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RESEARCH ARTICLE

Inhibition of calcium uptake during hypoxia in developing zebrafish is mediated by hypoxia-inducible factor

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ABSTRACT

The present study investigated the potential role of hypoxia-inducible factor (HIF) in calcium homeostasis in developing zebrafish (*Danio rerio*). It was demonstrated that zebrafish raised in hypoxic water (30 mmHg; control, 155 mmHg P_{O_2}) until 4 days post-fertilization exhibited a substantial reduction in whole-body Ca^{2+} levels and Ca^{2+} uptake. Ca^{2+} uptake in hypoxia-treated fish did not return to pre-hypoxia (control) levels within 2 h of transfer back to normoxic water. Results from real-time PCR showed that hypoxia decreased the whole-body mRNA expression levels of the epithelial Ca^{2+} channel (*ecac*), but not plasma membrane Ca^{2+}-ATPase (*pmca2*) or Na^+/Ca^{2+}-exchanger (*ncx1b*). Whole-mount *in situ* hybridization revealed that the number of *ecac*-expressing ionocytes was reduced in fish raised in hypoxic water. These findings suggested that hypoxic treatment suppressed the expression of *ecac*, thereby reducing Ca^{2+} influx. To further evaluate the potential mechanisms for the effects of hypoxia on Ca^{2+} regulation, a functional gene knockdown approach was employed to prevent the expression of HIF-1αb during hypoxic treatment. Consistent with a role for HIF-1αb in regulating Ca^{2+} balance during hypoxia, the results demonstrated that the reduction of Ca^{2+} uptake associated with hypoxic exposure was not observed in fish experiencing HIF-1αb knockdown. Additionally, the effects of hypoxia on reducing the number of *ecac*-expressing ionocytes was less pronounced in HIF-1αb-deficient fish. Overall, the current study revealed that hypoxic exposure inhibited Ca^{2+} uptake in developing zebrafish, probably owing to HIF-1αb-mediated suppression of *ecac* expression.

KEY WORDS: Calcium, ECaC, Ion regulation, HIF, Fish, Ionocyte

INTRODUCTION

A reduction in dissolved oxygen levels (hypoxia) is a global environmental issue which has been reported in many freshwater ecosystems (Jenny et al., 2016). In freshwater, fish are hyperionic to their environment and thus they must actively absorb ions from the environment to maintain whole-body ionic homeostasis. It is documented that exposure to hypoxia affects ionic balance in freshwater fish. For example, hypoxia inhibited the active uptake of Na^+ in rainbow trout (*Oncorhynchus mykiss*) (Iftikar et al., 2010) and Amazonian oscar (*Astronotus ocellatus*) (Wood et al., 2007). Plasma Na^+ levels were reduced in scaleless carp (*Gymnocypris przewalskii*) exposed to hypoxic water (Matey et al., 2008). The disturbance in Na^+ regulation during hypoxia appears to be caused, at least in part, by a suppression of gill Na^+/K^+-ATPase activity and/or a change in gill morphology (Matey et al., 2008; Wood et al., 2007).

Using a transcriptomics approach, it was demonstrated that hypoxia decreased the expression of multiple genes in developing zebrafish (*Danio rerio*), including genes that are responsible for aerobic metabolism and Ca^{2+} transport (e.g. Ca^{2+}-ATPases) (Ton et al., 2003). The reduced expression of these genes may potentially affect the active uptake of ions and whole-body ionic balance. However, no studies have yet investigated the impact of hypoxia on the ion-regulatory function in zebrafish.

The absorption of major cations (i.e. Na^+ and Ca^{2+}) in zebrafish is thought to occur via at least three types of ion-transporting cells termed ionocytes: H^+-ATPase-rich cells (HRCs), Na^+-Cl^--cotransporter-expressing cells (NCCCs) and Na^+/K^+-ATPase-rich cells (NaRCs). HRCs and NCCCs mediate the apical uptake of Na^+ via Na^+/H^+-exchanger (i.e. NHE3b) and Na^+-Cl^--cotransporter (i.e. NCC2), respectively (Esaki et al., 2007; Kumai and Perry, 2011; Shih et al., 2012; Wang et al., 2009). A subset of NaRCs facilitates the apical uptake of Ca^{2+} via the epithelial Ca^{2+} channel (ECaC) (Pan et al., 2005). In adult zebrafish, the gills are the predominant site of active ion uptake, but during larval stages before the gills are fully developed, regulation of ion uptake is primarily mediated by ionocytes found on the skin of the yolk sac (for reviews, see Hwang et al., 2011; Hwang and Chou, 2013; Kwong et al., 2016).

In vertebrates, the transcription factor hypoxia-inducible factor-1 (HIF-1) plays a critical role in promoting cellular responses to reduced oxygen levels (Jewell et al., 2001; Ratcliffe et al., 1998). HIF-1 is a heterodimer that consists of a hypoxia-responsive α subunit and the constitutively expressed subunit HIF-1β. Under normoxic conditions, HIF-1α is bound to the von Hippel–Lindau tumour-suppressor protein, which mediates ubiquitination of HIF-1α, which is subsequently targeted for proteasomal degradation (Ohh et al., 2000; Tanimoto et al., 2000). Under hypoxic conditions, however, HIF-1α becomes stable and binds HIF-1β to form the active dimer form of HIF. This post-translational stabilization of HIF-1α under hypoxic conditions is thought to be the primary mechanism for the regulation of HIF-1 activity, and thereby its actions on various downstream signalling targets (Huang et al., 1998).

