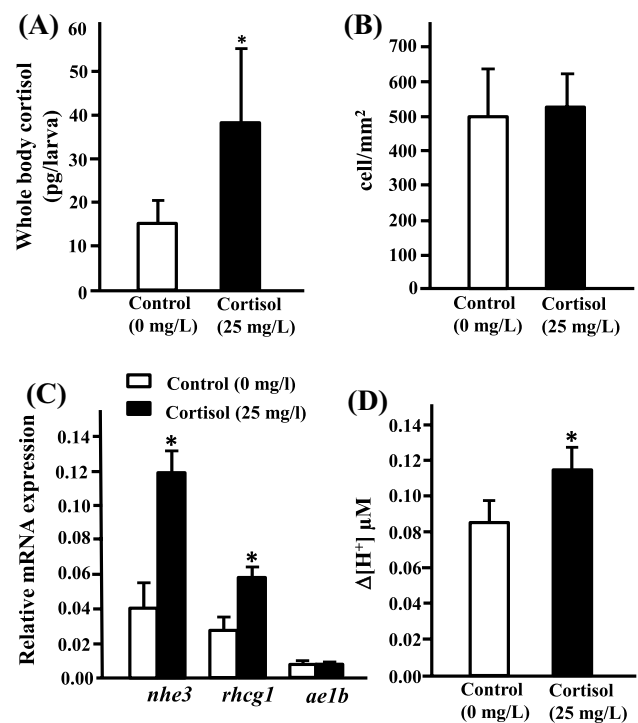

Fig. 2 Cortisol increases acid transporter expression and acid secretion in medaka larvae. Effects of exogenous cortisol (20 mg/L) on **A** whole body cortisol levels, **B** ionocyte cell density, **C** mRNA expression of transporter genes, and **D** proton secretion in the yolk skin of 7 dpf medaka larvae. The mRNA expression levels were analyzed by qPCR, with *rpl7* serving as the internal control. SIET was used to measure the H$^+$ activity on the skin. Values are mean ± SD ($n = 6$–12). Student's t test, $*p < 0.05$

found that ionocyte density was similar in medaka larvae with and without exogenous cortisol treatment (Fig. 2B). In addition, exogenous cortisol treatment did not change *ae1b* expression. However, the treatment did significantly stimulate expression of *nhe3* and *rncg1* and proton secretion in medaka larvae (Fig. 2C, D).

Effects of MR and GR knockdown on mRNA expression of acid-secretion genes and proton secretion in 7 dpf medaka larvae

Since cortisol often exerts its physiological functions via MR and GR, we next tested whether these receptors can influence proton secretion in medaka larvae. Medaka fertilized eggs were injected with MOs targeting MR, GR1 or GR2, and cell densities of ionocytes, transporter expression and proton secretion were analyzed in 7 dpf larvae. MR and GR1 knockdown did not alter ionocyte density, transporter expression or proton secretion (Figs. 3, 4). However, GR2 knockdown downregulated ionocyte density and proton secretion (Fig. 5A, C). In addition, *nhe3* and *rhcg1*
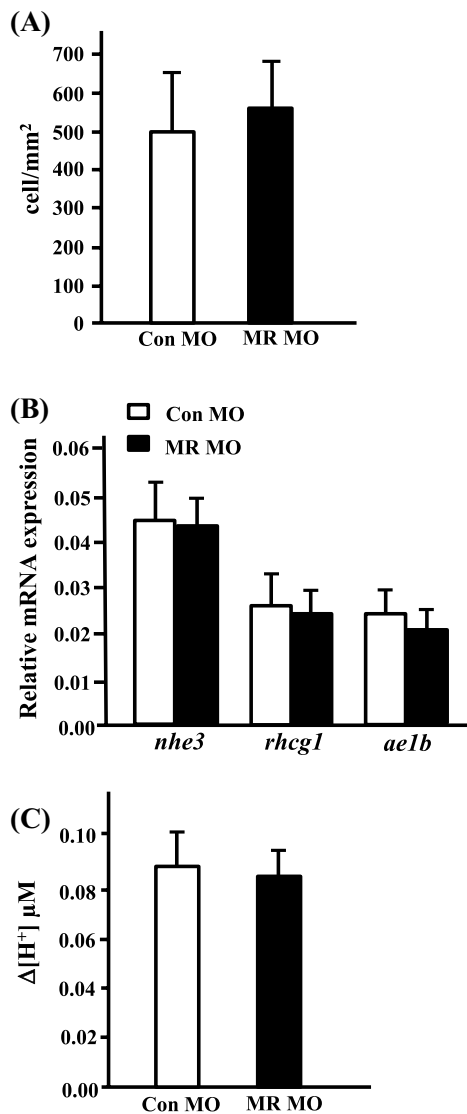
Fig. 3 MR is not required for acid secretion in medaka larvae. Effects of mineralocorticoid receptor (MR) knockdown on **A** ionocyte cell density, **B** mRNA expression of transporter genes, and **C** proton secretion in the yolk skin of 7 dpf medaka larvae. The mRNA expression levels were analyzed by qPCR, with *rpl7* serving as the internal control. SIET was used to measure the H^+ activity on the skin. Values are mean $\pm$ SD ($n = 6$–12)

