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Insulin-like growth factor 1 triggers salt secretion machinery in fish under acute salinity stress

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Abstract

Timely adjustment of osmoregulation upon acute salinity stress is essential for the survival of euryhaline fish. This rapid response is thought to be tightly controlled by hormones; however, there are still questions unanswered. In this work, we tested the hypothesis that the endocrine hormone, insulin-like growth factor 1 (Igf1), a slow-acting hormone, is involved in the activation of salt secretion mechanisms in euryhaline medaka (*Oryzias melastigma*) during acclimation to acute salinity stress. In response to a 30-ppt seawater (SW) challenge, Na⁺/Cl⁻ secretion was enhanced within 0.5 h, with concomitant organization of ionocyte multicellular complexes and without changes in expression of major transporters. Igf1 receptor inhibitors significantly impair the Na⁺/Cl⁻ secretion and ionocyte multicellular complex responses without affecting transporter expression. Thus, Igf1 may activate salt secretion as part of the teleost response to acute salinity stress by exerting effects on transporter function and enhancing the formation of ionocyte multicellular complexes. These findings provide new insights into hormonal control of body fluid ionic/osmotic homeostasis during vertebrate evolution.

Key Words

- ▶ IGF1
- ▶ salt secretion
- ▶ ionocytes
- ▶ salinity
- ▶ euryhaline fish

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Introduction

The development of mechanisms for intracellular and extracellular ionic homeostasis was an essential step in the evolution of many vertebrates, allowing continued operation of cellular activities and physiological processes under new environmental conditions. Vertebrates likely originated in seawater (SW) habitats (Robertson 1957), and some groups of them then conquered freshwater (FW) habitats, necessitating the evolution of iono-/osmoregulation mechanisms that were conserved in successive vertebrates, such as terrestrial mammals. Following the evolution from SW to FW and terrestrial habitats, some groups of vertebrates, like teleosts, further developed physiological traits that allow the organisms to survive drastic salinity

changes. Euryhaline teleosts developed sophisticated mechanisms for transporting water and ions, and most of these traits were conserved in advanced vertebrates, including mammals (Evans *et al.* 2005, McCormick & Bradshaw 2006, Takei *et al.* 2014). Timely adjustment of osmoregulation upon acute salinity stress is required for survival of euryhaline teleosts, and efficient iono-/osmoregulation upon chronic salinity stress also affects energy consumption, which is a major determinant of growth and sustainability (Takei *et al.* 2014, Takei & Hwang 2016). As such, the development of adaptive mechanisms for acute response to environmental salinity changes represents a critical step in the evolution of vertebrate species.

According to current knowledge of organismal response to salinity challenges, certain hormones exert rapid actions to activate salt secretion through regulation of existing transporters and ionocytes in the gills during the acute phase (minutes to hours) of exposure; thereafter, hormonal signaling may also produce long-term effects on transporter synthesis and ionocyte proliferation/differentiation to increase salt secretion capacity in the acclimation phase (hours to days) (McCormick & Bradshaw 2006, Takei *et al.* 2014). Among the hormones known to mediate these effects, the growth hormone (Gh)/insulin-like growth factor 1 (Igf1) axis is one of the most-studied regulators of osmoregulation in fish gill response to salinity stress. Gh/Igf1, considered as an SW-adapting hormone with slow actions, is known to increase fish SW tolerance by regulating gill Na⁺-K⁺-ATPase (NKA) activity (McCormick 1996), expression of salt-secreting transporters (Pelis & McCormick 2001, Tipsmark & Madsen 2009) and ionocyte density (Madsen 1990, Prunet *et al.* 1994), although the diversity in Gh/Igf1 actions was also reported in teleosts (Takei *et al.* 2014).

IGF1 has been shown to acutely stimulate epithelial Na⁺ channel (ENaC)-mediated Na⁺ reabsorption in renal cells of mammals (Gonzalez-Rodriguez *et al.* 2007, Staruschenko *et al.* 2007, Ilatovskaya *et al.* 2015) and toads (Blazer-Yost & Cox 1988, Blazer-Yost *et al.* 1989). Thus, we propose that acute actions of Igf1 on ion transporters (independent of effects on transcription and/or translation of the transporters) may have developed in early vertebrates, such as fish. This regulation of salt secretion machinery is expected to be of high physiological importance and determines fish survival in the hours following acute salinity stress (Hwang & Hirano 1985, Hwang 1987, Hwang *et al.* 1989, Moorman *et al.* 2015). Exploration of the mechanisms underlying this acute response to salinity changes should provide important insights into the physiological traits that were developed by early vertebrates during evolution to cope with variable environments.

