Angiotensin-II promotes Na^+ uptake in larval zebrafish, *Danio rerio*, in acidic and ion-poor water

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Abstract

The contribution of the renin–angiotensin system (RAS) to Na^+ uptake was investigated in larval zebrafish (*Danio rerio*). At 4 days post fertilization (dpf), the level of whole-body angiotensin-II (ANG-II) was significantly increased after 1- or 3-h exposure to acidic (pH=4.0) or ion-poor water (20-fold dilution of Ottawa tapwater), suggesting rapid activation of the RAS. Long-term (24 h) treatment of 3 dpf larvae with ANG-I or ANG-II significantly increased Na^+ uptake which was accompanied by an increase in mRNA expression of the $\text{Na}^+\text{-Cl}^-$ cotransporter (*zslc12a10.2*). Induction of Na^+ uptake by exposure to ANG-I was blocked by simultaneously treating larvae with lisinopril (an angiotensin-converting enzyme inhibitor). Acute (2 h) exposure to acidic water or ion-poor water led to significant increase in Na^+ uptake which was partially blocked by the ANG-II receptor antagonist, telmisartan. Consistent with these data, translational knockdown of renin prevented the stimulation of Na^+ uptake following exposure to acidic or ion-poor water. The lack of any effects of pharmacological inhibition (using RU486), or knockdown of glucocorticoid receptors on the stimulation of Na^+ uptake during acute exposure to acidic or ion-poor environments, indicates that the acute effects of RAS occur independently of cortisol signaling. The results of this study demonstrate that the RAS is involved in Na^+ homeostasis in larval zebrafish.

Key Words

- ▶ angiotensin-II
- ▶ cortisol
- ▶ zebrafish
- ▶ osmoregulation
- ▶ ion-poor water
- ▶ acidic water

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Introduction

To maintain their body fluids hypertonic to the dilute environment, freshwater teleosts actively absorb ions through specialized epithelial cells termed ionocytes. The molecular mechanisms underlying the active absorption of Na^+ , Cl^- , and Ca^{2+} have been investigated extensively over the past 80 years (for recent reviews see Evans (2011), Hwang *et al.* (2011), Dymowska *et al.* (2012) and Kumai & Perry (2012)). Based on these previous studies, it is clear that ion uptake is tightly regulated by several hormones, including prolactin (Pickford & Phillips

1959, Breves *et al.* 2010), cortisol (Laurent & Perry 1990, Lin *et al.* 2011, Kumai *et al.* 2012a), stanniocalcin (Tseng *et al.* 2009), vitamin D (Lin *et al.* 2012) and isotocin (Chou *et al.* 2011), as well as being under neurohumoral control (Perry *et al.* 1984, Kumai *et al.* 2012b). The existence of multiple hormonal regulators of ionic regulation highlights the importance of body fluid ionic homeostasis.

In mammalian kidney, which shares several similar functions with the fish gill, angiotensin-II (ANG-II) is recognized as a major regulatory hormone, controlling

salt reabsorption (Crowley & Coffman 2012). Angiotensinogen is converted into biologically inactive ANG-I by the enzyme renin, which in turn is converted into biologically active ANG-II by angiotensin-converting enzyme (ACE). Although recent studies have identified additional proteins interacting with ANG-II and renin, such as prorenin, renin receptors, and ACE-2, the enzymatic reactions resulting in the synthesis of ANG-II constitute the renin–angiotensin system (RAS; Santos *et al.* 2013). Based on *in vivo* and *in vitro* studies, ANG-II is known to increase the expression and/or activities of all major transporters involved with Na⁺ and acid transport in the various segments of the nephron, including Na⁺/H⁺ exchanger 3 (NHE3; Geibel *et al.* 1990, Cano *et al.* 1994), H⁺-ATPase (Rothenberger *et al.* 2007), epithelial Na⁺ channel (Peti-Peterdi *et al.* 2002), and thiazide-sensitive Na⁺-Cl⁻ cotransporter (Sandberg *et al.* 2007, San-Cristobal *et al.* 2009, Castaneda-Bueno *et al.* 2012). Although there are two distinct types of ANG-II receptors, referred to as type-I and -II (AT₁ and AT₂) receptors, expression of AT₂ is much lower than that of AT₁ in adult mammalian tissues, and consequently, the majority of the physiological effects of ANG-II, including the above-mentioned transporter activation, are attributed to AT₁-mediated signaling (Stegbauer & Coffman 2011).

