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Original Research

mRNA Expression of Select Hypoxia-Inducible Genes and Apoptotic Control Genes in Zebrafish Exposed to Hypoxia during Development

Zhonghai Ding[1], Ping Sun[1]*, Xin Hua[1], Yiyao Bai[1], Eva H. H. Shang[2], Rudolf S. S. Wu[2], Yuhui Zuo[1]**

[1]State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing 210046, China
[2]Center for Coastal Pollution and Conservation, City University of Hong Kong, Hong Kong SAR, China

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Abstract

Hypoxia is well known to occur in contaminated water environments, but it is still unclear about its effects on transcription of hypoxia-inducible genes and apoptotic control genes in zebrafish during development. In this study, the expression of select genes during zebrafish embryonic development was detected at mRNA level to investigate spatio-temporal changes of hypoxia-inducible genes and apoptosis control genes, as well as the correlation between these genes. Results showed that up-regulation of hypoxia-inducible factor 1 (HIF-1α) mRNA occurred within the first 2 h of exposure to hypoxia, followed by up-regulation of other hypoxia-inducible genes controlled by HIF-1α, such as erythropoietin and vascular endothelial growth factor. 48 hpf (the critical period for embryonic development, especially for the development of a cardiac system) and 40 dpf (the critical period for sex differentiation and development) were found to be the two sensitive windows to hypoxia, at which time significant changes in the mRNA expression of all selected hypoxia-inducible genes were clearly evident. A higher ratio of pro-apoptotic gene (Bax) vs. anti-apoptotic gene (Bcl-2) transcriptions was found in the head as compared with in the tail under hypoxia. A higher Bax/Bcl-2 ratio was found in hypoxic males than in hypoxic females, suggesting that hypoxia potentially favors the formation of testes by inducing apoptosis in ovaries during the hermaphroditism in zebrafish, which in turn causes a male-biased sex ratio.

Keywords: aquatic toxicity, development, gene expression, hypoxia, zebrafish

Introduction

It is well known that hypoxia may occur in various contaminated water environments, especially in the presence of organic pollutants. Growing evidence derived from *in vitro* studies in mammalian systems has revealed that hypoxia-inducible factor 1 (HIF-1), a heterodimeric basic helix-loop-helix transcription factor, is the master regulator for many hypoxia-responsive genes [1], and this is also ascertained on fish [2]. HIF-1 is a ubiquitous DNA binding protein composed of two subunits of α and β. Subunit α is unstable and rapidly degraded under normoxia [3], and therefore is only found in significant amounts under hypoxia. Upon receiving a signal from the molecular oxygen sensor under hypoxia [4], HIF-1α then activates the transcription of a number of hypoxia-inducible genes according to different strategies adopted by the organism.

*e-mail: sunping@nju.edu.cn
**e-mail: zuoyh@nju.edu.cn

For example, HIF-1α can up-regulate vascular endothelial growth factor (VEGF) to enhance the proliferation of blood vessels so as to increase oxygen supply [5], and up-regulate erythropoietin (EPO) to increase red blood cell production [6]. *In vitro* studies on normal and tumor human cells also showed that transcription of telomerase, which is important in controlling cell proliferation, also is regulated by HIF-1α [7].

Furthermore, HIF-1α also plays a role in development by affecting apoptosis and is regarded as a master regulator of cellular and developmental oxygen homeostasis [8]. Apoptosis is central to normal embryonic development since programmed cell death determines morphogenesis of developing tissues as well as homeostasis of adult organs and tissues. Developing organisms commonly produce excess cells that are subsequently removed by cell death during later stages of normal development [9, 10]. Our earlier work [2] showed that a significantly higher percentage of malformation (+77.7%) was found in hypoxic fish and the apoptosis pattern was changed by hypoxia. However, the mechanisms by which hypoxia may affect normal apoptosis remain unknown. Notably, effects of hypoxia on apoptosis should be most pronounced and significant during embryonic development, when these complex processes are moderated by apoptosis. No study has attempted to relate apoptosis to malformation caused by hypoxia, although apoptosis is a key process in modulating histogenesis and organogenesis, and hypoxia has been shown to affect apoptosis in rats, both *in vitro* and *in vivo* [1, 4]. It is therefore possible that hypoxia may disrupt vital developmental processes through alteration of apoptosis.

