Cortisol regulates epithelial permeability and sodium losses in zebrafish exposed to acidic water

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Abstract

The effects of cortisol on epithelial permeability and sodium (Na^+) handling during acid exposure were investigated in larval zebrafish (*Danio rerio*). The results demonstrated that the whole-body absorption of the paracellular permeability marker polyethylene glycol-4000 (PEG-4000) decreased with increasing levels of exogenous cortisol. Western blot analysis revealed that the abundance of the epithelial tight junction proteins occludin-a and claudin-b was increased after cortisol treatment. Furthermore, translational gene knock-down of claudin-b using an antisense morpholino oligonucleotide caused an increase in the permeability to PEG-4000, which was mitigated by cortisol treatment, further suggesting a role for cortisol in reducing paracellular permeability. Exposure to acidic water (pH 4.0 vs 7.6) caused an expected increase in the diffusive loss of Na^+ and a decrease in whole-body Na^+ levels. These disruptive effects of acute acid exposure on Na^+ balance were reduced by treatment of larvae with exogenous cortisol. Translational knockdown of the glucocorticoid receptor (GR) abolished the effects of cortisol on epithelial PEG permeability, suggesting that activation of GR was probably the major signaling pathway for reducing epithelial permeability. During acid exposure, the epithelial PEG permeability in the GR morphants was significantly higher than in the control fish. Additionally, GR morphants exhibited a more pronounced diffusive loss of Na^+ than the control fish during acid exposure. These findings suggest that cortisol may help to minimize the negative consequences of acid exposure on Na^+ homoeostasis via GR-mediated reductions in epithelial permeability and paracellular Na^+ loss.

Key Words

- cortisol
- efflux
- epithelial permeability
- sodium
- tight junction proteins
- zebrafish

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Introduction

Cortisol is the major corticosteroid in fish, and its effects on transcellular ion transport are well documented (see review by McCormick (2001)). Increasing evidence suggests that cortisol may also regulate paracellular permeability (Tipsmark *et al*. 2009, Bui *et al*. 2010, Chasiotis *et al*. 2010, Chasiotis & Kelly 2011). In vertebrates, paracellular permeability is governed primarily by tight junctions (TJs), which regulate the intercellular passage of water, ions and neutral solutes (Anderson & Van Itallie 2009). Three major trans-membrane protein families are found in TJs: claudins, occludins and junction adhesion molecules. To date, more than 20 members of claudins and two members of occludins have been identified in mammals (Furuse & Tsukita 2006) and in teleost fish (Loh *et al*. 2004, Tipsmark *et al*. 2008, Clelland & Kelly 2010, Kumai *et al*. 2011). Modulation of the expression of claudins and occludins by cortisol has been reported in several fish species

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(Tipsmark *et al.* 2009, Bui *et al.* 2010, Chasiotis *et al.* 2010, Chasiotis & Kelly 2011). For example, cortisol injection in freshwater (FW) Atlantic salmon (*Salmo salar*) was found to increase the expression of certain claudins (Tipsmark *et al.* 2009). Similarly, claudin and occludin expressions were increased in goldfish (*Carassius auratus*) and rainbow trout (*Oncorhynchus mykiss*) gill cell cultures after cortisol treatment (Chasiotis & Kelly 2011). Chasiotis *et al.* (2010) also demonstrated that cortisol treatment increased the abundance of occludin, which was associated with a reduced epithelial permeability in a trout gill cell culture.

In vertebrates, claudins are thought to be the regulators of paracellular fluxes of ions and uncharged solutes across epithelia and endothelia (Van Itallie & Anderson 2006). In particular, claudin-4 is known to form a charge-selective barrier to regulate paracellular Na^+ movement in mammalian renal collecting tubules (Van Itallie *et al.* 2001). Goldfish and zebrafish claudin-b, and salmon claudin-30, are orthologous to the mammalian claudin-4. In adult zebrafish and salmon, claudin-b/claudin-30 is expressed predominantly in major ion-regulatory organs including gill and kidney (Tipsmark *et al.* 2008, Clelland & Kelly 2010, Kumai *et al.* 2011). The branchial expression of claudin-b increased in goldfish and zebrafish exposed to ion-poor water (Chasiotis *et al.* 2012, Kwong *et al.* 2013), and this increase appeared to be associated with a reduction in diffusive Na^+ loss (Kwong *et al.* 2013). In zebrafish larvae, claudin-b is also expressed between cell–cell contacts in the skin (Kollmar *et al.* 2001, Haas & Gilmour 2006), and knockdown of claudin-b protein expression markedly increased paracellular permeability and diffusive Na^+ loss (Kwong & Perry 2013). Additionally, Engelund *et al.* (2012) recently showed that transfection of claudin-30 in a MDCK cell line increased transepithelial resistance and decreased paracellular permeability to Na^+. In contrast to claudins, occludins are ubiquitously expressed in various tissues of fish (Clelland & Kelly 2010, Kumai *et al.* 2011). Several recent studies have provided evidence that occludin is critical in determining epithelial tightness in fish. For example, the expression of occludin was found to increase in the gill of rainbow trout and goldfish after acclimation to ion-poor water (Chasiotis *et al.* 2009, 2012, Chasiotis & Kelly 2011). In addition, knockdown of occludin expression using siRNA in a goldfish gill cell preparation increased the absorption of polyethylene glycol-4000 (PEG-4000), a paracellular permeability marker (Chasiotis *et al.* 2012). Collectively, these studies suggest that claudin-b/claudin-30 and occludin are important components regulating epithelial tightness in fish.