We recently used medaka, an emerging model with similarities to zebrafish, to propose a new model for iono-/osmoregulation in SW fish gills and embryonic skin (Hwang & Lin 2013, Guh & Hwang 2017). A non-invasive scanning ion-selective electrode technique (SIET) was utilized to probe the real-time ion currents of ionocytes in an intact embryo (Shen *et al.* 2011). This technological development allows medaka to serve as a competent model for studying acute responses to salinity stress. In the present study, we use this approach to test if Igf1 is involved in the activation of salt secretion

machinery in ionocytes of Indian medaka (*Oryzias melastigma*) under acute salinity stress. Indian medaka can survive direct transfers from FW to 30-ppt SW, and the species is able to mature and reproduce in either FW or SW environments. The experiments were designed to study (1) the changes in function and expression of salt-secreting transporters and intercellular organization of ionocytes in medaka embryos after acute SW exposure and (2) the effects of blocking Igf1 signaling on these physiological changes.

Materials and methods

Experimental animals

Mature Indian medaka (*Oryzias melastigma*) were reared in tanks with circulating tap water at 27°C, with a ratio of 14 h light:10 h darkness photoperiod. Fertilized eggs were collected for the experiments. During the experiments, the larvae were not fed and the water was changed daily to maintain water quality. The experimental protocol was approved by the Academia Sinica Institutional Animal Care and Utilization Committee (approval no.: BSF0415-00003197), and all methods were performed in accordance with relevant guidelines and regulations.

Acclimation experiments

SW with 30-ppt salinity was prepared by adding appropriate amounts of sea salt (KENT Marine, Franklin, WI, USA) to double deionized water. For acclimation experiments (Fig. 1), 6-day-post-fertilization (dpf) embryos kept in FW were first dechorinated with hatch enzyme (National BioResource Project Medaka, Okazaki,

Figure 1

Detailed experimental scheme. The experimental design and timing of experiments are shown. All experimental embryos were dechorinated at the 6-dpf stage by treating with hatching enzyme. After dechorination, embryos were transferred to freshwater or seawater for 30 min before further experimental analyses. For the inhibitor experiment, picropodophyllin or BMS754807 was added to the water immediately after dechorination of embryos.

Japan) for 15 min and then moved to experimental petri dishes with FW or SW.

cDNA preparation

For total RNA extraction, 15–20 individuals of 6-dpf medaka embryos or the gills from one adult fish were collected as one sample. Samples were homogenized in TRIzol reagent (Invitrogen), and total RNA was purified following the manufacturer's protocol. Nanodrop 2000 (Thermo Scientific) was then used to determine the quality and quantity of the RNA. To synthesise cDNA, genomic DNA was first removed by treating total RNA with DNaseI (Roche Applied Science) at 37°C for 2 h, then 2 µg of total RNA was used to synthesise cDNA with SuperScript IV reverse transcriptase (Thermo Scientific) following manufacturer's protocol.

Quantitative RT-PCR

Real-time PCR was performed with a LightCycler real-time PCR system (Roche Applied Science) in a final volume of 10 µL. The reaction consisted of 5 µL 2× SYBR Green I Master (Roche Applied Science), 300 nM each primer, and 20–30 ng cDNA. *rpl7* was used as the internal control following a previous study (Zhang & Hu 2007). The primer sets used for qRT-PCR are shown in Supplementary Table 1 (see section on [supplementary materials](#) given at the end of this article). The specificity of primers was confirmed by Sanger sequence of each amplicon, and the amplification efficiency was confirmed by serious dilution of cDNA for each set of primer. Results were calibrated and normalized to control group using the method of $\Delta\Delta Ct$.

Scanning ion-selective electrode technique (SIET)

Na⁺ and Cl[−] gradients in the yolk skin of 6-dpf embryos were measured by SIET, following a previous study (Shen *et al.* 2011). Na⁺ and Cl[−] selective microelectrodes were used to measure ionic activities on the yolk skin surface of intact medaka embryos. The micropipettes with tip diameters of 3–5 µm were backfilled with a 1-cm column of electrolytes and frontloaded with a 20- to 30-µm column of a liquid ion exchanger cocktail (Sigma-Aldrich) to create an ion-selective microelectrode (probe). The ionophore cocktails (and electrolytes), Na⁺ ionophore II cocktail A (100 mM NaCl) and Cl[−] ionophore I cocktail A (100 mM NaCl), were used. The ion-selective microelectrode was connected to an operational amplifier (IP Amp ion polarographic amplifier; Applicable Electronics, East

Falmouth, MA, USA) via an Ag/AgCl wire electrode holder (World Precision Instruments, Sarasota, FL, USA). Electrode vibration and positioning were achieved with a stepper motor-driven 3D positioner (Applicable Electronics). Data acquisition, preliminary processing, and control of the 3D electrode positioner were performed with ASET software (Science Wares, East Falmouth, MA, USA). The Nernstian property of each microelectrode was measured to show the Nernst slopes of 55.4 ± 2 ($n=6-12$) for Na⁺ and -57.75 ± 1 ($n=4-12$) for Cl[−].