Zebrafish have two copies of the HIF-1α gene (HIF-1αa and HIF-1αb) and one copy of HIF-1β. The functional importance of each HIF-1α paralogue is not completely understood. Previous studies have proposed that HIF-1αa is involved in development, while HIF-1αb plays a role in responses to hypoxia (Rytkönen et al., 2013, 2014). In zebrafish, the mRNA expression of *hif-1αb* and *hif-1β* was detected starting at 0.5 h post-fertilization (Kajimura et al., 2006).

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Results from *in situ* hybridization suggested that *hif-1αb* mRNA is ubiquitously expressed in larval zebrafish, including notochord, brain and branchial region (Kajimura et al., 2006; Rojas et al., 2007). It was also demonstrated that the protein levels of HIF-1αb were elevated in larval zebrafish exposed to hypoxia (Köblitz et al., 2015; Robertson et al., 2014), suggesting that in zebrafish, HIF1αb may play an important role in the responses to hypoxia.

In the present study, we used developing zebrafish to test the hypothesis that (i) exposure to hypoxia disrupts Ca^{2+} balance by reducing active Ca^{2+} uptake; and (ii) the effects of hypoxia on Ca^{2+} homeostasis are associated with the activation of HIF-1αb. The findings demonstrated that exposure to hypoxia substantially reduced Ca^{2+} uptake and whole-body Ca^{2+} levels in developing zebrafish, effects which were probably associated with HIF-1αb-mediated inhibition of *ecac* expression.

MATERIALS AND METHODS

Animals and hypoxic treatments

Adult zebrafish, *Danio rerio* (Hamilton 1822) were maintained in aerated, dechloraminated City of Ottawa tap water at 28 °C (in mmol l^{-1}; 0.25 Ca^{2+}, 0.78 Na^{+}, 0.02 K^{+}, 0.15 Mg^{2+}; pH 7.6). Fish were subjected to a constant 14 h light:10 h dark photoperiod and fed daily until satiation with No.1 crumble-Zeigler (Aquatic Habitats, Apopka, FL, USA). Embryos were collected and immediately transferred to flow-through tanks supplied with normoxic (155 mmHg P_{O_2}) or hypoxic (30 mmHg P_{O_2}) water. Hypoxia was achieved by bubbling a mixture of air and nitrogen into the tank using a gas mixer (model GF-3/MP, Cameron Instruments, Inc., Port Aransas, TX, USA). Exposure to similar hypoxic water tension has previously been shown to induce expression of various HIF isoforms (Köblitz et al., 2015; Kopp et al., 2011). Water P_{O_2} was continuously monitored using a fibre optic oxygen electrode (FOXY AL300, Ocean Optics, Dunedin, FL, USA) over the entire course of experiments, and the measured P_{O_2} values were always within 10% of the target. Preliminary experiments demonstrated that fish raised in hypoxic water (30 mmHg P_{O_2}) exhibited a delay in development (~24 h delay). Therefore, all experiments were developmentally staged and matched as described by Kimmel et al. (1995). Thus, the age of the larvae are presented in this study as 'corrected days post-fertilization' (dpf), whereby developmentally similar (rather than chronologically similar) larvae were compared between the normoxic and hypoxic treatments. Essentially, the larval length, head–trunk angle and number of myotome segments were used to assess the developmental ages of fish. The experiments were conducted in compliance with guidelines of the Canadian Council of Animal Care (CCAC) and following approval of the University of Ottawa Animal Care Committee (Protocol BL-226).

Measurement of whole-body ion levels

To examine the effects of hypoxia on ionic balance, whole-body levels of Na^{+}, Ca^{2+} and K^{+} were measured in fish raised in normoxic (control) or hypoxic water. At 3 and 4 dpf, fish were killed with an overdose of tricaine methanesulphonate (MS-222), and then briefly rinsed in double-deionized water. Twenty fish were pooled as one sample, and a total of six samples (N=6) were analysed in this experiment. The fish were digested with 5 mol l^{-1} HNO_3 at 65°C for 48 h, and diluted appropriately with deionized water. The total ion concentration was measured by flame emission spectrophotometry (Spectra AA 220FS, Varian), and verified using certified standards (Fisher Scientific).

Measurement of Ca^{2+} influx

Because we observed a substantial reduction in whole-body Ca^{2+} levels following hypoxic treatment (see Results), influx of Ca^{2+} was evaluated using a radiotracer method as described previously (Kwong et al., 2014). In brief, fish reared in normoxic or hypoxic water were exposed for 2 h to 0.2 µCi ml^{-1} $^{45}Ca^{2+}$ (as $CaCl_2$; PerkinElmer) at 4 dpf. In some experiments, influx of Ca^{2+} in hypoxia-treated fish was measured immediately after transferring them back to normoxic water (post-hypoxia). At the end of the flux period, fish were killed with an overdose of MS-222 and rinsed in isotope-free water. Two fish were pooled as one sample, and a total of six samples (N=6) were analysed. Fish were digested with a tissue solubilizer (Solvable; Perkin Elmer) and later neutralized using glacial acetic acid. The radioactivity of the digest and the water samples was measured using a liquid scintillation counter (LS-6500, Beckman Coulter, Canada) following the addition of a scintillation cocktail (BioSafe-II, Research Products International). The Ca^{2+} influx (J_{in}; pmol $fish^{-1}$ h^{-1}) was determined using the formula: $J_{in}=F/(SA\times n\times t)$, where F is the total radioactivity counted in the fish (counts min^{-1}), SA is the specific activity of the water (cpm $nmol^{-1}$), n is the number of fish and t is the duration of the experiment in hours.