In mammals, cortisol is believed to exert its actions primarily by interacting with the glucocorticoid receptor (GR) and to a lesser extent the mineralocorticoid receptor (MR); the MR is more specifically activated by aldosterone (Hellal-Levy *et al.* 2000). Fish lack the capacity to synthesize aldosterone (Prunet *et al.* 2006), and it has been proposed that deoxycorticosterone, by stimulating the MR, may have a similar function in teleosts as aldosterone in mammals (Bury & Sturm 2007). In mammals, treatment with aldosterone but not dexamethasone (a GR agonist) reduced the paracellular movement of mannitol in a collecting duct cell line, indicating a role for the MR in regulating TJ properties (Le Moellic *et al.* 2005). By contrast, Kelly & Chasiotis (2011) demonstrated that aldosterone did not affect epithelial permeability in a trout gill cell preparation. Nevertheless, the MR antagonist spironolactone partially blocked the action of cortisol on reducing epithelial permeability, suggesting the potential role of the MR (Kelly & Chasiotis 2011). However, the interpretation of this latter result is complicated because spironolactone (a presumed antagonist) was found to activate the MR in COS-7 cells transfected with trout MR (Sturm *et al.* 2005). Similarly, a mammalian GR antagonist RU486 was found to act as a GR agonist in trout gill cell preparations (Kelly & Chasiotis 2011). In an Atlantic salmon gill cell culture, however, it was shown that the increased expression of claudins by cortisol was blocked by RU486 (Tipsmark *et al.* 2009). These results call into question the specificity of the mammalian GR and MR antagonists when used in fish models. Additionally, to our knowledge, there is as yet no direct evidence that cortisol can regulate piscine epithelial permeability in the more complex *in vivo* condition.

It is well documented that exposure to acidic water causes an increase in diffusive ion loss in FW fish (Gonzalez & Wilson 2001, Gonzalez *et al.* 2002, Kumai *et al.* 2011). While several FW fish species are tolerant of exposure to acidic water (Gonzalez *et al.* 2002), the mechanisms whereby fish can adapt to an acidic environment remain unclear. In zebrafish, it has been suggested that increases in Na^+ uptake and acid secretion are the major responses maintaining Na^+ homoeostasis and acid–base balance following chronic acid exposure (Horng *et al.* 2009, Kumai *et al.* 2011, 2012). Acid exposure was found to increase plasma or whole-body cortisol levels in FW fish (Goss & Wood 1988, Kakizawa *et al.* 1996, Kumai *et al.* 2012). In addition, cortisol treatment was shown to ameliorate the effects of acid exposure on plasma Na^+ levels in the Japanese medaka (*Oryzias latipes*; Yada & Ito 1999). The precise mechanism by which cortisol

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promotes whole-body Na^+ balance during acid exposure is unclear. Nevertheless, elevated cortisol levels have been shown to increase whole-body Na^+ uptake (Flik & Perry 1989, Laurent & Perry 1990, Kumai *et al*. 2012) via activation of the GR (Kumai *et al*. 2012), which may be beneficial during acclimatization to a low pH environment. In cultured gill cell models, cortisol treatment can reduce the paracellular movement of Na^+ (Chasiotis *et al*. 2010). However, the potential regulatory function of cortisol on diffusive Na^+ movements during acid exposure has not yet been addressed.

With the above background, this study examined the effects of cortisol on epithelial permeability to PEG and the potential modulation of the epithelial TJ proteins. The effects of cortisol on Na^+ handling in zebrafish larvae exposed to acidic water were also evaluated. We hypothesized that cortisol treatment would reduce epithelial PEG permeability and increase the expression of TJ proteins. We also tested the hypothesis that cortisol treatment can promote Na^+ balance during acid challenge. The objectives of this study were fourfold: i) to examine the effects of exogenous cortisol on epithelial permeability using the paracellular marker PEG-4000; ii) to evaluate the effects of cortisol on the transcript and protein levels of occludin-a and claudin-b; iii) to examine the interactive effects of cortisol and acid exposure on Na^+ handling; and iv) to assess the potential involvement of the GR in promoting the cortisol-mediated responses.

Materials and methods

Fish

Adult zebrafish (*Danio rerio*) were purchased from Big Al's Aquarium Services (Ottawa, ON, Canada) and were supplied with aerated, 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). Embryos were collected and reared in 50 ml petri dishes supplemented with dechloraminated City of Ottawa tap water (approximately pH 7.6) supplemented with 0.05% methylene blue. The petri dishes were kept in incubators at 28 °C. 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).

Effects of cortisol on whole-body epithelial permeability

Cortisol (as hydrocortisone; Sigma–Aldrich) was dissolved in dimethyl sulphoxide (DMSO) and stored as aliquots at −20 °C until use. The final concentration of DMSO vehicle in the exposure water was ≤0.1% and had no observable effects on zebrafish survival, behaviour or the absorption of a paracellular permeability marker PEG-4000 (detailed below). Concentrations of 0–50 μM cortisol were used based on previous studies (Lin *et al*. 2011, Kumai *et al*. 2012). Because a significant reduction in PEG-4000 absorption was observed at 10 μM cortisol (see Results section), this concentration was used for all subsequent experiments except where mentioned otherwise. Fish were incubated with cortisol starting at 2 days post-fertilization (dpf) and were sampled at 4 dpf in all experiments, except where mentioned otherwise. The incubation medium was changed daily and before any flux measurements along with fresh cortisol. [^{3}H]PEG-4000 (American Radiolabeled Chemicals, Inc., St Louis, MO, USA) was used to examine whole-body paracellular permeability. At 4 dpf, control (DMSO only) or cortisol-treated fish were transferred to a 2 ml microfuge tube and incubated in 2 μCi/ml PEG-4000 for 6 h. The final concentration of PEG-4000 in the water was 0.5 mM. Water samples were collected 10 min after adding the PEG-4000 and at the end of the flux period. After incubation, fish were killed with an overdose of MS-222 (4 mg/ml) and briefly washed in isotope-free water. Three fish were pooled as one sample ($n=1$) and were digested with 1 N HNO_3 at 60 °C for 48 h. The radioactivity in the digest as well as the water samples was measured using a liquid scintillation counter (LS-6500; Beckman Coulter Co., Mississauga, ON, Canada) following addition of scintillation cocktail (BioSafe-II; RPI co., Mt Prospect, IL, USA).