Pharmacological treatment

The stocks of 2 mM PPP (picropodophyllin, Santa Cruz Biotechnology) and 4.8 mM BMS754807 (Asooq Bioscience, Irvine, CA, USA) were prepared in DMSO. Dechorionated embryos were first transfer to FW either containing 2 µM PPP, 4.8 µM BMS754807, or 0.1% DMSO for 1 h then exposed to SW which contained inhibitor or DMSO vehicle control for 0.5 h (Fig. 1). The dose of 2 µM of PPP was selected based on a previous study in zebrafish (Bohnsack *et al.* 2011).

In situ hybridization

In situ hybridization followed a previous study (Guh *et al.* 2014). The cDNA fragments from marine medaka *igf1ra* (ENSOMEG00000006048) were obtained by PCR using the following primer set: forward, 5'-AGTGAC-GCTCTTCATCGCCA-3'; and reverse, 5'-GCCCCGACCT-TCCTGGAGATC-3', and *igf1rb* (ENSOMEG00000018503) were obtained by the following primer set: forward, 5'-CGTTTACCGCATCGACGTTTC-3'; and reverse, 5'-GTC-TCGGTGGACGAACCTTGT-3'. The amplicon size of *igf1ra* and *igf1rb* were 952 and 1003 bp, respectively. The PCR product was used to synthesize digoxigenin (DIG)-labeled probes, or sense RNA probes. After hybridization, embryos were washed with 2× SSC (at 65°C for 20 min), 0.2× SSC (at 55°C for 30 min, twice), and PBST (PBS with 0.1% Tween 20) at room temperature for 10 min several times. An alkaline phosphatase (AP)-coupled anti-DIG antibody and BM Purple (Roche Applied Science) were used to detect the signals. Images were obtained on an upright microscope (Axioplan 2 Imaging; Carl Zeiss).

Immunocytochemistry

Immunocytochemistry followed a previous study (Guh *et al.* 2014). A polyclonal antibody against human NKA alpha1 subunit (1:100 dilution; sc-28800, Santa Cruz

Biotech) and an Alexa Fluor 568 goat anti-rabbit IgG (1:200 dilution; Molecular Probes) were used to detect ionocytes. To stain tight junctions, samples were treated with a monoclonal antibody against human ZO-1 fusion protein (1:600 dilution; 33-9100, Thermo Fisher Scientific) and an Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG) antibody (1:200 dilution; Molecular Probes). To detect Na⁺-K⁺-2Cl⁻ cotransporter (NKCC) expressing ionocytes, a monoclonal antibody against human NKCC (1:200 dilution; T4, Developmental Studies Hybridoma Bank, Iowa, USA) and an Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG) antibody (1:200 dilution; Molecular Probes) were used. Cell nuclei were stained by DAPI. Images were acquired with TCS-SP5 confocal laser scanning microscope (Leica Lasertechnik), FV3000 inverted confocal laser scanning microscope (Olympus), or an upright microscope (Axioplan 2 Imaging; Carl Zeiss).

Cell counting

Fiji was used to analyze high-resolution, whole-mount embryo images taken with a TCS-SP5 confocal laser scanning microscope (Leica Lasertechnik). Different types of ionocytes in a fixed area of the dorsal-posterior region of each embryo were counted and averaged. The cell numbers within a square millimeter (mm²) were calculated.

Statistical analysis

Values are presented as mean ± s.d. (*n*), unless otherwise noted. Values from each condition were analyzed using one-way ANOVA followed by Turkey's pairwise comparisons. For simple comparisons, Student's unpaired *t*-test (two-tailed) was used to compare two means. A significant difference was determined as *P*-value less than or equal to 0.05. All data were analyzed by using Prism 8.4.2 (GraphPad Software, LLC) without omitting any data points.