Fig. 4 GR1 is not required for acid secretion in medaka larvae. Effects of glucocorticoid receptor 1 (GR1) knockdown on **A** ionocyte cell density, **B** mRNA expression of transporter genes, and **C** proton secretion in the yolk skin of 7 dpf medaka larvae. The mRNA expression levels were analyzed by qPCR, with *rpl7* serving as the internal control. SIET was used to measure the H^+ activity on the skin. Values are mean $\pm$ SD ($n = 6$–12)

expression levels were diminished in the GR2-knockdown larvae, but *ae1b* expression was not modulated (Fig. 5B).

Discussion

In zebrafish, HRCs are a specific subtype of acid-secreting ionocytes with high expression of NHE3b, AE1b, and Rhcg1 (Yan et al. 2007; Lee et al. 2011; Shih et al. 2008). Knockdown of NHE3b and AE1b is known to decrease acid secretion in zebrafish larvae (Lee et al. 2011; Shih et al. 2012),
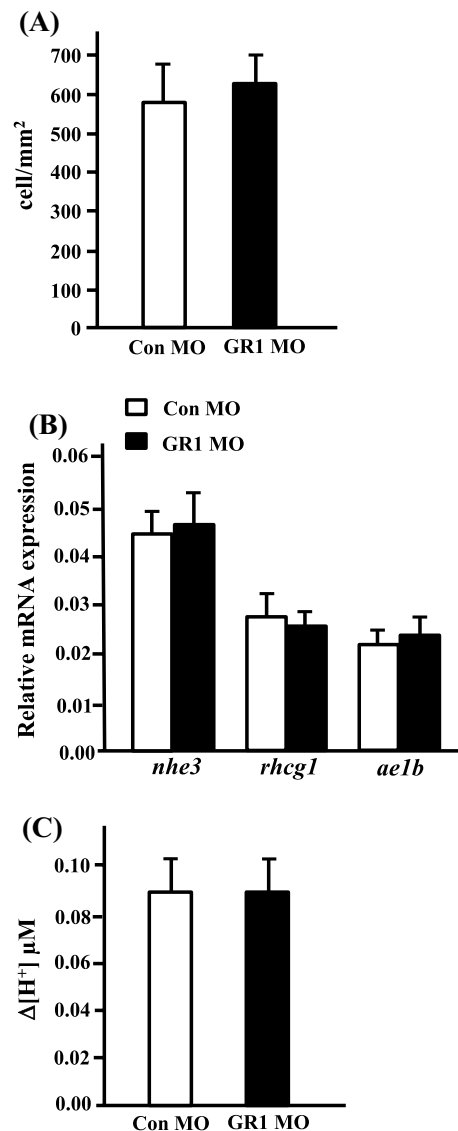
while the acid-trapping mechanism of coupled NHE3 and Rhcg1 actions provides a driving force for Na^+ uptake and acid secretion in zebrafish and medaka (Wu et al. 2010; Lin et al. 2012). In the present study, we found that AFW (pH 4.5) treatment upregulated mRNA expression of NHE3, Rhcg1 and AE1b in medaka larvae (Fig. 1A); this upregulation of transporters is expected to enhance the acid secretion capacity in medaka upon acidic stress. However, the present results indicate that acid secretion of medaka larvae was not modulated by AFW treatment (Fig. 1B), as was observed in
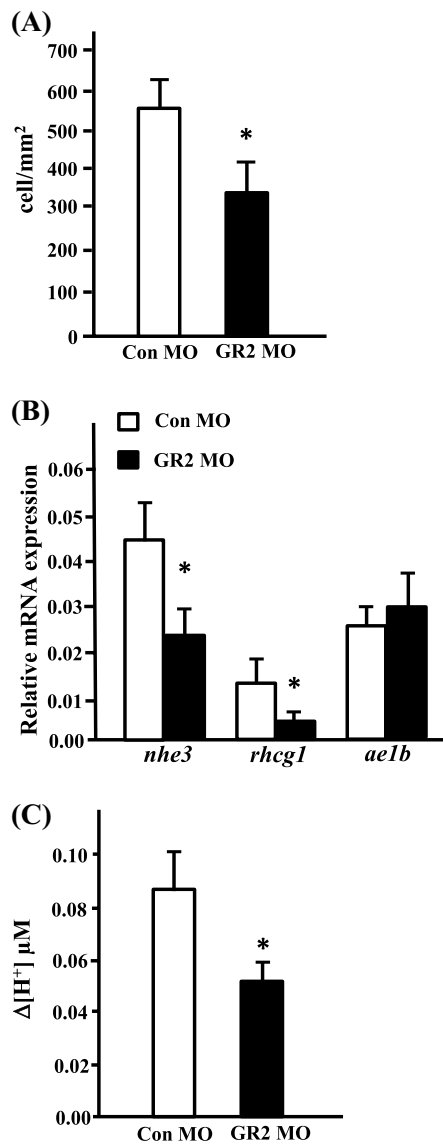
Fig. 5 GR2 is required for acid transporter expression and acid secretion in medaka larvae. Effects of glucocorticoid receptor 2 (GR2) knockdown on **A** ionocyte cell density, **B** mRNA expression of transporter genes, and **C** proton secretion in the yolk skin of 7 dpf medaka larvae. The mRNA expression levels were analyzed by qPCR, with *rpl7* serving as the internal control. SIET was used to measure the H^+ activity on the skin. Values are mean $\pm$ SD ($n = 6$–12). Student's *t* test, *$p < 0.05$