Western blot analysis

Methods for western blot analysis were similar to those described previously (Kwong *et al*. 2013). Briefly, proteins from ten larvae ($n=1$) were extracted using an RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, 1 mM EDTA and 1 mM phenylmethanesulfouyl fluoride) plus protease inhibitor cocktail (Roche). Samples (50 μg of protein) were loaded on a 10–12% SDS–PAGE and transferred to PVDF membrane (Bio-Rad). After transfer, the membrane was blocked with either 5% BSA (for occludin-a and claudin-b blotting) or 5% skimmed milk (for GR blotting) in Tris buffer plus 0.05% Tween 20 (TBST) for 1 h at room temperature. The membrane was then probed

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with occludin-a (1:1000 dilution; CHIKKMVGDYDRRA), claudin-b (1:250 dilution; CATPRSEASAPSGKNF) or GR (1:500 dilution; P-20, Santa Cruz Biotech) antibody in TBST with 2% BSA or 2% skimmed milk at 4 °C overnight. The specificity of these antibodies on zebrafish has been reported previously (Clelland & Kelly 2010, Kwong *et al.* 2013, Nesan *et al.* 2012). After washing with TBST (three times and 5 min each; 3×5 min), the membrane was probed with 1:5000–15 000 goat anti-rabbit antibodies (Pierce, Rockford, IL, USA) for 2 h at room temperature. The membrane was then washed (5×5 min) and the bands were detected using enhanced chemiluminescence (SuperSignal West femto chemiluminescent substrate; Pierce) with a ChemiDoc system (Bio-Rad). Subsequently, the membrane was re-probed with β-actin antibodies (1:4000; Sigma) for 2 h in room temperature after striping with Re-Blot Plus solution (Millipore, Billerica, MA, USA). The intensity of the bands was estimated using ImageJ (Rasband 2006, http://imagej.nih.gov/ij/), and the protein expression was normalized to that of β-actin.

Immunohistochemistry

For immunostaining of occludin-a, the fish were first fixed overnight in a 4% paraformaldehyde solution in PBS at 4 °C. After fixation, the fish were briefly rinsed with PBS with 0.1% Tween (PBST). The fish were then incubated with the occludin-a antibody (1:100 dilution) in PBST plus 1% BSA, 3% normal goat serum and 0.4% Triton-X at 4 °C overnight. Subsequently, the fish were incubated in an Alexa 488-coupled goat anti-rabbit IgG (Invitrogen; 1:500 dilution) for 2 h at room temperature in the dark. The images were acquired using a Zeiss LSM 5 Pascal/AxioVert 200 confocal microscope (with an Alexa 488 filter), which was fitted on an inverted microscope equipped with a 63× Plan-Apochromat 1.4 oil immersion objectives (Carl Zeiss, Jena, Germany).

Real-time PCR

RNA extraction, cDNA synthesis and real-time PCR analysis were performed as described previously (Kwong *et al.* 2013). Briefly, ten fish were pooled as one sample ($n=1$) and total RNA was extracted using TRIzol (Invitrogen). Following the removal of genomic DNA with DNase I (Invitrogen), this RNA was used to synthesize single-stranded cDNA using RevertAid H Minus reverse transcriptase (Fermentas, Hanover, MD, USA) and random hexamers. All RT-qPCR assays were performed on a real-time MX3000P qPCR system using Brilliant III SYBR Green Master Mix (Agilent Technologies, Palo Alto, CA, USA). Specific primers used to amplify the transcripts of claudin-b and occludin-a have been reported previously (Kumai *et al.* 2011). The expression of a housekeeping gene *18S* was also assessed. All RT-qPCR was performed using the following conditions: 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. The specificity of the PCR primers was checked at the end of each amplification using a DNA melting curve analysis with a ramping rate of 1 °C/min over a temperature range of 60–95 °C. All data were normalized to the expression of *18S*, and the relative mRNA expression was calculated based on the method described by Pfaffl (2001).

Effects of cortisol and acute acid exposure on sodium handling

The uptake and efflux of Na^+ were measured using radiotracer methods. To determine the uptake in control or cortisol-treated fish, the rearing water was replaced with either pH 7.6 (control) or pH 4.0 water (prepared by the addition of H_2SO_4) and the fish were then transferred to a 2 ml microfuge tube. Fish were exposed to 0.5 μCi/ml $^{22}Na^+$ (as $^{22}NaCl$; Perkin Elmer, Woodbridge, ON, Canada) for 1 h. Final concentrations of Na^+ in the water were 780 μM. At the end of the flux period, the fish were killed with an overdose of MS-222, briefly washed in isotope-free water and three larvae were pooled as one sample ($n=1$). The fish were digested, and radioactivity was measured as described previously.

To measure Na^+ efflux, 20 fish ($n=1$) were first incubated with 2.0 μCi/ml ^{22}Na for 6 h. After incubation, the fish were washed briefly in isotope-free water and then transferred to control (pH 7.6) or acidic (pH 4.0) water. The fish were placed in a 2.0 ml microfuge tube and water samples (250 μl) were removed at 20 min intervals for 1 h. Subsequently, ten fish were collected to measure radioactivity (as above) and another ten fish were used to determine the whole-body Na^+ concentration (detailed below) to determine the internal specific activity of Na^+.

To evaluate the whole-body Na^+ level, the fish were killed and briefly rinsed in deionised water. The fish were then digested with 1 N HNO_3 at 60 °C for 48 h. Subsequently, the digested sample was appropriately diluted and the total Na^+ content was measured by flame emission spectrophotometry (Spectra AA 220FS: Varian, Palo Alto, CA, USA).

Morpholino knockdown of claudin-b and GR

The zebrafish claudin-b morpholino (5′-CCG GTT GAT GCC ATG CTT TTT CGT T-3′; Gene Tools, Philomath, OR,

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USA) and GR morpholino (5′-CTC CAG TCC TCC TTG ATC CAT TTT G-3′; Gene Tools) were prepared with Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$ and 5.0 mM HEPES; pH 7.6) plus 0.05% phenol red before injection. The effectiveness of the claudin-b (Kwong & Perry 2013) and GR morpholino has been reported in previous studies (Kumai *et al*. 2012, Nesan *et al*. 2012). A 'sham' group was injected with a standard control morpholino (5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′; Gene Tools). Morpholino (2 ng) was injected into 1-cell stage zebrafish embryo using a micro-injector system (model IM 300; Narishige, Long Island, NY, USA). No significant effects on growth rate and survival were observed using these morpholino dosages. All morpholinos used were tagged with carboxyfluorescein; at 24 h post-injection, the embryos were screened for the presence of carboxyfluorescein using a SMZ1500 microscope (Nikon Instruments, Melville, NY, USA). Only carboxyfluorescein-positive embryos were used for the subsequent experiments.