As in mammals, the RAS is physiologically relevant in fish, where it is known to participate in fluid volume control and blood pressure regulation (Smith *et al.* 1991, Tierney *et al.* 1995, Bernier *et al.* 1999b, Takei & Tsuchida 2000, Nishimura 2001, Russell *et al.* 2001). While significant inter-species differences exist with respect to the mechanisms for blood pressure regulation by ANG-II (Bernier *et al.* 1999b), plasma [ANG-II] and renin activities have often been reported to be transiently or chronically elevated following transfer to seawater in several euryhaline fish species (Smith *et al.* 1991, Tierney *et al.* 1995, Anderson *et al.* 2006). Consequently, despite the extensive research related to ANG-II and renal salt reabsorption, surprisingly little is known about its potential role in freshwater fish ionic regulation. Smith *et al.* (1991) reported a decline in plasma renin activity in freshwater-acclimated rainbow trout fed with a salt-enriched diet, suggesting a possible role for ANG-II in the regulation of salt balance in freshwater fish. Subsequently, Hoshijima & Hirose (2007) reported an increase in renin mRNA expression during acclimation of zebrafish to ion-poor water (20-fold dilution of the control water), suggesting a role of the RAS in promoting salt absorption. Despite these studies, the question of whether the RAS promotes salt uptake in freshwater fish remains unexplored. Thus, the

main objective of this study was to test the hypothesis that ANG-II contributes to ionic homeostasis in a freshwater teleost. More specifically, it was proposed that ANG-II stimulates the absorption of Na⁺ when zebrafish are exposed to conditions that challenge Na⁺ uptake (e.g., low environmental pH and ion-poor water (Kumai & Perry 2011, Kumai *et al.* 2012b)). By monitoring ANG-II levels by RIA, treating larvae with inhibitors of various steps of RAS signaling, and knocking down the expression of renin, we revealed an important role of ANG-II in acutely inducing Na⁺ uptake in zebrafish larvae. The results of this study introduce RAS as an additional and important endocrine mechanism regulating ionic homeostasis in freshwater fish.

Materials and methods

Experimental animals

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 and dechloraminated City-of-Ottawa tap water at 28 °C. Fish were subjected to a constant 14 h light:10 h darkness photoperiod and fed daily until satiation with No. 1 crumble-Zeigler (Aquatic Habitats, Apopka, FL, USA). The embryos were collected and reared in 50 ml Petri dishes with dechloraminated City-of-Ottawa tap water (in µM; [Na⁺]=783±6.2, [Ca²⁺]=263±1.6, [Mg²⁺]=139±3.8, [K⁺]=24.5±0.4; Kwong *et al.* (2013)) supplemented with 0.05% methylene blue. The Petri dishes were kept in incubators at 28.5 °C. The dead embryos were removed and water was changed daily. 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 were purchased from Sigma.

Series 1: effect of acute exposure to acidic/ion-poor environments on whole-body ANG-II levels

Zebrafish larvae (4 days post fertilization (dpf)) were flash frozen in liquid N₂ after 1 or 3 h exposure to acidic or ion-poor water (50 larvae were pooled to generate one sample) and stored at -80 °C until extraction and analysis of whole-body ANG-II levels by RIA was carried out according to the protocol of Bernier *et al.* (1999a). Ion-poor water was prepared by diluting Ottawa tapwater 20-fold with

deionized water. Acidic water (pH ~4.0) was prepared by adding H₂SO₄ to Ottawa tapwater. Larval zebrafish were homogenized in 350 µl acidic acetone (a mixed solution of acetone, H₂O, and 1 M HCl at 40:5:1) and the homogenate was centrifuged for 10 min at 4 °C at 10 000 g. The supernatant was transferred to a 1.5-ml micro-centrifuge tube, and the extraction process was repeated on the remaining pellet. The supernatant collected from the two rounds of extractions was combined and lyophilized. The lyophilized samples were reconstituted in 350 µl RIA buffer (10 mM PBS, pH 7.4; 140 mM NaCl; 0.1% w/v NaN₃; 40 mM Na₂EDTA; 10 mM 6-aminogexanoic acid; 0.25% (v/v) Triton X-100; and 0.25% RIA-grade BSA fraction V).

For the RIA, samples for the standard curve were prepared by mixing serially diluted 0.1 ml standard [Asn¹, Val⁵]-ANG-II ligand, 0.1 ml antiserum raised against [Asp¹, Ile⁵]-ANG-II (1:3000 dilution; cat# T-4005, Bachem, Torrance, CA, USA), and 0.1 ml normal rabbit serum (1:250 dilution; cat# 869019; Calbiochem, Gibbstown, NJ, USA). After incubation for 20 h at 4 °C, 0.05 ml ¹²⁵I-labeled [Asp¹, Ile⁵]-ANG-II (~7500 c.p.m.; specific activity = 2200 Ci/mmol; Perkin Elmer, Woodbridge, ON, Canada) was added to the mixture and incubated for another 24 h at 4 °C. Antigen bound to ¹²⁵I ANG-II was precipitated by adding 0.1 ml PANSORBIN Cells (0.25%; Calbiochem) and incubating for 5 h at 4 °C. Subsequently, samples were centrifuged at 2000 g for 1 h at 5 °C and radioactivity of the precipitates was determined using a WIZARD2 gamma counter (Perkin Elmer). For measurement of ANG-II in extracted samples, 0.1 ml ANG-II standard was replaced with larval extract (the extraction efficiency was 89.8%). To confirm that the extraction protocol originally developed for plasma was also appropriate for larval tissue, tissue samples were 'spiked' with [Asn¹, Val⁵]-ANG-II. ANG-II was extracted from the 'spiked' sample and was serially diluted to confirm that the dilution curve ran parallel to the standard curve. All samples were measured in triplicate within a single assay.