Mammalian studies showed that a number of genes are closely related to apoptosis. It has been well characterized that the anti-apoptotic gene Bcl-2 protects cells against various types of apoptotic stimuli [11]. Bax, on the other hand, is a pro-apoptotic member that exerts its function by homodimerizing with each other and accumulating in the mitochondrial membrane [12]. It has been indicated that transcription factor p53 affects cell cycle arrest or apoptosis in response to a variety of genotoxic and physical stresses [13]. Apoptosis may be elicited, in part, by p53-stimulated transcription of the pro-apoptotic gene Bax and repression of the anti-apoptotic gene Bcl-2 [14, 15]. Additionally, under hypoxia, HIF-1α may stabilize p53 [16] and lead cells to apoptosis through the enhanced expression of Bax [17]. VEGF and EPO also are involved in apoptosis: VEGF plays a central role in the development of new blood vessels (angiogenesis), for establishing a functional vascular system during embryogenesis, and the survival of immature blood vessels by preventing the apoptosis of vascular endothelial cells (vascular maintenance); for EPO, recent work has extended the traditional role of EPO from a mediator of erythroid maturation to one that offers protection against apoptosis in a wide variety of tissues [18, 19].

Embryogenesis is a complex process that requires the intricate interactions of large groups of genes, with different and programmed timing in their expression. While current knowledge on molecular response to hypoxia is largely restricted to studies of adults, mammalian models, or *in vitro* studies. Very little is known on how hypoxia may affect gene transcription during development, albeit this is very important because:

(a) the gene transcription pattern will determine subsequent protein translation and individual development and
(b) generally speaking, embryos are more sensitive to adverse environmental conditions than adults [20].

Besides, our previous study [21] showed that hypoxia is a teratogen to fish embryos, and more effective in certain developmental windows. Therefore, timing of exposure to hypoxia during development appears to be critical, and understanding how hypoxia affects changes in gene transcription during development will contribute significantly to our knowledge of how vertebrates respond to hypoxia at the molecular level and the dynamic interactions between genes and the environment. Furthermore, it is still unclear about how early these genes would begin to respond to hypoxia; whether these genes are more sensitive during embryonic stages than adult stages. Besides, most teratogens have a very specific window, but the specific windows for hypoxia in terms of gene expression remain unknown.

The aims of this study are:

(1) to test the hypothesis that embryos are more sensitive to hypoxia than adults
(2) to identify the most sensitive developmental period during embryonic development of zebrafish
(3) to compare gene expression patterns in heads and tails, with a view to provide an explanation on the difference in apoptotic patterns observed in our earlier study.

Experiments also were carried out to examine if there is any difference between the gene expression patterns in different sexes. We chose zebrafish as the model because their embryos develop rapidly [22]. Zebrafish embryos remain transparent throughout most of embryogenesis, which simplifies the observation and analysis. Furthermore, zebrafish are small, inexpensive to maintain, and easily bred in large numbers, which can meet the requirement of a large amount of embryos for gene analysis.

Materials and Methods

Zebrafish Maintenance and Embryo Collection

Fish were kept at 28.5ºC in aerated water (60 mg 'Instant Ocean'/dH_2O) and subjected to a 14:10 h light/dark cycle. Fertilized eggs were collected by placing a plastic box (24 cm length × 12 cm width) at the bottom of each tank after group mating during the first 30 min of the light period. They were washed and transferred to either hypoxic or normoxic aquaria within 1 h and incubated at 28.5ºC in embryo medium (NaCl, 19.3 mM; KCl, 0.23 mM; $MgSO_4 \cdot 7H_2O$, 0.13 mM; $Ca(NO_3)_2$, 0.2 mM; HEPES, 1.67 mM; pH 7.2). At 5 hpf, all embryos were screened under a dissecting stereo-microscope (Carl Zeiss, 60x; Oberkochen, Germany). Only normal embryos that reached the blastula stage (30% epiboly) were selected for subsequent experiments.