Effects of cortisol on the whole-body epithelial permeability in claudin-b morphants

The effects of cortisol on PEG-4000 absorption were examined in claudin-b morphants. Sham or claudin-b morphants were exposed to 10 μM cortisol starting from 1 dpf. On 3 dpf, fish were sampled for measurements of PEG-4000 absorption as detailed earlier. The incubation media were changed daily and before any flux measurements along with fresh cortisol. Three dpf was chosen in this study because we observed that the paracellular permeability of PEG-4000 in the claudin-b morphants was significantly elevated on 3 dpf but appeared to be reduced to the control level at 4 dpf.

Examination on the role of GR in epithelial permeability

To evaluate whether activation of GR was the potential signaling pathway for the reduction of epithelial permeability, the effects of cortisol on PEG-4000 permeability were examined in fish following *gr* knockdown. Sham and GR morphants were incubated with 10 μM cortisol starting from 2 dpf, and measurements of PEG-4000 absorption were performed on 4 dpf as described earlier.

Effects of GR knockdown on epithelial permeability and sodium handling during acid exposure

It was previously demonstrated that chronic exposure to acidic water increases whole-body cortisol level in larval zebrafish (Kumai *et al*. 2012). To examine whether the elevated whole-body cortisol levels serve to reduce epithelial permeability and Na^+ loss, epithelial PEG permeability, Na^+ efflux and whole-body Na^+ levels were measured during acid exposure in GR morphants and shams. Sham and GR morphants were exposed to either pH 4.0 or 7.6 (control) water starting at 2 dpf, and PEG-4000 absorption and Na^+ measurements were performed at 4 dpf as detailed earlier.

Calculations and statistics

The influx (J_{in}) of PEG-4000 and Na^+ were calculated by

$$J_{in} = \frac{F}{SA \times n \times t},$$

where F is the total radioactivity incorporated into the fish (c.p.m.), SA is the specific activity in the water (c.p.m./fmol for PEG-4000 and c.p.m./pmol for Na^+), n is the number of fish and t is the duration of influx (h).

The efflux (J_{in}) of Na^+ was calculated by

$$J_{out} = \frac{1}{SA \times n} \times \frac{dW}{dt},$$

where SA is the internal specific activity of Na^+ (c.p.m./pmol) and W is the total radioactivity in the water (c.p.m.).

All statistical analyses were performed using Sigmaplot (version 11.2; Systat Software, Inc., Chicago, IL, USA). Data were either analysed using one-way or two-way ANOVA followed by a *post hoc* Holm–Sidak test. Except for the comparison in the expression levels of occludin-a and claudin-b, where a student's *t*-test was used. Data were either log or square-root transformed when the assumptions of equal variance or normal distribution were violated (determined automatically by the statistical software). Data are reported as the means ± S.E.M., and $P \leq 0.05$ was taken as the level of significance.

Results

Epithelial permeability decreases with increasing exogenous cortisol levels

The effects of exogenous cortisol on epithelial permeability were evaluated using a paracellular permeability marker PEG-4000. Cortisol treatment reduced the whole-body permeability of PEG-4000 in fish at 4 dpf; the absorption of PEG-4000 at 10–50 μM cortisol was significantly lower than that at 0 μM cortisol (Fig. 1).

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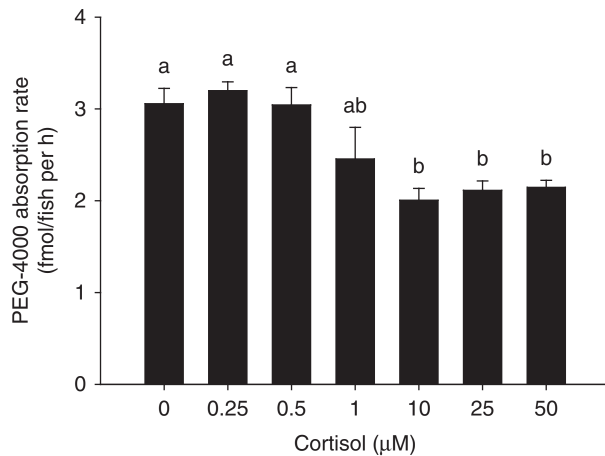


Figure 1
The epithelial permeability reduces with increasing exogenous cortisol levels in larval zebrafish. The effects of cortisol treatment on the absorption of a paracellular permeability marker PEG-4000 in larval zebrafish at 4 dpf. Bars labelled with different letters represent a statistical difference (one-way ANOVA; $P<0.05$). Values are means±S.E.M., $n=6$.

The TJ protein occludin-a is expressed between cell–cell contacts in the larval skin

Western blot analysis showed that the occludin-a antibody detected a single band at ~60 kDa (Fig. 2A). Immunohistochemistry demonstrated that the occludin-a antibody stained between cell–cell contacts in the larval skin (Fig. 2B).

Cortisol treatment increases the expression of occludin-a and claudin-b

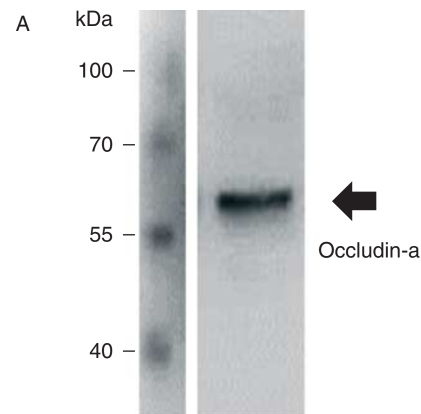
The effects of cortisol on mRNA and protein expression of occludin-a and claudin-b were examined using real-time PCR and western blot respectively. The protein level of occludin-a was increased after cortisol treatment, whereas its transcript levels remained unchanged (Fig. 3A). By contrast, both the transcript and protein levels of claudin-b were significantly increased following cortisol treatment (Fig. 3B). Representative western blots of occludin-a and claudin-b after cortisol treatment are shown in Fig. 3C and D respectively.

The increased epithelial permeability in the claudin-b morphants is mitigated by cortisol treatment

The effects of cortisol treatment on the epithelial PEG permeability were examined in the claudin-b morphants. Claudin-b morphants exhibited a significant increase in PEG-4000 absorption at 3 dpf (Fig. 4). Exposure to cortisol significantly reduced the absorption of PEG-4000 in the sham and morphants. Nevertheless, the absorption of PEG-4000 in cortisol-treated morphants remained significantly higher than that in cortisol-treated sham fish.