Series 2: consequences of treatment with waterborne ANG

Series 2.1: Na⁺ uptake To determine the effect of extended (24 h) elevation of ANG on Na⁺ uptake, larvae were treated with 100, 500, or 1000 nM ANG-I ([Asn¹, Val⁵, Asn⁹]-ANG-I) or ANG-II ([Asn¹, Val⁵]-ANG-II) between 3 and 4 dpf. After the 24-h exposure, Na⁺ uptake was measured in the control water using ²²Na. For the measurement, 0.25 µCi ²²Na in the form of NaCl (Perkin Elmer) was added to each tube to a final concentration

of 0.15 µCi/ml. Radioactivity was analyzed in the water samples (50 µl) at the beginning and at the end of a 2-h flux period as well as in digested larvae. Sample preparations and calculations for estimating Na⁺ uptake are described elsewhere (Kumai *et al.* 2012b). Unless stated otherwise, Na⁺ uptake was measured in the continual presence of pharmacological reagents.

To assess the effect of ANG-I treatment is due to modulation of RAS, another group of larvae exposed to 500 nM ANG-I was cotreated with 100 µM lisinopril (an ACE inhibitor) kept in the control water. The dosage of lisinopril used in this study (100 µM) was chosen based on a previous study on other species of teleosts (Bernier *et al.* 1999a,b). The Na⁺ uptake in these groups of larvae was measured at 4 dpf as described above.

Series 2.2: RNA extraction and real-time PCR for Na⁺-transporting genes

To determine the effects of ANG-II on the expression of Na⁺ transporting genes, mRNA levels of NHE3b (*zslc9a3b*), Na⁺-Cl⁻ cotransporter (NCC) (*zslc12a10.2*), and H⁺-ATPase (*zatp6v1a*) were analysed in 4 dpf zebrafish larvae after 24 h treatment with 500 nM ANG-II. After treatment, the larvae were killed by MS-222 overdose, flash frozen, and stored at -80 °C until RNA extraction. Total RNA (ten larvae were pooled for each sample) was extracted with TRIZOL (Invitrogen) according to manufacturer's instructions. cDNA was synthesized by treating 1 µg of extracted RNA with DNase (Invitrogen) and RevertAid M-MNuLV reverse transcriptase (Fermentas, Burlington, ON, Canada) according to the manufacturer's instructions. Real-time PCR was performed using a Bio-Rad CFX96 qPCR system with Brilliant III SYBR Green Master Mix (Agilent Technologies, La Jolla, CA, USA). PCR conditions for all primer sets were as follows; 95 °C for 3 min, 40 cycles of 95 °C for 20 s, and 58 °C for 20 s, with final extension for 5 min at 72 °C. Data were normalized to the expression of 18S, and were presented relative to the control group. For the list of primers, see Table 1.

Series 3: consequences of pharmacological inhibition of RAS during acute exposure to acidic or ion-poor water

To assess the potential role of the RAS in the regulation of Na⁺ uptake, zebrafish larvae (4 dpf) raised in control water were exposed for 30 min to 10 µM telmisartan (Santa Cruz Biotechnology; a selective AT₁ blocker). Following the 30-min preincubation, larvae were exposed to either acidic or ion-poor water for 2 h in the continual presence of telmisartan. After the acidic and ion-poor water exposures,

Table 1 Primer sets used in the present study

Gene	Sequence	Reference
NHE3 (<i>zslc9a3b</i>)	FWD: 5'-TGC AGA CAG CGC CTC TAG C-3' REV: 5'-TGT GGC CTG TCT CTG TTT GC-3'	Yan <i>et al.</i> (2007)
H ⁺ -ATPase (<i>zatp6v1a</i>)	FWD: 5'-GAG GAA CCA CTG CCA TTC CA-3' REV: 5'-CAA CCC ACA TAA ATG ATG ACA TCG-3'	Chang <i>et al.</i> (2009)
NCC (<i>zslc12a10.2</i>)	FWD: 5'-GCC CCC AAA GTT TTC CAG TT-3' REV: 5'-TAA GCA CGA AGA GGC TCC TTG-3'	Wang <i>et al.</i> (2009)
18S	FWD: 5'-GGC GGC GTT ATT CCC ATG ACC-3' REV: 5'-GGT GGT GCC CTT CCG TCA ATT C-3'	Kumai <i>et al.</i> (2011)

larvae were transferred back to the control water for Na⁺ uptake measurement.