Results

Changes in salt secretion mechanism after acute transfer from FW to SW

To determine how fast Indian medaka switch from salt absorption to salt excretion under acute salinity stress, the real-time changes in Na⁺/Cl⁻ secretion (indicated by Na⁺/Cl⁻ gradients) were measured with SIET in the skin

of 6-dpf medaka embryos over a period of 3 h after direct transfer from FW to SW (Fig. 2A and B). Surprisingly, alterations in Na⁺ and Cl⁻ gradients were observed as early as 0.5 h after SW transfer. The ion gradients in the SW group rose to a peak at 1 h and then were maintained at a higher level than the control group in FW. Next, we focused on the molecular and cellular mechanisms underlying this rapid activation of salt secretion at 0.5 h. The expression level of genes for major transporters in salt secretion, that is, Na⁺-K⁺-Cl⁻ cotransporter 1a (*nkcc1a*), Cystic fibrosis transmembrane conductance regulator (*cfr*), and NKA alpha1 subunits (*atp1a1b*, *atp1a2*, *atp1a3*, and *atp1a3b*), was not changed at 0.5 h SW transfer (Fig. 2C). Similarly, no changes were found in the mRNA expression of genes encoding Igf1 receptors (*igf1ra* and *igf1rb*) or *igf1* (Fig. 2C). Although the NKCC signals in NKA-labeled ionocytes obviously increased in 6-day-old SW acclimated embryos, the difference to the control FW embryos could not be observed after 0.5 h SW exposure (Fig. 3A, B, C, D, E, F, G, H, I, J, K and L). Moreover, the number of NKA-labeled ionocytes on the yolk of embryos also did not show any difference to the control at this early time-point (Fig. 2D, E and F).

To look for potential changes at the cellular level, we examined the organization of ionocytes in the skin of FW- and SW-acclimated medaka embryos. Three different types of NKA-labeled ionocytes were identified based on morphology (Fig. 4A, B, C, D and E): single ionocytes (s-IC) with a large ZO-1 (a tight junction marker)-labeled apical opening, multicellular ionocytes (mc-ICs) in groups of adjacent ionocytes sharing a smaller opening, and transforming ionocytes (t-IC) that exist with a cellular organization somewhere between s-ICs and mc-ICs. The leaky junction between mc-ICs is one of the key cellular features utilized in paracellular Na⁺ secretion (Evans *et al.* 2005). Accordingly, an increase of mc-ICs may constitute a morphological sign that activation of salt secretion has occurred. As shown in Fig. 4F, FW and SW (at 0.5 h after SW transfer) groups had the same total number of ionocytes, but the population of cell types changed. In the embryos of the FW control group, ionocytes comprised mostly of s-ICs with mc-ICs and t-ICs accounting for only a small percentage of the population. Strikingly, the populations of mc-ICs and t-ICs were intensively increased after SW transfer. The increased mc-IC and t-IC percentages likely resulted from conversion of s-ICs, which drastically decreased after SW transfer. Importantly, these changes in ionocyte morphology (Fig. 4) corresponded with the activation of salt secretion at 0.5 h of SW transfer (Fig. 2A and B).

Figure 2

Changes in salt secretion mechanism in 6-dpf medaka embryos after acute transfer from FW to SW. Embryos at 6-dpf were directly transferred from FW to SW. (A) Na^+ and (B) Cl^- gradients on the skin of yolk sac were examined by SIET at different time-points. (C) mRNA expression was measured by qPCR for *igf*, *igf1ra*, *igf1rb*, *nkcc1a*, *cltr*, and NKA alpha subunit isoforms (*atp1a1b*, *atp1a2*, *atp1a3*, and *atp1a3b*). (D) Number of Na^+ - K^+ -ATPase (NKA)-labeled ionocytes and (E, F) NKA immunocytochemical signals in the yolk sac skin at 0.5 h after SW transfer were compared with those in controls (FW-FW). In SIET analyses, lines represent the mean. In qPCR analysis and cell counting, values are presented as mean $\pm$ s.d. Sample size is shown in the parentheses. Different letters indicate a significant difference between time-points in SIET analyses (one-way ANOVA and Turkey's comparison, $P < 0.05$). Student's *t*-test: ** $P < 0.01$, **** $P < 0.0001$). A full colour version of this figure is available at <https://doi.org/10.1530/JOE-20-0053>.

Figure 3

Changes in Na^+ - K^+ - $2Cl^-$ cotransporter (NKCC) expression in 6-dpf medaka embryos after transfer from FW to SW. The expression of NKCC (A, E, and I) and Na^+ - K^+ -ATPase (NKA) (B, F, and J) in 6-day-old FW acclimated embryos (A-B), 0.5-h acute FW-SW transfer (E-F), and 6-day-old SW acclimated embryos (I-J) were analyzed by immunostaining of NKCC and NKA antibody. The NKCC signals were faintly detected in FW acclimated and acute FW-SW transferred embryos, but strongly expressed in SW acclimated embryos. Cell nuclei were labeled by DAPI staining (C, G, and K). The merge images of NKCC, NKA, and DAPI were shown (D, H, and L). A full colour version of this figure is available at <https://doi.org/10.1530/JOE-20-0053>.