Cortisol treatment attenuates the consequences of acute acid exposure on sodium homoeostasis

The effects of cortisol treatment on Na^+ handling during acute acid exposure (1 h at pH 4.0; control, pH 7.6) were evaluated. Cortisol treatment significantly increased the uptake of Na^+ at pH 7.6 (Fig. 5A). At pH 7.6, an increase in Na^+ loss was also observed in fish treated with cortisol (Fig. 5B). Control or cortisol-treated fish exhibited a reduction in Na^+ uptake rate during acute exposure to acidic water (Fig. 5A). Nevertheless, after cortisol



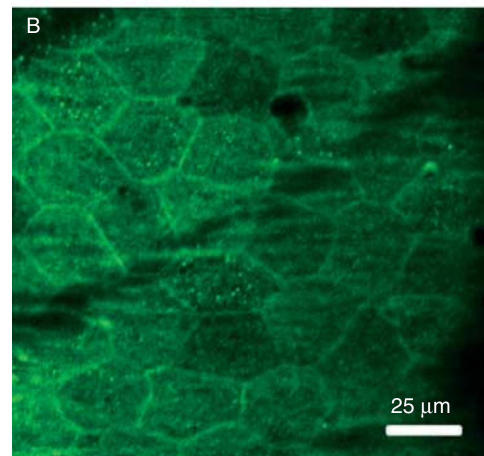


Figure 2
The TJ protein occludin-a is expressed between epithelial cell contacts in larval zebrafish. (A) A representative western blot showing an occludin-a antibody yielded a single immunoreactive band at ~60 kDa in lysates of 4 dpf zebrafish larvae. (B) Fluorescent immunohistochemistry and confocal microscopy revealed the expression of occludin-a between cell–cell contacts in the skin.

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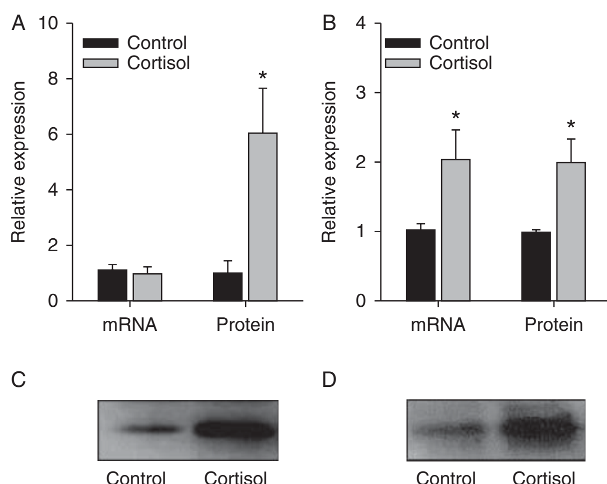

Figure 3
Cortisol treatment increases the expression of the TJ proteins occludin-a and claudin-b in larval zebrafish. The effects of cortisol treatment on the mRNA and protein expression levels of (A) occludin-a and (B) claudin-b in zebrafish larvae at 4 dpf. The mRNA and protein levels were determined by real-time PCR and western blot, using 18S and β-actin as the internal control respectively. An asterisk indicates a significant difference in mRNA or protein level between control and cortisol-treated fish (Student's *t*-test; $P<0.05$). Values are means ± S.E.M., $n=6$. The representative western blots for occludin-a and claudin-b following cortisol treatment are shown in (C) and (D) respectively.

treatment, the rate of Na^+ uptake in the acid-exposed fish appeared to be comparable to that in the control fish at pH 7.6. In the absence of cortisol, acute exposure to acidic water significantly increased Na^+ efflux (Fig. 5B). By contrast, there was no statistical difference in the Na^+ efflux between control and acid-exposed fish after cortisol treatment. Acid exposure also caused a significant reduction in the whole-body Na^+ levels in the control fish but not in the cortisol-treated fish (Fig. 5C).

Reduction of epithelial permeability by cortisol is associated with GR activation

To evaluate whether the reduction of epithelial PEG permeability by cortisol treatment was mediated by GR activation, the effects of cortisol on PEG permeability was examined in fish following *gr* knockdown. As revealed by western blot, the protein expression of GR was reduced in the lysates of 4 dpf fish after morpholino *gr* knockdown (Fig. 6A). In the absence of cortisol, the absorption of PEG-4000 between sham and GR morphants was comparable (Fig. 6B). Cortisol treatment significantly reduced PEG-4000 absorption in sham, whereas it had no apparent effect in GR morphants. Similarly, cortisol treatment did not reduce diffusive Na^+ loss in the GR morphants during acute acid exposure (the Na^+ efflux in cortisol-treated sham and cortisol-treated GR morphants during acute acid exposure were 768 ± 32 and 1311 ± 319 pmol/fish per h respectively ($P<0.05$; data not shown)).

The increased epithelial permeability during acid exposure is further elevated by *gr* knockdown

The potential involvement of GR in regulating epithelial PEG permeability following chronic exposure to acidic water (pH 4.0 for 2 days; control, pH 7.6) was investigated. The absorption of PEG-4000 was comparable between sham and GR morphants at pH 7.6 (Fig. 7). Acid exposure caused a significant increase in PEG-4000 absorption in both sham and GR morphants. However, GR morphants exhibited a more pronounced PEG-4000 absorption than shams during acid exposure.