Series 4: consequences of renin knockdown on Na⁺ uptake

To more directly determine the role of RAS in rapidly modulating Na⁺ uptake, a translation blocking morpholino targeting renin (5'-AGTCAAGCAGTGGATT-TTCATTCTC-3') was designed by Gene Tools; the 3' end was conjugated with carboxyfluorescein. Larvae received injections of renin MO at a dose of 4 ng/embryo at 1- to 2-cell stages and after 24 hours post fertilization (hpf) were screened microscopically for the widespread presence of fluorescein (SMZ1500 microscope; Nikon Instruments, Melville, NY, USA). Only fluorescein-positive embryos were used for subsequent experiments. To control for the effect of microinjection, a separate group of larvae received injections of the same dose of control MO (5'-CCTCTTACCTCAGTTACAATTATA-3') and handled as the renin MO-injected group. The Na⁺ uptake in sham and renin morphants following acute exposure to acidic and ion-poor water was determined as described above using 4 dpf larvae. Acidic water (pH ~4.0) was prepared by adding H₂SO₄ to Ottawa tapwater. Ion poor water was prepared by diluting Ottawa tapwater by tenfold with deionized water. No morphological abnormalities were observed in the renin morphants.

Effectiveness of knockdown was confirmed with western blotting using a renin antibody (ARP41409_T100; Aviva Systems Biology, San Diego, CA, USA) whose epitope shared 78% identity with zebrafish renin precursor amino acids 348–394; accession number NP_998025.1. Western blotting was performed as described in Kwong & Perry (2013), with the exception that total protein was extracted from MO- and sham-injected larvae using Tris buffer (10 mM Tris-HCl with 2% Triton X-100; pH adjusted to 7.4) supplemented with protease inhibitor tablet (Complete Mini, Roche).

Series 5: potential involvement of cortisol in mediation of the effects of the RAS on Na⁺ uptake

Because of the well-known interaction between angiotensin and aldosterone, we investigated whether cortisol might be involved in the regulation of Na⁺ uptake, potentially by interacting with ANG-II. The function of the glucocorticoid receptor (GR) was inhibited either by pharmacological blockade of the receptor using 1 μM RU-486 or translational gene knockdown of GR using a morpholino antisense oligonucleotide against zebrafish GR (5'-CTCCAGTCCTCCTTGATCCATTTTG-3') (Kumai *et al.* 2012a). For RU-486 treatment, larvae were exposed to 1 μM of RU-486 dissolved in DMSO for 30 min before being transferred to either acidic or ion-poor water for 2 h in the continued presence of inhibitor/DMSO. Final concentration of DMSO did not exceed 0.1%. Fish were subsequently transferred back to the control media and their Na⁺ uptake was measured.

GR morpholino and sham-injected larvae were raised in control water until 4 dpf and then directly challenged with acidic or ion-poor water for 2 h. Subsequently, they were transferred back to the control water and their Na⁺ uptake was measured. To verify the effectiveness of knockdown, separate groups of sham and GR morphants were treated with either DMSO or 500 nM cortisol for 2 days (between 2 and 4 dpf); Na⁺ uptake was measured in the control water at 4 dpf.

Statistical analysis

All statistical analyses were performed with SigmaPlot (v. 11, Systat, Inc., Chicago, IL, USA). Student's *t*-test was used to analyze data from Series 2.2. One-way ANOVA followed by the Tukey *post hoc* test was used to analyze data from Series 2.1 and 3. This *post hoc* test was chosen based on its conservativeness. Data from the RU-486 treatment experiment (Series 5) were analyzed using one-way ANOVA on ranks because the data could not be transformed to meet

Figure 1

The effect of acute exposure to acidic or ion-poor water on whole-body ANG-II content. Acute (1 and 3 h) exposure to acidic or ion-poor water significantly increased whole-body ANG-II content in 4 dpf zebrafish larvae. *Significant difference between the treatment and control groups (one-way ANOVA; $n=7-8$). Data are presented as means $\pm$ S.E.M.

the assumptions for normality. Data in Series 1 were analyzed by one-way ANOVA comparing all treatment groups with the control group using the Holm-Sidak *post hoc* test with critical values adjusted to 0.013–0.025. Two-way ANOVA followed by the Tukey *post hoc* test was used to analyze data from Series 4 and 5 (all data involving GR knockdown). When assumptions of normality or equal variance were violated, data were transformed using natural log- or square-root transformation. For all analyses, the level of statistical significance was set at $P \leq 0.05$.