Figure 4

Changes in skin ionocyte morphology in 6-dpf medaka embryos after acute transfer from FW to SW. Three types of ionocytes are shown: (A) single ionocyte (s-IC), (B) transforming ionocytes (t-IC), and (C) multicellular complex ionocyte (mc-IC). The types were identified by double labeling with Na⁺-K⁺-ATPase (red) and DAPI (blue) in embryos. (D) s-ICs and (E) mc-ICs showed apical opening with different sizes. Arrows and green signal indicate labeling of tight junction marker, ZO-1. (F) Numbers of different ionocyte types in the yolk sac of 6-dpf embryos at 0.5 h after SW transfer and in controls (FW-FW) were compared. Sample size is shown in the parentheses. Values are presented as mean ± s.d. **P* < 0.05, ****P* < 0.001; *****P* < 0.0001 (two-tailed unpaired Student's *t*-test). A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0053>.

Expression of *igf1r* in ionocytes

Localization of *igf1r* mRNA in salt-secreting ionocytes was assessed to understand if Igf1-Igf1r signaling may exert a direct action on ionocytes. *In situ* hybridization of *igf1ra* could not be detected in cells with ionocyte-like signals in the yolk of embryos, probably due to low expression levels. However, *in situ* hybridization with an *igf1rb* specific probe showed a 'pepper and salt' (i.e. ionocyte pattern) pattern in the skin of embryos (Fig. 5A and B). Double labeling with *in situ* hybridization of *igf1rb* mRNA and NKA immunocytochemistry showed that most NKA-labeled ionocytes also express *igf1rb* mRNA (Fig. 5C and D). A negative control of *in situ* hybridization with sense

Figure 5

Localization of *igf1rb* mRNA in skin ionocytes of SW acclimated 6-dpf medaka embryos. Whole-mount *in situ* hybridization of *igf1rb* mRNA (A, B) and double labeling with Na⁺-K⁺-ATPase (NKA, green) (C, D) in the yolk sac skin of embryos showed colocalization of the two signals (arrows). Some *igf1rb* mRNA signals were not localized to the ionocytes (arrowheads). (C) and (D) Magnifications of the indicated areas in (A) and (B), respectively. (E) Negative control with the sense probe for *igf1rb* shows no specific signals in the embryos. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-20-0053>.

probe (Fig. 5E) confirmed the aforementioned results. These results suggested that salt-secreting ionocytes may be target cells for Igf1 actions.

Effects of diminished Igf1 signaling on salt secretion mechanism after acute transfer from FW to SW

To clarify whether Igf1 signaling is involved in salt secretion of ionocytes after acute salinity stress without influencing the expression of salt transporters. Igf1r inhibitors, picropodophyllin (PPP) and BMS754807 (BMS), were used to block the phosphorylation of Igf1r (Bohnsack *et al.* 2011). As shown in Fig. 6, 6-dpf embryos treated with or without inhibitors were transferred to SW for 0.5 h and ion gradients were then measured by SIET.

Figure 6

Effects of IGF1R inhibitors on salt secretion in 6-dpf medaka embryos after acute transfer from FW to SW. Embryos treated with 2 μM picropodophyllin (PPP) or 4.8 μM BMS754807 (BMS) were directly transferred from FW to SW for 0.5 h. (A and C) Na^+ and (B and D) Cl^- gradients in the yolk sac skin were measured by SIET, and (E) mRNA expression levels of *nkcc1a* and *cfr* were measured by qPCR. (F) Cell number of $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter (NKCC) expressing Na^+-K^+ -ATPase (NKA)-labeled ionocytes were examined by double immunostaining. Ctrl, control embryos with vehicle (DMSO) only. In SIET analyses, lines represent the mean. In qPCR analysis and cell counting, values are presented as mean $\pm$ s.d. Sample size is shown in the parentheses. $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ (two-tailed unpaired Student's *t*-test).

The results showed significantly decreased Na^+ and Cl^- gradients in the PPP- and BMS-treated embryos compared with the respective controls (incubated with DMSO only) (Fig. 6A, B, C and D). However, the qPCR analysis revealed no significant differences in the mRNA levels of *nkcc1a* or *cfr* between PPP-treated and control embryos (Fig. 6E). The number of NKCC expressing NKA-labeled ionocytes also showed no significant differences between PPP-treated and control embryos (Fig. 6F and Supplementary Fig. 1A, B, C, D, E, F). These results suggested that the activation of salt secretion is triggered via Igf1 signaling upon acute salinity stress, but this action is probably not mediated by transcriptional regulation of salt transporters.