Evidence for the involvement of GR in reducing sodium loss during acid exposure

To investigate whether the increased whole-body cortisol levels during acid exposure (Kumai *et al*. 2012) potentially serve to reduce Na^+ loss via GR activation, Na^+ efflux and whole-body Na^+ levels in GR morphants following chronic exposure to acidic water (pH 4.0 for 2 days; control, pH 7.6) were investigated. Exposure to acidic water markedly increased diffusive Na^+ loss in shams,

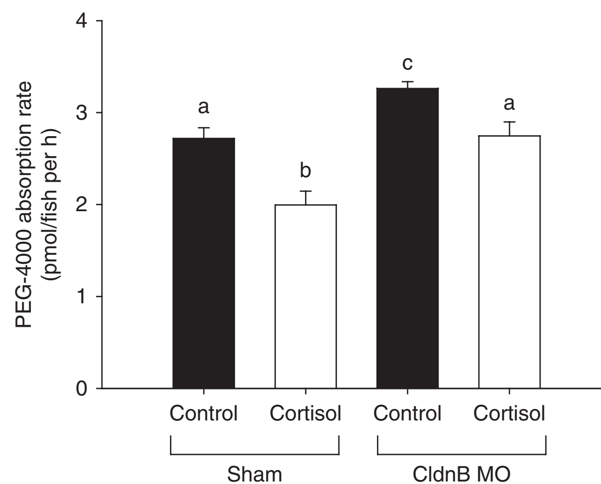

Figure 4
Cortisol treatment ameliorates the increased epithelial permeability in the claudin-b morphants. The effects of cortisol treatment on the absorption of a paracellular permeability marker PEG-4000 in sham and claudin-b morphants (CldnB MO) at 3 dpf. Bars labelled with different letters represent a statistical difference (two-way ANOVA followed by a *post hoc* Holm–Sidak test; $P<0.05$). Values are means ± S.E.M., $n=6$.

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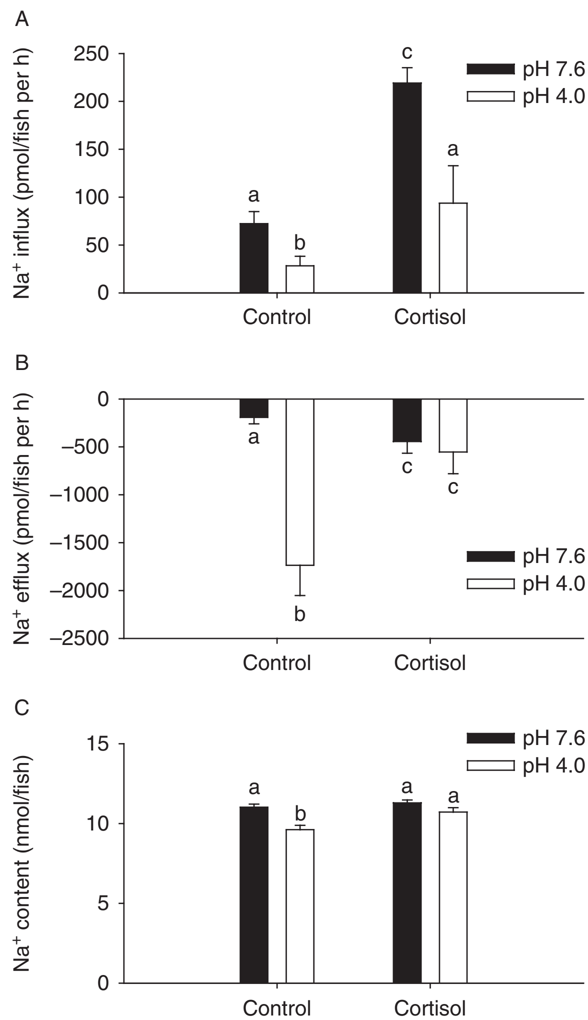

Figure 5
Cortisol treatment attenuates the consequences of acute acid exposure on sodium homoeostasis in larval zebrafish. The effects of cortisol treatment on the Na^+ (A) influx, (B) efflux and (C) whole-body Na^+ levels in zebrafish larvae at 4 dpf during acute exposure to pH 4.0 water for 1 h. Bars labelled with different letters represent a statistical difference (two-way ANOVA followed by a *post hoc* Holm–Sidak test; $P<0.05$). Values are means±S.E.M., $n=6$.

which was more severe in fish following *gr* knockdown (Fig. 8A). Similarly, the whole-body Na^+ level in the morphants was significantly lower than that in the shams following acid exposure (Fig. 8B).

Discussion

Overview

In this study, we showed that epithelial paracellular permeability to a non-ionic solute (PEG-4000) was reduced after cortisol treatment. The abundance of the epithelial TJ proteins occludin-a and claudin-b was also increased following cortisol treatment. Importantly, cortisol treatment was found to reduce diffusive Na^+ loss and to promote whole-body Na^+ retention during acute acid exposure. The effects of cortisol on reducing epithelial permeability were abolished in fish experiencing knockdown of the GR receptor, implicating its involvement in the cortisol-mediated responses. Additionally, GR morphants exhibited a higher epithelial PEG permeability and greater Na^+ losses than control fish during acid exposure. The results suggest that cortisol has the potential to attenuate the consequences of acid exposure on Na^+ homoeostasis, which is at least in part associated with the GR signaling pathway.

Cortisol treatment reduces epithelial permeability and increases the abundance of epithelial TJ proteins

Previous *in vitro* studies using gill cell cultures showed that cortisol exposure reduces the epithelial permeability of

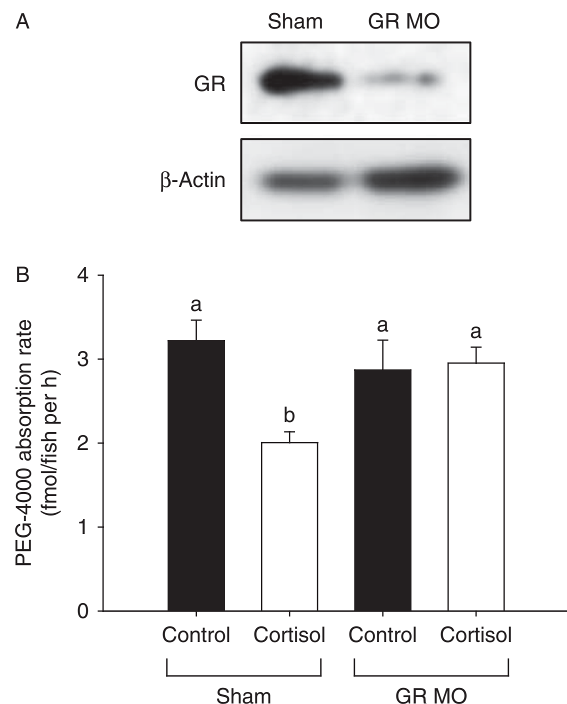

Figure 6
Knockdown of GR abolishes the effects of cortisol on epithelial permeability. (A) A representative western blot showing the reduced protein expression of GR in lysates of 4 dpf larval zebrafish following morpholino *gr* knockdown (GR MO). (B) The effects of cortisol treatment on the absorption of a paracellular permeability marker PEG-4000 in sham and GR morphants (GR MO). Bars labelled with different letters represent a statistical difference (two-way ANOVA followed by a *post hoc* Holm–Sidak test; $P<0.05$). Values are means±S.E.M., $n=6$.