Results

Effect of exposure to ion-poor or acidic water on whole body ANG-II levels

Acute (1 or 3 h) exposure to either acidic or ion-poor water significantly increased (roughly by fourfold) whole-body ANG-II levels compared with the control group (Fig. 1; one-way ANOVA; $n=7-8$; $F=5.534$; overall $P=0.002$; $P \leq 0.007$ for all comparisons).

Effect of chronic exposure to ANG-I and ANG-II on Na^+ uptake

Exposure to either ANG-I or ANG-II led to significant increases in Na^+ uptake by zebrafish larvae (Fig. 2A and B; one-way ANOVA; $n=6-12$; $F=7.684$ and $P < 0.001$ for Fig. 2A and $F=4.255$ and $P=0.018$ for Fig. 2B). In both cases, uptake was most robustly stimulated when fish were

treated with a 500 nM dose and the pattern of response appeared to be biphasic. The stimulatory effect of ANG-I was inhibited by cotreating the larvae with 100 μM lisinopril (Fig. 2C; one-way ANOVA; $n=7$; $F=9.244$; overall $P=0.002$). When the mRNA expression of three major Na^+ transporting genes was analyzed after 24-h treatment with ANG-II, the expression of only NCC (*zslc12a10.2*) was significantly elevated ($P=0.016$), whereas the changes in NHE3 (*zslc9a3b*) and H^+ -ATPase (*zatp6v1a*) expression levels were not statistically significant ($P=0.158$ and 0.114 respectively), indicating that the increase in Na^+ uptake after ANG-II treatment is at least partially mediated by NCC (Fig. 2D; Student's *t*-test, $n=6$).

Pharmacological inhibition of RAS during exposure to acidic or ion-poor water

Acute (2 h) exposure to acidic water caused a significant increase in Na^+ uptake (Fig. 3A and B). When larvae were pretreated with telmisartan, the stimulation of Na^+ uptake was attenuated during both acidic (Fig. 3A; one-way ANOVA; $n=5-12$; $F=7.182$; overall $P=0.004$) and ion-poor (Fig. 3B; one-way ANOVA; $n=6-12$; $F=28.134$; overall $P < 0.001$) water exposure. However,

Figure 2

The effect of chronic exposure to ANG on Na^+ uptake. Treatment of 3 dpf larvae for 24-h with 100–1000 nM ANG-I (A; $n=12$; one-way ANOVA) or ANG-II (B; $n=6$) significantly increased Na^+ uptake at doses above 500 nM. The induction of Na^+ uptake by 500 nM ANG-I was abolished when larvae were cotreated with 100 μM lisinopril (C; $n=7$; one-way ANOVA). The mRNA expression level of NCC was significantly elevated (D; $n=6$; Student's *t*-test); changes in NHE3b and H^+ -ATPase expression levels were not statistically significant. In A, B, and C, groups not sharing the same letters are significantly different from each other. Data are presented as means $\pm$ S.E.M.

Figure 3

Effect of AT₁ inhibition during acute exposure to acidic and ion-poor water. Exposure to acidic (Fig. 3A; $n=5-12$; one-way ANOVA) and ion-poor water (Fig. 3B; $n=6-12$; one-way ANOVA) in the presence of 10 μ M telmisartan (an AT₁ selective inhibitor) significantly reduced Na⁺ uptake. In A and B, groups not sharing the same letters are significantly different from each other. Data are presented as means $\pm$ S.E.M.

Na⁺ uptake remained significantly elevated in the group exposed to ion-poor water even after the treatment with telmisartan (Fig. 3B).

Consequences of renin knockdown on Na⁺ uptake in response to acute challenges

Effective knockdown of renin following morpholino injection was demonstrated by western blotting (Fig. 4A and B). The usual stimulatory effects of acute (2 h) exposure to acid- or ion-poor water on Na⁺ uptake were attenuated in the renin morphants (Fig. 4C and D; two-way ANOVA; $n=5-13$; F and $P=472$ and <0.001 for water treatment effect; 2.98 and 0.1 for knockdown effect and 9.217 and 0.007 for interaction effect for Fig. 4C; F and $P=13.31$ and <0.001 for water treatment effect; 1.157 and 0.288 for knockdown effect; and 4.045 and 0.05 for interaction effect for Fig. 4D). The renin knockdown caused greater inhibition on Na⁺ uptake in the group exposed to ion-poor water.