0.9-fold compared to the FW group (Fig. 1A), implying a high probability that secreted H^+ will combine with secreted NH_3 to form NH_4^+. This result provides further evidence for the suggestion previously made in Lin et al. (2012).

Many studies have demonstrated that acidosis stress increases circulating cortisol levels in fish (Brown et al. 1986; Gilmour et al. 2011; Kumai et al. 2012; Warren et al. 2004; Wood et al. 1999). Lin et al. (2015) also revealed that expression of *cyp11b*, which encodes the enzyme for the final step of cortisol synthesis, is stimulated upon acid stress in zebrafish. Similarly, our results in medaka larvae show that AFW treatment increased *cyp11b* expression and cortisol levels, and exogenous cortisol treatment upregulated acid secretion (Figs. 1A, 2C). On the basis of zebrafish studies, exogenous cortisol treatment is thought to stimulate acid secretion of HRCs and yolk skin by promoting HRC proliferation and mRNA expression of acid-secretion-related transporters (Cruz et al. 2013a, b; Lin et al. 2015). However, ionocyte density was not modulated by exogenous cortisol treatment after 4 dpf in medaka larvae (Fig. 2A, Trayer et al. 2013). Trayer et al (2013) performed a time-course analysis during embryogenesis to explore the effect of exogenous cortisol on the number of ionocytes in medaka embryos. They found exogenous cortisol only induced ionocyte differentiation to start earlier. However, exogenous cortisol treatment had no effect on the final ionocyte density. Trayer et al. (2013) suggested that exogenous cortisol treatment may induce ionocyte differentiation in early stages, but it does not regulate cell proliferation or differentiation rate in medaka. In the present study, we started to treat the medaka larvae with exogenous cortisol at 5 dpf and also did not find the change of ionocyte density. This result corresponded to Trayer et al. (2013). Hence, we suggest that cortisol may not be involved in the regulation of ionocyte density in medaka. Nevertheless, we found the expression levels of *nhe3* and *rhcg1* to be enhanced, but *ae1b* expression was not affected by exogenous cortisol treatment (Fig. 2B). Several studies also showed that corticosteroid hormone regulates the gene expression of some ion transporters. In trout and zebrafish, cortisol treatment increased the *nhe2* and *nhe3* expression levels (Ivanis et al. 2008; Lin et al. 2015). Dexamethasone treatment also stimulated *nhe3* expression in human epithelial colorectal adenocarcinoma cells (Wang et al. 2007). Moreover, aldosterone regulates *rhcg1* expression in mammalian intercalated cells (Izumi et al. 2011). For acid trapping, the coupling of NHE3 and Rhcg1 activities is thought to be vital for acid secretion in medaka (Wu et al. 2010; Lin et al. 2012). Taking all these findings together, it is likely that cortisol may upregulate acid secretion by stimulating *nhe3* and *rhcg1* expression, as observed in the present study. It also appears that the influence of cortisol on functional acid secretion mechanisms is conserved in vertebrates; however, there are subtle differences between stenohaline fish

Lin et al. (2012). This lack of measured effect suggests that increased NH_3 excretion via Rhcg1 may be coupled with increased H^+ concentrations driven by NHE3, resulting in the formation of NH_4^+ and a decreased H^+ gradient in medaka larvae with AFW treatment. This explanation would account for the lack of a detectable increase in acid secretion by SEIT. The results in this study revealed that AFW treatment of medaka larvae causes *rhcg1* expression to increase by about 2.5-fold, while *nhe3* expression is only about

species, which mainly utilize apical H^+-ATPase, and euryhaline species that preferentially use apical NHE. Cortisol stimulates acid secretion by upregulating differentiation of acid-secreting ionocytes and/or transporter expression in zebrafish, but in medaka, these actions seem to be mediated entirely by expression of the transporters, without any observable effect on cell proliferation/differentiation. This observation may be of evolutionary and comparative significance if it is confirmed by studies on other species.