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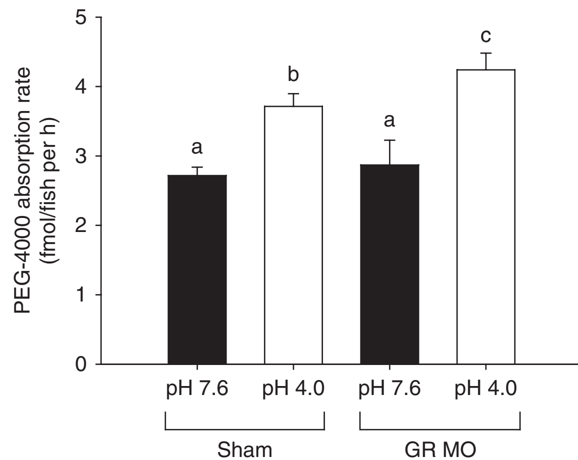

Figure 7
Knockdown of GR causes a more pronounced increase in epithelial permeability in larval zebrafish exposed to acidic water. The effects of chronic acid exposure (pH 4.0; control, pH 7.6) on the absorption of a paracellular permeability marker PEG-4000 in sham and GR knockdown morphants (GR MO). The fish were exposed to acidic water starting at 2 dpf, and the absorption of PEG-4000 was measured at 4 dpf. Bars labelled with different letters represent a statistical difference (one-way ANOVA; $P<0.05$). Values are means ± S.E.M., $n=12$.

PEG-4000 (Chasiotis *et al*. 2010, Chasiotis & Kelly 2011). Similarly, this study demonstrated that cortisol treatment reduced the absorption of PEG-4000 in the zebrafish larvae, providing evidence that cortisol may regulate paracellular permeability *in vivo*. Exposure to cortisol was reported to increase the mRNA and protein expression of occludin in a rainbow trout gill cell culture, which was associated with a decrease in epithelial permeability (Chasiotis *et al*. 2010, Chasiotis & Kelly 2011). In addition, knockdown of occludin in a trout gill cell culture caused an increase in the epithelial permeability to PEG-4000, suggesting a critical role of occludin in maintaining a tight epithelium (Chasiotis *et al*. 2012). Here, we observed that occludin-a was expressed between cell–cell contacts in the larval skin. Additionally, the levels of occludin-a protein were increased after cortisol treatment, suggesting its potential contribution in reducing paracellular permeability. Although we observed that the mRNA levels of occludin-a were unchanged by cortisol treatment, the fish were sampled 2 days after the exposure and thus we cannot rule out the possibility that cortisol caused a transient increase in occludin-a mRNA expression. On the other hand, cortisol treatment was found to have no effect on the mRNA expression of claudin-b in a cultured goldfish gill cell preparation (Chasiotis & Kelly 2011). By contrast, injection of cortisol in Atlantic salmon was reported to increase the mRNA expression of claudin-30, an ortholog of the zebrafish claudin-b (Tipsmark *et al*. 2009). Previously, we observed that claudin-b is also expressed in the skin of zebrafish larvae and that morpholino knockdown of claudin-b expression increased the paracellular permeability to PEG-4000 (Kwong & Perry 2013). In this study, both the mRNA and protein levels of claudin-b were found to increase after cortisol treatment. Additionally, cortisol treatment was found to reduce PEG-4000 permeability in the claudin-b morphants, possibly by increasing the abundance of other epithelial TJ proteins such as occludin-a. Overall, the results suggest that the increased PEG permeability in the claudin-b morphants can be mitigated by cortisol treatment.

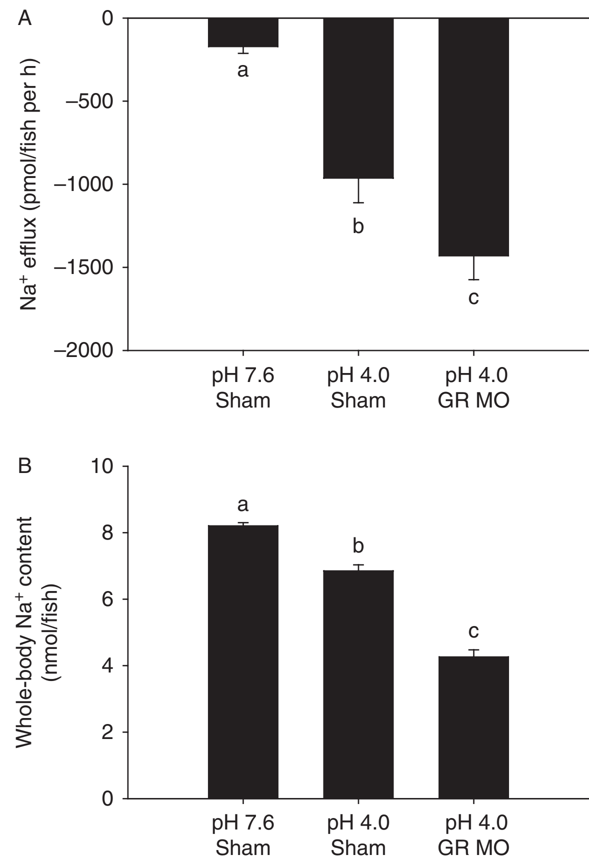

Figure 8
Knockdown of GR causes a more severe loss of sodium in larval zebrafish exposed to acidic water. The effects of chronic acid exposure (pH 4; control: pH 7.6) on the (A) Na^+ efflux and (B) whole-body Na^+ level in sham and GR knockdown morphants (GR MO). The fish were exposed to acidic water starting at 2 dpf, and the Na^+ measurements were performed at 4 dpf. Bars labelled with different letters represent a statistical difference (one-way ANOVA; $P<0.05$). Values are means ± S.E.M., $n=10$–12.