Lack of effect of cortisol on Na⁺ uptake during acute exposure to acidic or ion-poor water

Pretreating larvae with 1 μ M RU-486 before and during acute (2 h) exposure to acidic or ion-poor water did not influence the usual stimulation of Na⁺ uptake (Fig. 5A and B; $n=5-6$; one-way ANOVA on Ranks; $Q=2.78$ for Fig. 5A and >2.49 for Fig. 5B). A similar result was observed in GR morphants; acid- or ion-poor water-exposure increased Na⁺ uptake equally in both sham-treated and GR morphants (Fig. 5D and E; $n=6$; two-way ANOVA; for Fig. 5D and F and $P=223.46$ and <0.001 , 0.0668, and 0.799 and 7.143 and 0.015 for water treatment, knockdown, and interaction effect respectively, and for Fig. 5E and F and $P=72.34$ and <0.001 , 1.83, and 0.19 and 1.142 and 0.297 for water treatment, knockdown, and interaction effect respectively). However, the previously reported prominent role of GR in the regulation of Na⁺ uptake following chronic (48 h) elevation of cortisol was reconfirmed in this study; unlike sham-treated fish, the GR morphants did not respond to treatment with 500 nM cortisol (Fig. 5C, $n=6$; two-way ANOVA; F and $P=19.98$ and <0.001 , 13.68, and 0.001 and 11.047 and 0.003 for cortisol, knockdown, and interaction effect respectively).

Figure 4

The effect of renin knockdown on Na⁺ uptake during acute acid and ion-poor water exposure. Western blotting with a renin antibody detected a band corresponding to the expected size of renin in a protein derived from sham-injected 4 dpf larvae (~ 35 kDa; A; lane 'S'). This band was not observed in the protein derived from the renin morphants, confirming the successful knockdown of renin (A; lane 'M'). The same membrane was blotted with an anti- β -actin antibody, which was used as the loading control (B). Na⁺ uptake in renin morphants was not significantly induced following acute exposure to acid- or ion-poor water (C and D; two-way ANOVA; $n=5-13$). *Significant difference between groups indicated by brackets over the data points as revealed by *post hoc* test. Data are presented as means $\pm$ S.E.M.

Figure 5

Cortisol does not contribute to the stimulation of Na^+ uptake during acute exposure to acidic- or ion-poor water. Treatment of 4 dpf larvae with 1 μM RU-486 (a GR antagonist) did not affect stimulation of Na^+ uptake during acute (2 h) exposure to acidic (A; $n=5-6$; one-way ANOVA) or ion-poor water (B; $n=5-6$; one-way ANOVA). Na^+ uptake by 4 dpf GR morphants was not affected by 48 h-long waterborne treatment with 500 nM cortisol (between 48 and 96 hpf; C; $n=6$; two-way ANOVA), thus confirming the effectiveness of the knockdown. Furthermore, GR knockdown did not

impair the ability of 4 dpf larvae to stimulate their Na^+ uptake in response to acute (2 h) acid or ion poor water exposure (D and E; $n=6$; two-way ANOVA). In A and B, groups not sharing the same letters are significantly different from each other. *Significant difference between groups indicated by brackets over the data points as revealed by *post hoc* test. In the case of E, owing to the absence of interaction between the two factors, *post hoc* analysis was restricted to comparing the global effects of exposure to ion-poor water. Data are presented as means $\pm$ S.E.M.

Discussion

Based on the increase in whole-body levels of ANG-II during exposure of larvae to acidic or ion-poor water and the marked attenuating effects of RAS inhibition on Na^+ uptake, this study provides the first direct evidence, to our knowledge, that the RAS is involved in the stimulation of Na^+ uptake in freshwater fish. In addition, chronic (24 h) waterborne treatment of larvae with ANG-I or ANG-II significantly elevated Na^+ uptake, indicating that RAS might affect Na^+ uptake in freshwater fish during both acute and chronic environmental stress. Because cortisol does not appear to acutely stimulate Na^+ uptake, the RAS, at least during acute exposure to acidic or ion-poor water, apparently exerts its effects independent of cortisol.

The RAS promotes Na^+ uptake in zebrafish larvae

The physiological significance of the RAS in the regulation of salt reabsorption in the mammalian kidney has been firmly established (see Introduction). Although previous studies (e.g., Hoshijima & Hirose 2007) indicated a role for

the RAS in the activation of ion uptake in freshwater fish, no convincing physiological data have been published to support this idea. The rapid increase in whole-body ANG-II content in response to two experimental treatments known to induce uptake of Na^+ (Fig. 1) as well as the elevation of Na^+ uptake following chronic treatment with either ANG-I or ANG-II (Fig. 2) demonstrate the potential of the RAS to stimulate Na^+ uptake in zebrafish. Although ANG is a hydrophilic peptide hormone which normally would not be expected to readily cross epithelia, the results of this study suggest that it could enter zebrafish larvae. Indeed, the prevention of the effects of waterborne ANG-I by pretreatment with the ACE inhibitor lisinopril (Fig. 2C) strongly indicates that ANG-I enters zebrafish in which it is converted to ANG-II. Rather than transcellularly, ANG may enter larvae via tight junctions (Salama *et al.* 2006). The dosage of ANG used in this study was higher than in previous studies, in which isolated kidney segments were infused with ANG-II (1–100 nM; Houillier *et al.* 1996, Rothenberger *et al.* 2007); the requirement for higher levels in this study may reflect the reduced permeability of ANG into larval zebrafish. Although the exact route of entry