Effects of HIF stabilization on Ca^{2+} regulation

The potential effects of HIF on Ca^{2+} regulation were assessed using a HIF-stabilizing drug, dimethyloxaloylglycine (DMOG), in normoxic conditions. Fish were treated with 100 µmol l^{-1} DMOG (Santa Cruz Biotech) immediately after fertilization, and the exposure water was refreshed daily. Whole-body Ca^{2+} levels and Ca^{2+} influx were measured at 4 dpf as described above.

Microinjection of antisense morpholino oligonucleotide

A morpholino oligonucleotide (5′-CAT CTG CAA AAT CGA ATA ACA TCC C-3′; Genetools, OR, USA) was designed to target the splice junctions between intron 2 and exon 3 of the zebrafish *hif-1αb* subunit (Ensembl gene ID: ENSDARG00000006181). The morpholino was diluted in a Danieau buffer [58 mmol l^{-1} NaCl, 0.7 mmol l^{-1} KCl, 0.4 mmol l^{-1} $MgSO_4$, 0.6 mmol l^{-1} $Ca(NO_3)_2$, 5.0 mmol l^{-1} Hepes (pH 7.6)] plus 0.05% Phenol Red before injection into embryos at the 1-cell stage. A 'sham' group was injected with a standard control morpholino (5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′; GeneTools) prepared as for the HIF-1αb morpholino. In preliminary trials, we observed that injection of 4 ng morpholino did not induce developmental defects, and effectively abolished the increased HIF-1αb protein expression following hypoxic treatment for 4 h (see Results). Therefore, this dose was used in all subsequent experiments. Fish injected with control or HIF-1αβ morpholino were raised in either normoxic or hypoxic water until 4 dpf. Whole-body Ca^{2+} levels and Ca^{2+} influx were measured as described above.

Protein extraction and western blotting

Shock-frozen zebrafish larvae were covered with 2× Laemmli sample buffer (Bio-Rad). Samples were boiled at 95°C for 30 min and dispersed by frequent and rapid pipetting. Undissolved proteins and pigment were separated by centrifugation (10 min, 16,000 rpm; Eppendorf Centrifuge 5415R). Protein concentration was determined by measurement of total protein absorption at 280 nm with a NanoDrop 2000c (Thermo Scientific) in triplicate.

Western blotting was performed as described previously (Köblitz et al., 2015; Kopp et al., 2011); 400 µg of protein per sample was loaded onto 12% pre-cast SDS-PAGE gels (18-well Criterion™ TGX Stain-Free™ Gels, Bio-Rad), and blotted onto PVDF

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membranes. Equal loading was verified by UV exposure in a Chemidoc XRS+ (Bio-Rad) imaging system and comparison of overall intensity of the protein staining. Non-specific protein-binding sites were blocked by pre-incubating the membranes in Tris-buffered saline containing 5% skimmed milk powder and 0.1% Tween 20 (blocking buffer) for 45 min at room temperature. Primary polyclonal HIF antibodies (Kopp et al., 2011) were diluted 1:1000 in blocking buffer. Incubation with primary antibody was performed overnight at 4°C. Binding of the primary antibody was detected with a secondary antibody conjugated to horseradish peroxidase (Abcam, Cambridge, UK) diluted 1:10,000 in blocking buffer for 1 h at room temperature followed by enhanced chemiluminescence detection (Amersham™ ECL™ Select Western Blotting Detection Reagent). Band densities were analysed with Image Lab 4.1 software (Bio-Rad).

Real-time PCR analysis

Because exposure to hypoxia was found to significantly reduce Ca^{2+} influx, the expression of mRNA encoding various Ca^{2+} channels/transporters and hypocalcaemic hormones was further evaluated by real-time PCR. Methods for RNA extraction, cDNA synthesis and PCR analysis were similar to those described by Kwong et al. (2014). In brief, total RNA from fish exposed to normoxia or hypoxia at 4 dpf was extracted using an RNeasy kit (Qiagen). After treatment with DNase I (Biolabs, USA), cDNA was synthesized with 1 µg of RNA using RevertAid H-minus reverse transcriptase (Thermo Scientific) and random hexamer primers. The mRNA levels of *ecac*, plasma membrane Ca^{2+}-ATPase isoform 2 (*pmca2*), Na^{+}/Ca^{2+} -exchanger isoform 1b (*ncx1b*), stanniocalcin-1 (*stc1*) and calcitonin (*ct*) were examined using real-time PCR (N=6). A previous study showed that *pmca2* and *ncx1b* are expressed in *ecac*-positive ionocytes (Liao et al., 2007) and thus these isoforms were chosen for analysis in this study. Primer sets used in the present study are summarized in Table 1. Real-time PCR analysis was performed on a Bio-Rad CFX96 qPCR system as described previously (Kwong et al., 2014), and 18S RNA was used as an internal control.

Whole-mount *in situ* hybridization

To examine the potential effects of hypoxic exposure on the number of *ecac*-expressing ionocytes, whole-mount *in situ* hybridization was performed as described previously (Kwong et al., 2014). In brief, a fragment of zebrafish *ecac* mRNA from 4 dpf larval zebrafish cDNA was PCR amplified (see Table 1 for primer sequences), cloned into a pDrive cloning vector (Qiagen, USA) and sequenced. After plasmid purification and linearization, an *ecac* RNA probe was synthesized by *in vitro* transcription in the presence of digoxigenin (dig)-UTP (Roche, Penzberg, Germany). Fish exposed to normoxia (4 dpf) or hypoxia (5 dpf) were fixed in 4% paraformaldehyde overnight at 4°C, and washed several times with phosphate-buffered saline plus 0.1% Tween-20 (PBST) before gradual dehydration with methanol. After rehydration with PBST, the fish were permeabilized in acetone for 20 min at −20°C and then washed with PBST. The fish were first pre-hybridized in a hybridization buffer supplemented with 500 µg ml^{-1} yeast tRNA and 50 µg ml^{-1} heparin (Sigma) for 2 h at 65°C, and then incubated with 100 ng of *ecac* RNA probe overnight at 65°C. After serial washing with hybridization buffer and PBST, the fish were incubated in a blocking solution containing 10% calf serum in PBST for 2 h before incubation with an alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2000 dilution for 2 h at room temperature). Subsequently, fish were washed with PBST and incubated in a NBT/BCIP staining buffer until the desired coloration intensity was obtained. The total number of *ecac*-positive ionocytes on the skin of the yolk sac was counted (N=10). The density of NaRCs following hypoxic treatment was quantified by immunohistochemistry as described by Kwong and Perry (2015).