Figure 7

Effects of IGF1R inhibitors on morphology of ionocytes in 6-dpf medaka embryos after acute transfer from FW to SW. Embryos treated with 2 μM picropodophyllin (PPP) or 4.8 μM BMS754807 (BMS) were directly transferred from FW to SW for 0.5 h. (A–F) Ionocytes were morphologically categorized as single ionocytes (s-ICs, arrows), transforming ionocytes (t-ICs), and multicellular complex ionocytes (mc-ICs, arrowheads) by double labeling with Na^+-K^+ -ATPase (NKA, red) and DAPI (blue). (G) The cells numbers were quantified. Ctrl, control embryos with vehicle (DMSO) only. Sample size is shown in the parentheses. Values are presented as mean $\pm$ s.d. (two-tailed unpaired Student's *t*-test, $**P < 0.01$, $*P < 0.05$). A full colour version of this figure is available at <https://doi.org/10.1530/JOE-20-0053>.

As previously mentioned, our data suggest that an increase of mc-ICs is one of the major events underlying the activation of salt secretion after acute SW stress. Therefore, we further used PPP to examine if Igf1 signaling affects the intercellular organization of ionocytes during acclimation to acute salinity stress. The cell numbers of s-ICs, t-ICs, and mc-ICs were compared between PPP-treated and control (incubated with DMSO only) embryos at 0.5 h after SW transfer (Fig. 7A, B, C, D, E and F). The total numbers of ionocytes were not different between the two groups of embryos; however, an increase in the percentage of s-IC with a concomitant decrease in both t-ICs and mc-ICs were found in PPP-treated group compared with controls after SW transfer (Fig. 7G). These results provided morphological evidence to support our

physiological data, reinforcing the idea that Igf1 signaling plays an important role in the activation of salt secretion in medaka coping with acute salinity stress.

Discussion

Activation of salt secretion machinery in the gills is one of the most vital responses for euryhaline teleosts to cope with acute salinity stress, as a timely and efficient activation of this machinery is known to be necessary for fish survival (Bath & Eddy 1979, Hwang 1987, Hwang *et al.* 1989). According to current thought, the most important responses in gills during salinity stress are the activation of Cl⁻ secretion by apical CFTR and basolateral transporters, such as NKCC and NKA, as well as the activation of paracellular secretion of Na⁺ through leaky junctions between ionocytes and accessory cells down the NKA-generated Na⁺ gradient (Evans *et al.* 2005, Hwang *et al.* 2011, Hwang & Lin 2013). The cellular and physiological responses underlying these processes were found to occur rapidly after salinity change. For example, Cl⁻ current in isolated opercular epithelium, as measured with a Ussing chamber, was increased in killifish 1 h after transfer to SW and in tilapia 1 day after 1/3 SW treatment (Foskett *et al.* 1981, Shaw *et al.* 2008). Moreover, the effluxes of Na⁺ and Cl⁻ radioisotopes were respectively increased in rainbow trout 24 and 2 h after transfer to 2/3 SW (Bath & Eddy 1979). In Japanese medaka (*Oryzias latipes*), real-time SIET electrophysiology measurements revealed that Na⁺ and Cl⁻ currents were elevated at 4 and 0.5 h, respectively, in skin ionocytes after transfer to 25-ppt SW (Shen *et al.* 2011). The present study, in concordance with those previous studies, revealed that a rapid activation of ionocyte Na⁺ and Cl⁻ secretion (measured by SIET electrophysiology) in Indian medaka occurred as early as 0.5 h after exposure to 30-ppt SW (Fig. 2A and B). Furthermore, this activation coincides with the cellular events that enhance salt secretion. In ayu (*Plecoglossus altivelis*) embryos, skin ionocytes were found to reorganize to form multicellular complexes with leaky junctions between SW-type ionocytes and accessory cells as early as 3 h after transfer to 33-ppt SW (Hwang & Hirano 1985). In Indian medaka, the vast majority (about 86%) of ionocytes were s-ICs, with only 6–9% t-ICs and mc-ICs under FW conditions, while the portion of mc-ICs was increased about 6-fold after transfer to SW for 0.5 h (from 6 to 38%) (Fig. 4F). A previous study indicated that mc-ICs excrete more Na⁺ and Cl⁻ than s-ICs in medaka embryonic skin (Shen *et al.* 2011). These observations