warrants clarification, ultimately the penetration of ANG is no different than the apparent entry of numerous other hydrophilic compounds, such as catecholamines and propranolol, into zebrafish larvae (Steele *et al.* 2011, Kumai *et al.* 2012b). It is also interesting to note that waterborne treatment with both ANG-I and ANG-II appeared to have a biphasic effect, rather than simple dose-dependent stimulation. A similar biphasic effect of the RAS on transepithelial Na^+ transport was reported for ANG-II (Houillier *et al.* 1996) and angiotensin 1–7 (Garcia & Garvin 1994). The proposed role of the RAS in osmoregulation by larval zebrafish is further supported by our observation that knockdown of renin, an enzyme responsible for initiating the enzymatic reactions that synthesize ANG-II, blunts stimulation of Na^+ uptake in response to acute exposure to both acidic and ion-poor water (Fig. 4). However, because renin morphants still increased their Na^+ uptake in response to acid exposure, there may be other physiological mechanisms contributing to the response, including the possible involvement of adrenergic receptors (Kumai *et al.* 2012b). In contrast, renin knockdown abolished the stimulation of Na^+ uptake associated with ion-poor water exposure (Fig. 4D). While these findings indicate that the RAS is the sole mechanism responsible for stimulating Na^+ uptake in ion-poor water, the interpretation is confounded because knockdown of β_{2A} receptors also attenuates Na^+ uptake stimulation in ion-poor water exposure (Kumai *et al.* 2012b). Potential interaction between the RAS and adrenergic receptors in the regulation of Na^+ uptake, especially under ion-poor conditions, is interesting for future investigations.

ANG-II exerts its physiological effects by interacting with two distinctive receptor sub-types, AT_1 and AT_2 . In fish, AT_1 receptors have been identified in several species, including eel, *Anguilla anguilla*; toadfish, *Opsanus beta*; and rainbow trout, *Oncorhynchus mykiss* (Nishimura 2001). Although Tucker *et al.* (2007) reported the presence of putative AT_1 receptors in zebrafish, there is debate as to whether the ligand for this receptor is actually apelin rather than ANG-II (Zeng *et al.* 2007). Based on immunohistochemical staining with a mouse MAB, the AT_1 receptor was localised to osmoregulatory tissues, including ionocytes from eel gill (for review, see Nishimura (2001) and Russell *et al.* (2001)). The results of this study revealed an inhibitory effect of AT_1 blockade (using telmisartan) on Na^+ uptake, indicating the probable involvement of AT_1 in mediating the stimulatory effect of ANG-II on Na^+ uptake in zebrafish. The dosage of telmisartan used in this study (10 μM) was higher than that used in a previous study that used this chemical on adult medaka (1 μM ; Kuwashiro *et al.* 2011),

although in that study, the fish were treated chronically (several weeks). In an attempt to visualize the ANG-II binding sites in zebrafish larvae, fish were incubated with fluorescently conjugated ANG-II, which was used previously to visualize ANG-II receptors in the posterior cardinal vein of rainbow trout (Bernier & Perry 1997). However, no clear staining was observed (Y Kumai and S F Perry, unpublished observations) and to our knowledge there is no commercial antibody able to recognize the zebrafish ANG-II receptor. Wong & Takei (2013) recently have reported the sequence of the AT_2 receptor from *A. japonica* and observed its high expression in spleen and gill; no particularly strong staining was observed in branchial ionocytes based on *in situ* hybridization. Because the same study suggested the presence of an AT_2 -like gene in zebrafish based on sequence analysis, it is possible that AT_2 is also involved with Na^+ uptake regulation in zebrafish larvae. Although the lack of an AT_2 blocker whose specificity has been verified on zebrafish makes this idea difficult to test experimentally, the potential role of AT_2 in osmoregulation (and other function of the RAS in fish physiology) warrants further investigation.