Table 1. Primer sets used in the present study

Gene	Primer	Reference
Real-time PCR		
ecac	F: TCC TTT CCC ATC ACC CTC T	Lin et al., 2011
	R: GCA CTG TGG CAA CTT TCG T	
pmca2	F: AAG CAG TTC AGG GGT TTA C	Liao et al., 2007
	R: CAG ATC ATT GCC TTG TAT CA	
ncx1b	F: TAAAGTGGCAGCGATACAGG	Liao et al., 2007
	R: CAGATCAAGGCGAAGATGG	
stc1	F: CCA GCT GCT TCA AAA CAA ACC	Tseng et al., 2009
	R: ATG GAG CGT TTT CTG GCG A	
ct	F: CTA CGA GGC GAG AAG ATT GCT T	Lafont et al., 2011
	R: TGG ATA CGT CTG CAG CTT GTG	
In situ hybridization		
ecac	F: TGG CTC AGG ATG CAG AAC AG	Kwong et al., 2014
	R: TAG GGT CCC AGC ATC TCG AA	

ecac, epithelial Ca^{2+} channel; *pmca2*, plasma membrane Ca^{2+}-ATPase isoform 2; *ncx1b*, Na^{+}/Ca^{2+}-exchanger isoform 1b; *stc1*, stanniocalcin isoform 1; *ct*, calcitonin.

Statistical analysis

All statistical analyses were performed using Sigmaplot® (version 11.2, Systat Software, Inc., USA). Data were analysed using Student's *t*-test, one-way or two-way analysis of variance (ANOVA; morpholino knockdown and hypoxic treatment as two independent variables) followed by a *post hoc* Holm–Sidak test. 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 means±s.e.m., and $P \leq 0.05$ was taken as the level of significance.

RESULTS

Exposure to hypoxia disrupts whole-body ion levels

Whole-body Ca^{2+} levels were significantly reduced in fish exposed to hypoxic water at both 3 and 4 dpf (Fig. 1A). Under normoxic conditions, a significant increase in both Ca^{2+} and Na^{+} levels was observed over development (Fig. 1A,B). Hypoxic exposure did not affect whole-body levels of Na^{+} at either 3 or 4 dpf. Whole-body K^{+} levels were reduced in fish exposed to hypoxic water at 4 dpf (Fig. 1C).

Ca^{2+} influx and *ecac* mRNA levels are reduced by hypoxic treatment

A significant reduction in Ca^{2+} influx was observed in fish raised in hypoxic water at 4 dpf (Fig. 2A) compared with those raised in normoxia. After transfer back to normoxic water (i.e. post-hypoxia), Ca^{2+} influx in hypoxia-treated fish remained reduced when compared with that of fish raised in normoxia.

The mRNA expression of genes encoding various Ca^{2+} channels/transporters and hypocalcaemic hormones following hypoxic treatment was examined using real-time PCR. The mRNA levels of *ecac* were substantially decreased in fish raised in hypoxic water (Fig. 2B). However, the mRNA levels of *pmca2* and *ncx1b* were not affected by hypoxic treatment. Exposure to hypoxia also did not affect the mRNA expression of *stc1* and *ct* (Fig. 2C).

Number of *ecac*-positive ionocytes is reduced by exposure to hypoxia

To evaluate the impact of hypoxia on the number of Ca^{2+}-transporting ionocytes, whole-mount *in situ* hybridization of *ecac*

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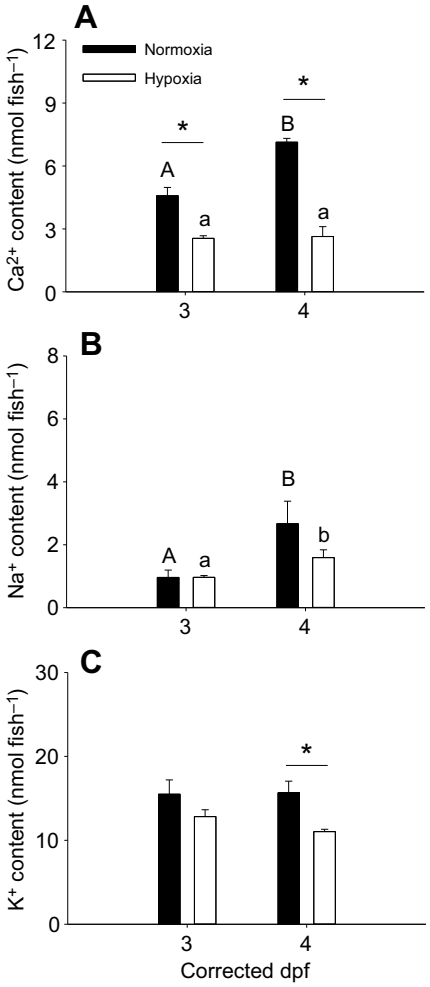

Fig. 1. Effects of hypoxia on whole-body ion levels. Whole-body (A) Ca^{2+}, (B) Na^+ and (C) K^+ content in developing zebrafish raised in normoxic or hypoxic water. Note that the ages of fish were developmentally matched (i.e. corrected days post-fertilization, dpf). Asterisks indicate a statistical difference between normoxia and hypoxia within the same developmental age. Different letters indicate a statistical difference between 3 and 4 dpf within the same normoxia or hypoxia treatment (P<0.05; two-way ANOVA, N=6). Data are presented as means±s.e.m.