In a previous study on medaka larvae treated with AFW (pH 5.0), increased acid secretion could not be detected because excreted H^+ combined with NH_3 to form NH_4^+ through an acid-trapping mechanism (Lin et al. 2012). Herein, we found exogenous cortisol treatment on medaka larvae induced higher relative expression of *nhe3* (~3-fold) than that of *rhcg1* (~0.8-fold) (Fig. 2B). In addition, the fish were reared in FW, where operation of NHE3 may face less resistance from a H^+ gradient across the cell membrane. These reasons may explain why acid secretion was increased in medaka larvae with cortisol treatment but not after AFW exposure.

According to previous in vitro studies (Greenwood et al. 2003; Sturm et al. 2005; Bury et al. 2003), cortisol may execute its ion regulation function through GR and/or MR in fish. Moreover, Trayer et al. (2013) showed that ionocyte number was reduced in GR2 medaka morphants, but it was not changed in GR1 or MR morphants at 82 h post fertilization (82 hpf). Concordantly, we also found the ionocyte density was downregulated in GR2 medaka morphants, but it was not changed in GR1 or MR morphants at 7 dpf (Figs. 3A, 4A, 5A). In zebrafish, Bone Morphogenetic Protein (BMP) plays an essential role in the maintenance of epidermal ionocyte progenitors, as it can induce p63, an important factor in the proliferation of epidermal stem cells (Hsiao et al. 2007; Kwon et al. 2010; Chang and Hwang 2011). Furthermore, the mRNA expression of BMP is reduced in zebrafish GR morphants (Nesan et al. 2012). Therefore, Trayer et al. (2013) hypothesized that medaka GR2 may regulate ionocyte number through BMP signaling. In the present study, we found acid secretion of medaka larvae was inhibited in GR2 morphants, but not changed in GR1 and MR morphants (Figs. 3C, 4C, 5C). The decreased acid secretion may result from the downregulation of ionocyte density in GR2 morphants. Lin et al. (2015) showed that cortisol and GR may directly regulate mRNA expression of acid-secretion-related transporters to stimulate acid secretion in HRCs of zebrafish. In the present study, *nhe3*, *ae1b* and *rhcg1* expression levels were not modulated in GR1 or MR morphants (Figs. 3B, 5B). In contrast, the knockdown of GR2 caused decreased *nhe3* and *rhcg1* expression, but it did not affect *ae1b* expression (Fig. 4B). These results further suggest that only GR2 is necessary for acid secretion in medaka. From the present data, it remains unclear whether

GR expression is colocalized with ionocytes in medaka, however, previous studies showed that GR expression can be found in ionocytes of both tilapia and zebrafish (Aruna et al. 2012; Kumai et al. 2012; Cruz et al. 2013b; Lin et al. 2016). Thus cortisol may act via GR2 to stimulate acid secretion in medaka ionocytes. Nonetheless, further experiments are still required to test this hypothesis.

In conclusion, as with zebrafish (Lin et al. 2015), the present results showed that the cortisol-synthesis enzyme, *cyp11b*, was upregulated upon AFW challenge, and cortisol acted via GR to upregulate expression of acid-secretion transporters and stimulate acid secretion in medaka (Figs. 1A, 2). However, the cortisol-GR pathway that upregulates acid secretion in medaka does not involve increased ionocyte density (Fig. 2A). Taken these together, these finding suggest that cortisol-mediated stimulation of acid secretion via GR may be conserved in fish with different acid-secretion strategies. While the mechanism may be conserved, the effects of cortisol-GR signaling on acid secretion may exhibit subtle differences between fish species. Whether these subtle differences are related to a divergent mechanism of acid secretion in fish is worthy of clarification in the future. Recent studies also demonstrated that NHE-mediated NH_4^+ excretion may play an important role in acid–base regulation in zebrafish and medaka (Ito et al 2014; Tseng et al. 2020). How cortisol signaling might also regulate NH_4^+ excretion in fish coping with an acidic environment is an interesting question to be explored in future studies.

Author contributions CHL and PPH were involved in conception and study design; CHL, HJH, HJC, and YLT carried out the experiments and data collection; CHL, HJH, HJC, and PPH did the data analysis and interpretation; CHL and PPH wrote the manuscript; All the authors reviewed and revised the manuscript, and gave final approval for publication.

Declarations

Conflict of interest The authors declare no conflict of interests.

Ethical approval All applicable national and/or institutional guidelines for the care and use of animals were followed.

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