As discussed in the Introduction, in mammalian kidney ANG-II activates H^+ -ATPase, NHE3, and NCC, all of which have been suggested to play a role in Na^+ uptake by zebrafish (for recent reviews see (Hwang *et al.* 2011, Kumai & Perry 2012). As an initial attempt to determine the transporter(s) activated by ANG-II treatment, we assessed the mRNA expression level of NCC, *NHE3b*, and *H⁺-ATPase* in 4 dpf zebrafish larvae following a 24-h treatment with ANG-II. Interestingly, only the expression level of NCC was significantly elevated following chronic ANG-II treatment (Fig. 2D), indicating that at least part of the increase in Na^+ uptake following chronic ANG-II treatment is mediated by NCC, although it is possible that other transporters are also being regulated, but solely through posttranslational regulation. It is interesting to note that stimulation of Na^+ uptake following exposure to low Cl^- water was attenuated in the presence of telmisartan (data not shown), indicating that NCC might also be activated by ANG-II within hours. If such regulation indeed takes place, it is most probably post-translational (e.g., phosphorylation; van der Lubbe *et al.* (2011)). Clearly, the exact identity of the Na^+ transporters being regulated by ANG-II warrants further investigation.

Does RAS interact with cortisol in zebrafish?

Although recent studies have demonstrated independent effects of aldosterone and the RAS on salt reabsorption in

the mammalian kidney (van der Lubbe *et al.* 2011, 2012), the two endocrine systems are intricately linked and indeed the RAS is sometimes referred to as the renin-angiotensin-aldosterone system (RAAS). Although fish possess mineralocorticoid receptors (MR; Sturm *et al.* 2005), they lack the capacity to synthesize significant amounts of aldosterone (Colombo *et al.* 1972). Thus, in fish, cortisol binds to both MR and GR to activate their downstream signaling (McCormick & Bradshaw 2006, Takahashi & Sakamoto 2013). The physiological role of cortisol in promoting ionic uptake in freshwater fish is extensively documented (Laurent & Perry 1990, Lin *et al.* 2011, Kumai *et al.* 2012a). It was recently demonstrated that waterborne treatment with cortisol between 48 and 96 hpf promotes Na⁺ uptake in zebrafish larvae through signaling via GR (Kumai *et al.* 2012a). To test whether the stimulation of Na⁺ uptake by ANG-II is mediated by secondary induction of cortisol, the function of GR was inhibited by treatment with 1 µM RU-486 (a dose that was shown to abolish the stimulatory effect of cortisol) and injection of 2-ng GR-morpholino (a dose that was shown to effectively knockdown GR-expression) (Kumai *et al.* 2012a, Kwong & Perry 2013). Inhibition of GR function using either approach did not appear to impede the increase in Na⁺ uptake in response to acute acidic or ion-poor water exposure; note, however, that there was a significant interaction effect shown in Fig. 5D, indicating that the magnitude of Na⁺ uptake stimulation with acid-exposure differed between the sham and GR MO groups and thus the absence of a significant difference between the acid-treated sham and GR MO groups should be interpreted with caution. Moreover, because of the lack of a significant interaction shown in Fig. 5E, we could not conclude that there was significant difference between fish of the sham and GR-MO groups exposed to the ion-poor water. These observations indicate that, during acute (2 h) experimental challenges, signaling via GR is unlikely to play a major role in inducing Na⁺ uptake. Although Kumai *et al.* (2012a) suggested that MR does not play a significant role in the regulation of Na⁺ uptake in zebrafish during chronic exposure to acidic water, in this study we tested the hypothesis that cortisol might interact with MR during acute challenge with acidic or ion-poor water. Treating larvae with 10 µM eplerenone, a recently developed MR inhibitor known to be effective in zebrafish (Pippal *et al.* 2010), did not hinder the capacity of fish to increase Na⁺ uptake during acute exposure to acidic or ion-poor water (data not shown). Thus, the effect of ANG-II on Na⁺ uptake reported in this study is likely to be cortisol-independent. Whether the acute physiological

responses mediated by ANG-II eventually trigger, over a longer term, a cortisol-mediated increase in Na⁺ uptake is an interesting area for further research.

Perspectives

Despite the well-documented role of the RAS in promoting salt reabsorption in the mammalian kidney, its role in promoting ionic uptake in freshwater fish has been largely unexplored. With the current study providing the first evidence, to our knowledge, implicating the RAS in the regulation of Na⁺ uptake in zebrafish, a number of issues emerge including i) whether ANG-II receptors are expressed in osmoregulatory tissues (adult gill, and kidney, and larval skin), and the relative contribution of AT₁ and AT₂ receptors, ii) how ANG-II interacts with other known regulatory mechanism of ion uptake in freshwater fish, iii) what are the downstream signaling cascades activated by ANG-II receptors, that ultimately lead to the increase in Na⁺ uptake, and iv) what are the mechanisms underlying the detection of rapid alterations in water chemistry and that trigger the synthesis of ANG-II. Development of more specific inhibitors for AT₁ and AT₂ receptors, as well as homologous antibodies directed against these receptors, would allow some of these questions to be addressed in the future.

Declaration of interest

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

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