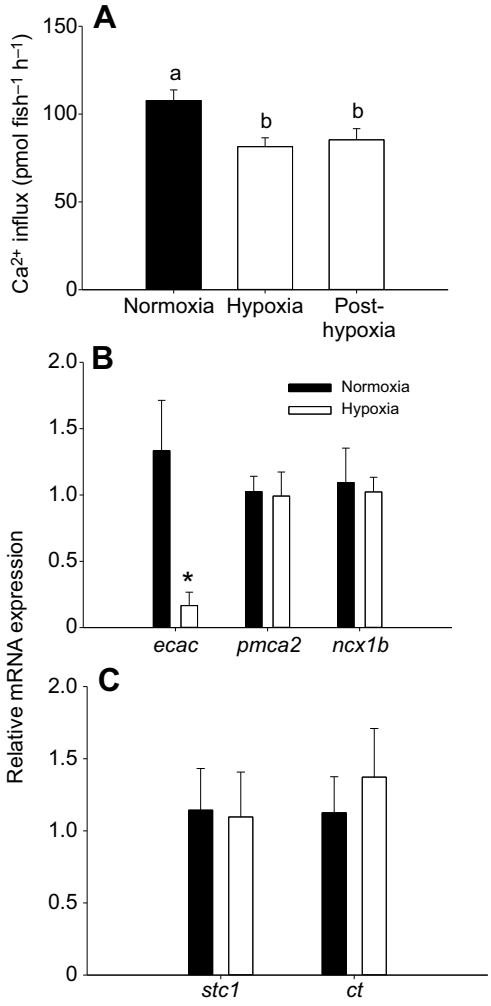

Fig. 2. Effects of hypoxia on Ca^{2+} influx and Ca^{2+} transport-related genes. (A) Influx of Ca^{2+} in developing zebrafish raised in normoxic or hypoxic water. Ca^{2+} influx in hypoxia-treated fish was also measured in normoxic water (post-hypoxia). Different letters indicate a statistical difference between treatments (P<0.05; one-way ANOVA, N=6). (B,C) The mRNA expression levels of (B) epithelial Ca^{2+} channel (*ecac*), plasma membrane Ca^{2+}-ATPase isoform 2 (*pmca2*), Na^+/Ca^{2+}-exchanger isoform 1b (*ncx1b*), and (C) stanniocalcin isoform 1 (*stc1*) and calcitonin (*ct*) in fish raised in normoxia or hypoxia. Data were normalized to 18S RNA expression, and are expressed relative to fish in normoxia. Asterisks indicate a statistical difference (P<0.05; Student's t-test, N=6). All data are presented as means±s.e.m.

at 4 dpf was performed. Compared with fish raised in normoxia, a significant reduction in the number of *ecac*-expressing ionocytes was observed in fish raised in hypoxic water (Fig. 3A). Fig. 3B,C and Fig. 3D,E are representative images showing the *ecac* mRNA signals in the skin of the yolk sac after exposure to normoxia and hypoxia, respectively. The density of NaRCs was also reduced in fish exposed to hypoxia at 3 or 4 dpf (Fig. 4).

The increase in HIF1-αb protein expression is prevented by morpholino gene knockdown

The effectiveness of morpholino gene knockdown on HIF1-αb protein expression was evaluated by western blotting at 4 dpf. In the sham group, exposure to hypoxia for 4 h markedly increased HIF1-αb protein expression (Fig. 5). However, such an increase was not observed in HIF1-αb morphants (i.e. HIF MO).

The role of HIF1-αb in Ca^{2+} uptake

Wild-type fish treated with the HIF-stabilizing drug DMOG exhibited a significant reduction in both Ca^{2+} influx and whole-body Ca^{2+} levels at 4 dpf (Fig. 6). In controls (sham group), exposure to hypoxia reduced Ca^{2+}influx (Fig. 7A). However, this was not observed in fish following HIF1-αb knockdown. The reduced whole-body Ca^{2+} content during hypoxia was also significantly less pronounced in HIF1-αb morphants (percentage reduction in whole-body Ca^{2+} content during hypoxia: sham group, 62.6±5.5%; HIF1-αb morphants, 28.7±2.8%; data not shown). Results from whole-mount *in situ* hybridization suggested that the number of *ecac*-expressing ionocytes was reduced by HIF1-αb knockdown or by exposure to hypoxia (Fig. 7B). However, the reduction in the number of *ecac*-expressing ionocytes during hypoxia was less pronounced in fish experiencing HIF1-αb knockdown.