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Cortisol treatment attenuates the effects of acute acid exposure on sodium homoeostasis

A recent study has shown that cortisol exposure promotes Na^+ uptake via activation of GR in zebrafish larvae (Kumai *et al*. 2012). Similarly, we observed that cortisol treatment dramatically increased the influx of Na^+ in zebrafish larvae, which appeared to be accompanied with an increase in Na^+ efflux. Similar to several other FW species (Wilson *et al*. 1999, Gonzalez *et al*. 2002, 2005), zebrafish larvae acutely transferred to acidic water (pH 4.0) exhibit a pronounced decrease in Na^+ uptake and an increase in diffusive Na^+ loss. The whole-body content of Na^+ was also reduced during acid exposure. Stimulation of diffusive Na^+ loss by acid exposure was probably due to the disruption of TJs integrity (McDonald *et al*. 1980, Kumai *et al*. 2011), thereby increasing paracellular Na^+ efflux. Notably, exposure to acidic water for over 24 h was found to increase Na^+ uptake (Kumai *et al*. 2011) and did not disrupt the whole-body Na^+ content in zebrafish larvae (Horng *et al*. 2009). Therefore, it appears that zebrafish increase Na^+ uptake to compensate for the elevated Na^+ loss during long-term exposure to acidic water. It is also noteworthy that although acute exposure to acidic water reduced Na^+ uptake, the uptake of Na^+ in cortisol-treated fish at pH 4.0 was comparable to that in control fish at pH 7.6. More importantly, we also observed that cortisol treatment reduced diffusive Na^+ loss during acid exposure. The reduction in the whole-body Na^+ levels during acute acid exposure was also found to be ameliorated by cortisol treatment. These results indicate that cortisol has the potential to promote Na^+ uptake and reduce Na^+ loss during acute acid exposure, effects that would ultimately attenuate the consequences of acid exposure on Na^+ homoeostasis. However, the underlying mechanism for the protective effects of cortisol against Na^+ loss during acid exposure was unclear but was likely associated with the enhanced paracellular barrier formed by TJ proteins. For example, Engelund *et al*. (2012) have recently shown that the expression of claudin-30 (orthologous to zebrafish claudin-b) in a MDCK cell line decreases the paracellular permeability to Na^+. Similarly, knockdown of claudin-b expression in larval zebrafish increases diffusive Na^+ loss and causes a reduction in whole-body Na^+ levels (Kwong & Perry 2013). Therefore, it seems possible that the increased expression of claudin-b by cortisol treatment may subsequently serve to reduce Na^+ loss during acute acid challenge.

Several recent studies have shown that diffusive movements of PEG and Na^+ exhibit similar patterns of change when general epithelial 'tightness' is altered through modifications of paracellular permeability. For example, exposure of adult zebrafish to acidic water was found to increase diffusive Na^+ loss and epithelial PEG-400 permeability (Kumai *et al*. 2011). Studies with cultured gill cells have also demonstrated that cortisol treatment reduces epithelial permeability to both PEG-4000 and Na^+ (Wood *et al*. 2002, Chasiotis *et al*. 2010, Kelly & Chasiotis 2011). However, Wood *et al*. (2009) showed that branchial Na^+ efflux in Amazonian oscar (*Astronotus ocellatus*) was reduced under hypoxic conditions, whereas the permeability of PEG-4000 remained unchanged. The whole-body absorption of a smaller solute PEG-400 in adult zebrafish was also found to increase after prolonged exposure to ion-poor water, although diffusive Na^+ loss was decreased (Kwong *et al*. 2013). These results suggest that general epithelial tightness and ion permeability through TJs are not always coupled. In this study, we observed that cortisol treatment (in neutral water) lowered PEG-4000 permeability while simultaneously increasing diffusive Na^+ loss. A possible explanation is that a portion of overall Na^+ loss occurs via transcellular routes that are also influenced by cortisol treatment. Alternatively, cortisol might have a greater effect on TJ proteins regulating large molecular weight uncharged solutes than TJ proteins regulating the permeability of cations. It is worth noting here that the hydrodynamic radii of PEG-4000 (12.5 Å) is considerably larger than Na^+ ions (<2 Å; Kiriukhin & Collins 2002), and different claudins have been suggested to exhibit different control on the size of the paracellular pore (see review by Anderson & Van Itallie (2009)). Therefore, we could not rule out the possibility that the paracellular movement of PEG-4000 and Na^+ occurred through different physical pathways.

Evidence for the involvement of the GR in reducing epithelial permeability and diffusive sodium loss during acid exposure

Previous *in vitro* studies with fish gill models have suggested that cortisol treatment reduces epithelial permeability at least in part via the GR (Tipsmark *et al*. 2009, Kelly & Chasiotis 2011). In this study, we observed that morpholino knockdown of *gr* expression completely abolished the effects of cortisol on reducing PEG-4000 absorption, indicating that activation of the GR is an important signaling pathway. It has been reported that the plasma cortisol levels in FW rainbow trout increases during acid exposure (Goss & Wood 1988,

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Kakizawa *et al*. 1996). Kumai *et al*. (2012) have also shown that chronic exposure to acidic water increases whole-body cortisol levels in larval zebrafish, suggesting a potential role of cortisol during acclimation to an acidic environment. To examine whether the elevated whole-body cortisol levels serve to attenuate the increased epithelial permeability and Na^+ loss during acid exposure, epithelial PEG permeability and Na^+ homoeostasis were investigated in acid-exposed GR morphants. We observed that the increased PEG-4000 absorption by acid exposure was more pronounced in the GR morphants. Moreover, the GR morphants exhibited a greater diffusive loss of Na^+ than sham fish after acid exposure. Additionally, the reduction in the whole-body Na^+ content by acid exposure was more severe in the GR morphants when compared with the sham fish. These findings indicate that the elevated whole-body cortisol levels in acid-exposed fish (Kumai *et al*. 2012) have the potential to mitigate the increased epithelial permeability and diffusive Na^+ loss via GR signaling pathways. It is also noteworthy that knockdown of GR prevents the compensatory increase in Na^+ uptake during acid exposure (Kumai *et al*. 2012). Therefore, the reduction in the whole-body Na^+ content in the acid-exposed GR morphants was probably a result of both the increased diffusive Na^+ loss and a decreased ability to increase Na^+ uptake. Overall, the results provide direct functional evidence that GR activation is involved in reducing epithelial permeability and paracellular Na^+ loss in zebrafish larvae during acid challenge.

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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