reinforce the long-held model of ionocyte morphology in SW conditions and also implicate the timely and efficient activation of salt secretion with simultaneous formation of multicellular ionocyte complexes (to provide more leaky paracellular pathways) as key events for euryhaline teleosts to cope with salinity stress. Moreover, the activating events appear to be unrelated to the transcriptional regulation of salt-secreting ion transporters or differentiation of salt-secreting ionocytes. No changes were observed in the expression levels of *nkcc1a*, *cfr*, and NKA alpha subunit isoforms (Fig. 2C), the number of ionocytes (NKA as the marker; Fig. 2D, E and F), and the NKCC signals in ionocytes (Fig. 3A, B, C, D, E, F, G, H and Supplementary Fig. 2A, B, C, D, E, F) in medaka embryos at 0.5 h after SW transfer. These data suggested that acute activation of salt secretion must be achieved by the regulation of transporter activity and/or translocation of preexistent transporters, reinforcing the conclusions of previous studies. For example, in killifish, CFTR and NKCC are respectively redistributed toward apical and basolateral surfaces of opercular ionocytes at 24 h after exposure to SW (Marshall *et al.* 2002), and acute upregulation of opercular Cl⁻ secretion at 1 h post salinity stress involves the translocation of CFTR from the intracellular pool to the plasma membrane (Shaw *et al.* 2008).

Quantitative RT-PCR analysis of gene transcript in the whole embryos may not be able to reflect the actual gene expression in the ionocytes. The expression of *nkcc1a* and *cfr* in adult medaka gills (Supplementary Fig. 3B) and *atp1a1b* in medaka embryos (Supplementary Fig. 3A) did elevate after SW acclimation for 6–7 days, being consistent with previous studies in other teleosts (Flemmer *et al.* 2010, Hsu *et al.* 2014, Bollinger *et al.* 2016, Lv *et al.* 2016, Wong *et al.* 2017). The expression of *nkcc1a* and *cfr* showed no differences in medaka embryos after a 6-day SW acclimation (Supplementary Fig. 3A); however, the NKCC protein signals in NKA-labeled ionocytes in embryonic skin obviously intensified after a 6-day SW acclimation (Fig. 3I, J, K, L and Supplementary Fig. 2G, H, I). As such, immunocytochemistry (or *in situ* hybridization) of target genes is an alternative approach, other than whole-embryo RT-PCR, to analyze the protein (or mRNA) expression of target genes in embryonic skin ionocytes for studying related iono- and osmoregulation mechanisms.

GH and IGF1 are widely known to be involved in body fluid ionic and osmotic homeostasis in mammals and other vertebrates (Kamenicky *et al.* 2014, Takei *et al.* 2014), suggesting the physiological significance

of the development of this trait during the evolution of vertebrates. IGF1 is an independent regulator or mediator of GH and was reported to regulate glomerular and tubular function in mammalian kidney, including reabsorption of Na^+ , PO_4^{3-} , and Ca^{2+} (Kamenicky *et al.* 2014). The main transporters targeted by GH/IGF1 in mammalian renal Na^+ reabsorption are ENaC and NKA (Gonzalez-Rodriguez *et al.* 2007, Kamenicky *et al.* 2014). IGF1 is also known to be involved in the development and growth of mammalian kidney through its effects on proliferation, differentiation, and survival of renal cells (Bach & Hale 2015). The actions of IGF1 on body fluid ionic and osmotic homeostasis appear to have developed in early vertebrates that evolved the traits of iono- and osmoregulation. In teleosts, Igf1 has long been known as a mediator of Gh and has been well studied with regard to its effects on salt secretion during acclimation to a SW environment. The actions of Igf1 in this context were thought to mainly affect gill NKA expression and activity, as well as the differentiation of gill ionocytes (Madsen & Bern 1993, McCormick 1996, 2001, Pelis & McCormick 2001, Evans *et al.* 2005, Tipsmark & Madsen 2009, Takei *et al.* 2014).

In mammalian renal tubules, IGF1 was reported to rapidly (within several minutes) regulate the activities of various ion transporters, including ENaC, potassium Kir4.1/5.1 channels, and Cl^- channel (CIC-Kb/2) (Gonzalez-Rodriguez *et al.* 2007, Zaika *et al.* 2016a,b). It is reasonable to think that such a rapid action of IGF1 on ion transporters would have been developed in early vertebrates equipped with iono-/osmoregulation traits, as discussed previously; however, there was no evidence to support this hypothesis until the present study. First, salt-secreting ionocytes were identified as target cells for Igf1 signaling by localizing *igf1rb* mRNA in NKA-labeled ionocytes in medaka embryonic skin (Fig. 5C and D). In the acclimation experiments, acute transfer to SW for 0.5 h neither changed the mRNA expression of *igf1* and the receptor genes in the whole embryos (Fig. 2C), nor affected the *igf1rb* mRNA signals in embryonic skin ionocytes (Supplementary Fig. 4). Similarly in zebrafish, Igf1 signaling activated and *igfbp5a* expressing cells increased after acclimation to a low calcium environment for 72 h, but the expression of *igf1ra* and *igf1rb* in isolated *igfbp5a* expressing cells was not changed (Liu *et al.* 2018). Therefore, altering the expressing of Igf1 receptors may not be essential for the activation of Igf1 signaling. We then further analyzed the actions of Igf1 signaling by using pharmacological and real-time electrophysiological approaches. PPP and BMS, specific IGF1R inhibitors,