DISCUSSION

Overview

The present study demonstrated that zebrafish raised in hypoxic water exhibit a significant reduction in whole-body Ca^{2+} levels and

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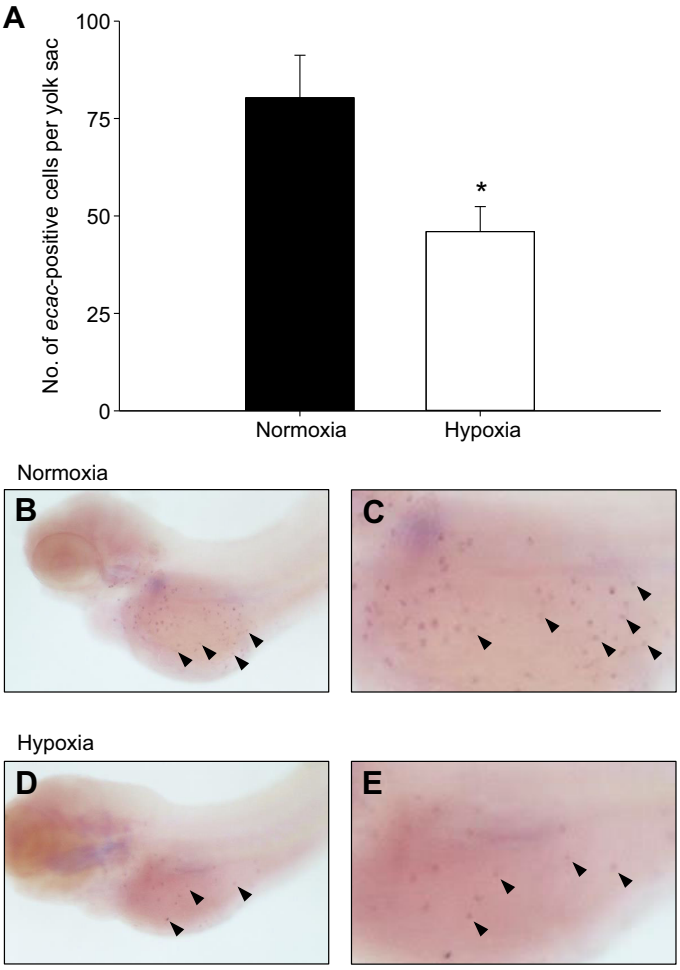

Fig. 3. Effects of hypoxia on the number of *ecac*-expressing ionocytes. (A) The number of *ecac*-expressing ionocytes on the skin of the yolk sac following exposure to normoxia or hypoxia at 4 dpf (corrected). Asterisks indicate a statistical difference (P<0.05; Student's t-test, N=6). Data are presented as means±s.e.m. (B–E) Representative images of whole-mount *in situ* hybridization showing the *ecac* mRNA signals (arrowheads) in fish raised in normoxic (B,C) or hypoxic (D,E) water.

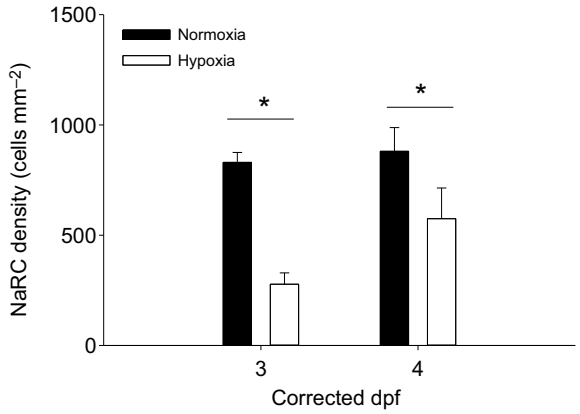

Fig. 4. Effects of hypoxia on the density of Na^+/K^+-ATPase-rich cells (NaRCs). NaRC density was measured in developmentally matched (i.e. corrected dpf) zebra fish. Asterisks indicate a statistical difference between normoxia and hypoxia within the same developmental age (P<0.05; two-way ANOVA, N=6). Data are presented as means±s.e.m.

an impairment of Ca^{2+} uptake. The reduction in Ca^{2+} uptake was associated with a decrease in *ecac* expression and number of *ecac*-expressing ionocytes. Interestingly, the inhibitory effects on Ca^{2+} uptake of hypoxic exposure were reduced in fish experiencing HIF-1αb knockdown, suggesting that activation of HIF-1αb suppressed Ca^{2+} uptake under hypoxic conditions.

Impact of hypoxia exposure on ionic regulation

In the present study, fish raised in hypoxic water exhibited a delay in development by about 24 h. Previous findings have also demonstrated that hypoxic treatment results in developmental delay in larval zebrafish (Manchenkov et al., 2015; Robertson et al., 2014). For this reason, results were compared for larvae exhibiting similar developmental ages.

Our results showed that zebrafish raised in hypoxic water until 4 dpf (corrected) exhibited a substantial reduction in whole-body Ca^{2+} and K^+ levels. In contrast, hypoxia did not affect whole-body Na^+ content in developing zebrafish. A previous study showed that exposure to hypoxia for 24 h significantly reduced plasma levels of Na^+ in scaleless carp, *G. przewalskii* (Matey et al., 2008). The apparent discrepancy may reflect stage- or species-specific differences in responses to hypoxia, and/or differences in the hypoxia protocols that were used. Because we observed that hypoxia severely affected whole-body Ca^{2+} balance in developing zebrafish, we opted to focus our study on the interactive effects of hypoxia and HIF1αb on Ca^{2+} influx. The results demonstrated that Ca^{2+} uptake was markedly reduced in fish raised in hypoxic water. Importantly, Ca^{2+} uptake did not recover to control levels after transfer of the fish back to normoxic water. These findings suggest that the inhibition of Ca^{2+} uptake was not caused by the acute effects of low water O_2 levels on Ca^{2+} influx, but rather reflected possible long-term changes in the Ca^{2+}-transport function (e.g. reduction in the abundance of Ca^{2+} transporters; discussed below) that persisted when hypoxia was withdrawn. Such long-term impairment in Ca^{2+} uptake may have important implications for the development of cartilage/skeleton (Kwong and Perry, 2015; Vanoevelen et al., 2011). Further investigations are required to address this issue.

Interaction of hypoxia and HIF-1αb with Ca^{2+} transport

In larval zebrafish, uptake of Ca^{2+} is thought to occur in a subset of NaRCs, which express ECaC at the apical membrane (for Ca^{2+}

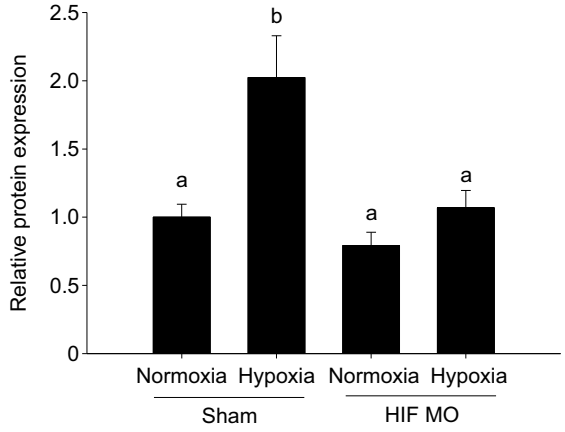

Fig. 5. Effects of morpholino gene knockdown on HIF-1αb protein expression. The relative protein expression levels of HIF-1αb in the sham group and HIF-1αb morphants (HIF MO) exposed to hypoxic water for 4 h. Total protein stain was used as a loading control, and data are expressed relative to the sham group in normoxia. Different letters indicate a statistical difference (P<0.05; one-way ANOVA, N=6). All data are presented as means±s.e.m.

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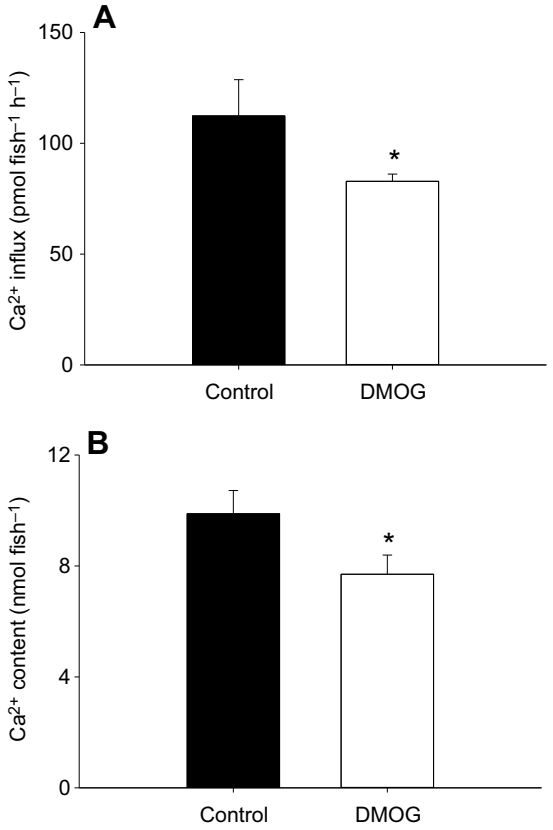

Fig. 6. Effects of HIF stabilization on Ca^{2+} regulation. (A) Ca^{2+} influx and (B) whole-body Ca^{2+} content in developing zebrafish treated with 100 μmol l^{-1} dimethyloxaloylglycine (DMOG). Asterisks indicate a statistical difference (P<0.05; one-tail Student's t-test, N=6). All data are presented as means±s.e.m.

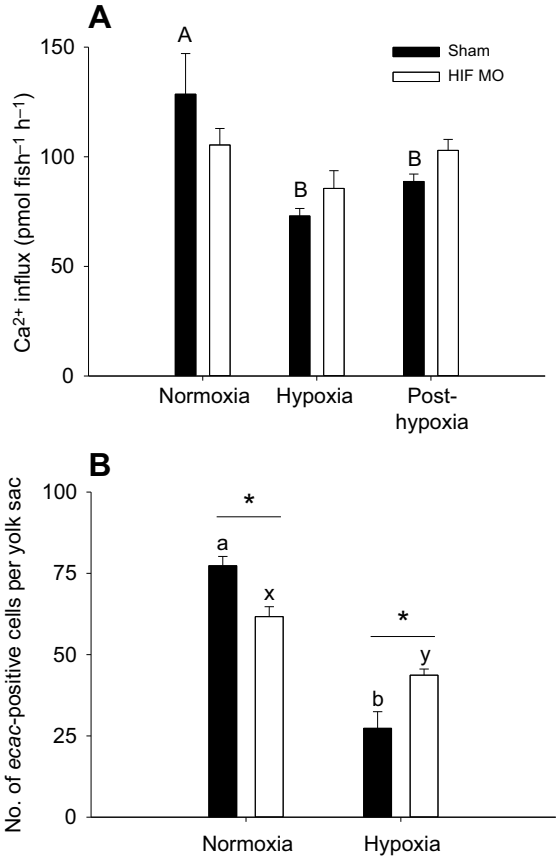

Fig. 7. Effects of HIF-1αb knockdown on Ca^{2+} regulation. (A) Influx of Ca^{2+} in the sham group and HIF-1αb morphants (HIF MO) raised in normoxic or hypoxic water. Ca^{2+} influx in hypoxia-treated fish was also measured in normoxic water (post-hypoxia). Different letters indicate a statistical difference (P<0.05; two-way ANOVA, N=6). (B) The number of *ecac*-expressing ionocytes in the shams group and HIF MO raised in normoxic or hypoxic water. Asterisks indicate a statistical difference between sham and HIF MO within the same normoxia or hypoxia treatment. Different letters indicate a statistical difference between normoxia and hypoxia within sham or HIF MO (P<0.05; two-way ANOVA, N=6). All data are presented as means±s.e.m.