suppressed salt secretion without affecting the expression of ion transporters in the whole embryos and the number of NKCC expressing NKA-labeled ionocytes in embryonic skin at 0.5 h after acute SW transfer (Fig. 6 and Supplementary Fig. 1), demonstrating a fast action of Igf1 signaling on salt secretion machinery in euryhaline teleosts during acclimation to acute salinity stress. We found that PPP inhibited the intercellular reorganization of ionocytes (i.e. formation of mc-ICs) in the embryos after acute SW transfer (Fig. 7). The appearance of mc-ICs is a key event that allows paracellular Na^+ secretion, and is rapidly triggered by salinity stress to activate Na^+ secretion function in teleost gills (Hwang & Hirano 1985, Cozzi *et al.* 2015). Interestingly, IGF1 is widely known to modulate paracellular transport (transepithelial permeability) by regulating the formation of tight junctions in various mammalian tissues (Paye *et al.* 2007, Ko *et al.* 2009, Mitsui *et al.* 2010). IGF1 also affects liver architecture and intercellular junctions by regulating the expression of genes encoding components of the cytoskeleton, extracellular matrix, and cell-cell adhesion complexes (Tutau *et al.* 2009, Lara-Diaz *et al.* 2017). Taken together, the traits of fast-acting IGF1-regulated epithelial transport, which occur via effects on ion transporters and intercellular organization of ionocytes (for paracellular transport), appear to be conserved from fish to mammals.

The impairment of intercellular organization of ionocytes by blocking Igf signaling in medaka embryonic skin (Fig. 7) may be reasonably considered as an effect on the microtubule, since the behaviors of ionocytes were previously reported to be mediated by microtubule assembly in fish gills (Tsai & Hwang 1998, Yang *et al.* 2016). Many studies used similar or higher concentrations of PPP or BMS than the present study to block Igf signaling in fish embryos (Bohnsack *et al.* 2011, Dai *et al.* 2014, Lu *et al.* 2016). A known off-target effect caused by high concentrations of PPP or BMS on cell lines (Carboni *et al.* 2009, Waraky *et al.* 2014) may also need to be considered to affect the behavior of ionocytes. Furthermore, it was noted that PPP and BMS, the two specific IGF1R inhibitors, did not fully inhibit secretory ion currents (Fig. 6A, B, C and D). There are several hormones, such as vasotocin and atrial natriuretic peptide, which have been reported to exert actions on ion regulation mechanisms after acute salinity stress (Takei *et al.* 2014). These hormones may compensate the defective functions caused by blocking Igf1 signaling. Nevertheless, how these fast-reacting hormones interact to regulate salt secretion mechanism to cope with acute salinity stress is a challenging issue and remains to be studied in the future.

Vertebrates developed transepithelial water and ion transport functions to achieve body fluid homeostasis when they evolved into iono- and osmoregulators, and hormonal regulation appears to have co-evolved with these transport machineries. Euryhaline teleosts (or other iono- and osmoregulatory fish) initially developed diverse and sophisticated traits for transporting water and ions (absorption and secretion of water and ions), and these traits are mostly conserved in other advanced vertebrates, including mammals; although, the precise transport functions and target tissues have changed during evolution (Evans *et al.* 2005, McCormick & Bradshaw 2006, Takei *et al.* 2014). Dissecting the hormonal control mechanisms underlying those transport functions enables us to further understand how iono-/osmoregulation traits may have been shaped during vertebrate evolution. The present study reveals that Indian medaka, an emerging model for fish osmoregulation, activates Na⁺/Cl⁻ secretion, without transcriptional regulation of transporters, but with remodeling of ionocyte organization to cope with acute salinity stress. Moreover, Igf1 was found to exert action on transporter activities and to enhance the formation of multicellular complexes of ionocytes to increase paracellular transport. These new findings not only describe how euryhaline teleosts activate timely and efficient salt secretion machineries to cope with acute salinity stress but also highlight the physiological significance of Igf1-regulated ion transport for body ionic and osmotic homeostasis as a key trait during the evolution of vertebrate species.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-20-0053>.

Declaration of interest

The authors declared that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J-J Y and P-P H conceived and designed the research. J-J Y, Y-C L, and Y-C T carried out the acclimation experiments and molecular/cellular analyses.

Y-L T performed SIET analyses. J-J Y and P-P H wrote the manuscript draft. P-P H supervised the project and finalized the manuscript. All authors approved the manuscript for publication.

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