uptake) and Na^{+}/Ca^{2+}-exchanger (i.e. NCX1b) and Ca^{2+}-ATPase (i.e. PMCA2) at the basolateral membrane (for Ca^{2+} extrusion out of the cell) (Liao et al., 2007; Pan et al., 2005). To evaluate the potential molecular mechanisms underlying the inhibition of Ca^{2+} uptake during hypoxia, the mRNA expression of these genes was measured using real-time PCR. The results suggested that the mRNA levels of *ecac*, but not *pmca2* and *ncx1b*, are substantially reduced in fish raised in hypoxic water. This finding suggested a specific inhibitory effect of hypoxia on *ecac* expression. In rats, expression of the transient receptor potential vanilloid (TRPV) 5 and TRPV6 (orthologues of ECaC) was also found to decrease in the duodenum of animals exposed to hypoxia (Yang et al., 2013). In the present study, we also evaluated the effects of hypoxia on two hypocalcaemic hormones, stanniocalcin-1 (stc-1) and calcitonin (ct). These hormones were shown to inhibit Ca^{2+} uptake by decreasing the expression of *ecac* in larval zebrafish (Lafont et al., 2011; Tseng et al., 2009). Both *stc-1* and *ct* were found to be expressed before 1 dpf, and continued to be expressed over development (Lafont et al., 2011; Tseng et al., 2009). Hypoxic treatment was reported to stimulate STC-1 mRNA and/or protein levels in various mammalian cell lines (Ito et al., 2014; Yeung et al., 2005). Additionally, we identified a putative hypoxia-regulatory element (HRE) in the upstream region of the *stc-1* gene (data not shown). However, our results demonstrated that hypoxic treatment does not affect mRNA expression of *stc-1* or *ct*. These findings suggest that the decreased *ecac* expression accompanying hypoxia was unlikely to be associated with *stc-1* and *ct* expression, although we cannot rule out the possibility that hypoxia modulated their protein levels. We further examined *ecac* expression using whole-mount *in situ* hybridization, and observed that the number of *ecac*-expressing ionocytes was significantly reduced following hypoxia. Notably, we also found that the density of NaRCs was decreased in fish exposed to hypoxia, suggesting a possible inhibitory effect of hypoxia on the differentiation of *ecac*-expressing NaRCs. Overall, the results indicate that the reduction in Ca^{2+} uptake by hypoxia was caused by a decrease in *ecac* expression associated with a reduction in the number of *ecac*-expressing ionocytes. It is also feasible that the expression of *ecac* per *ecac*-expressing ionocyte was reduced.

In the present study, we observed that fish treated with DMOG (a HIF-stabilizing drug; van Rooijen et al., 2009) also exhibited a reduction in both Ca^{2+} uptake and whole-body Ca^{2+} content under normoxic conditions, suggesting that HIF may play an inhibitory role in Ca^{2+} regulation. A previous study showed that larval zebrafish exposed to hypoxia exhibited an increase in protein levels of HIF-1αb, but not HIF-2α or HIF-3α (Köblitz et al., 2015), suggesting that HIF-1αb may play a more important role in hypoxic responses in this species in comparison to the other HIF genes. To examine the potential involvement of HIF-1αb in Ca^{2+} regulation, a functional gene knockdown approach was used to prevent activation of HIF-1αb during hypoxia. The reduction in Ca^{2+} influx during hypoxia

was no longer observed in fish experiencing HIF-1αb knockdown. Additionally, the decrease in the number of *ecac*-expressing ionocytes associated with hypoxia was less pronounced in the HIF-1αb-deficient fish. Together, these findings indicate that activation of HIF-1αb during hypoxia was inhibitory to Ca^{2+} uptake, probably via its interaction with *ecac*. The physiological significance of the HIF-1αb-mediated reduction in active Ca^{2+} uptake remains unclear. Because active uptake of Ca^{2+} is costly, the reduced Ca^{2+} uptake may help to reserve energy for other regulatory functions that may be more critical during early development, such as the cardiovascular and respiratory systems. In fact, hypoxia exposure has been shown to increase breathing frequency, stroke volume, cardiac output and the number of red blood cells in developing zebrafish (Kopp et al., 2014; Porteus et al., 2014). Clearly, more investigations are required to understand the precise role of HIF-1αb during early development, particularly in hypoxic environments.

Conclusions and perspectives

The current study demonstrated that larval zebrafish raised in hypoxic water exhibit a disturbance in Ca^{2+} balance. Hypoxia reduced Ca^{2+} uptake by reducing the number of *ecac*-expressing ionocytes, which, in turn, presumably contributed to the decrease in whole-body *ecac* expression. These effects appeared to be associated with the activation of HIF-1αb during hypoxia. The present study revealed an important inhibitory role of HIF-1αb in Ca^{2+} transport functions in zebrafish. However, the precise molecular mechanisms underlying the actions of HIF-1αb on ionocyte numbers and thereby *ecac* expression are unknown. It is possible that activation of HIF-1αb suppressed the differentiation of epidermal stem cells into *ecac*-expressing ionocytes. Long-term impairment of Ca^{2+} regulation may have significant implications for cartilage/skeleton development (Kwong and Perry, 2015; Vanoevelen et al., 2011). Future investigations should also address the potential chronic effects of hypoxia on bone formation in larval fish.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

R.W.M.K., Y.K., V.T., E.A., N.H. and C.R. conducted the research; R.W.M.K., Y.K., N.H., B.P. and S.F.P. designed the research; R.W.M.K., Y.K., V.T., E.A., N.H., B.P. and S.F.P. analysed and interpreted the data; all authors revised the manuscript, and gave final approval for publication.

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