Set-up of Hypoxic and Normoxic Systems

Two circulating systems, one with hypoxic water and the other with normoxic water, were set up to provide a constant level of dissolved oxygen for zebrafish throughout the experimental period. In the hypoxic system, water in the reservoir tank (300 L) was pumped through a stripping column with plastic balls (4 cm in diameter), where an appropriate amount of nitrogen/air and water were allowed to mix to achieve the desirable oxygen levels (i.e. 0.8 mg $O_2 \cdot l^{-1}$ and 5.8 mg $O_2 \cdot l^{-1}$ for the hypoxic and normoxic systems, respectively). The desired level of dissolved oxygen was controlled and maintained by a dissolved oxygen controller (Cole-Parmer 01972-00, Illinois, USA) (within±0.1 mg $O_2 \cdot l^{-1}$) throughout the entire experimental period. Dissolved oxygen in the systems was checked every day using a dissolved oxygen (DO) meter (YSI model 52, Yellow Springs Instrument Company, OH, USA). 45 net cages (16.5 cm length × 12.5 cm width ×13 cm height) were suspended in each of the hypoxic and normoxic systems. Approximately 5,000 zebrafish eggs for each treatment were randomly put in the small nets equally and reared for 120 days. During the first month of development, fish were fed with Azoo *Artemia* cysts twice a day and 50% of the water in the system was renewed once a week. After that, fish were fed with *Artemia* cysts in the morning and TetraMin (baby) completed food in the afternoon every day, and 50% of the water (pre-mixed with nitrogen to reach the oxygen level desired) was changed every 2-3 days.

Selection of Genes and Time Points for Detection

Hypoxia-inducible genes, HIF-1α, VEGF, EPO, p53 and apoptosis control genes Bax and Bcl-2 were selected to investigate mRNA expression changes under hypoxia. Temporal changes in mRNA expression pattern of select genes were studied throughout the whole development of zebrafish, covering different developmental stages: 24 hpf (Prim-5 stage, Pharyngula period), 48 hpf (Long-pec stage, Hatching period), 120 hpf (the beginning of Larval stage), 10 dpf (Larval stage), 40 dpf (Juvenile stage), and 60 dpf (Adult stage) (according to the criteria described in www.zfin.org). mRNA expression of many hypoxia-related genes is controlled by HIF-1a. It is therefore likely that up-regulation of HIF-1a will precede those genes regulated by HIF-1a. As such, mRNA expression of HIF-1a at 1, 2, 4, and 12 hpf was also determined in this study. In addition, transcription of hypoxia-inducible genes and apoptosis control genes at 36 hpf (Prim-25 stage, Pharyngula period during organogenesis) in the head and tail regions was also investigated.

RNA Extraction

At 1, 2, 4, 12, 24, 48, and 120 hpf (100-120 embryos/larvae for each time point), as well as 10 and 40 dpf (about 50 larvae for each time point), fish were sampled from each of the hypoxic and normoxic groups and pooled as one replicate, and total RNA was extracted from each replicate (n=4). At 36 hpf, the chorions of the embryos were first removed using forceps and then dissected into two parts into the head and tail regions. 100-120 heads or tails were pulled together as one replicate (n=4), and RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's protocol. After 60 days, liver from 3-4 male and 3-4 female fish were dissected and pooled together as one replicate. RNA was extracted from 4 such replicates using the same kit described above.

DNAse I Digestion of RNA

In order to eliminate residual genomic DNA in the total RNA preparation, all RNA samples were treated with amplification-grade DNase I (Invitrogen Life Technologies, Carlsbad, CA, USA) prior to real-time RT-PCR analysis. The yield and purity of extracted RNA in each sample was determined using a BioPhotometer spectrophotometer (260 nm reading and 260 nm/280 nm ratio; Eppendorf Scientific, Germany), and the integrity of RNA was verified by gel electrophoresis.

First-Strand cDNA Synthesis

cDNA was synthesized from 2 µg RNA from each sample using the Superscript First-Strand Synthesis System for real-time RT-PCR analysis (Invitrogen Life Technologies) according to the manufacturer's instructions. mRNAs were specifically transcribed by priming with oligo $(dT)_{12-18}$ primers (Invitrogen Life Technologies) in 20 µl reactions.

Real Time RT-PCR Reagents and Cycling

RT-PCR primers were selected based on their respective genomic sequence (GenBank AF277675) (Table 1). For each selected gene, RT-PCR reactions (n=4 for each gene in each sample) were performed on 4 replicate samples.

Table 1. RT-PCR primers used for quantification of the targeted genes in this study.

Gene name	Sequence of the primers (5'-3')
HIF-1α	forward-5'CGAACATCGAGGTGCCATTG3' reverse-5'TCGTACACTGATCTGTTCAAGAGG3'
EPO	forward-5'TGGTGCTGGAGTGGACCCGTC3' reverse-5'AGCCTCTGCATCCCATGCCTCC3'
VEGF	forward-5'GGACCTTTCTTTACGAACAGTAGC3' reverse-5'CCCTAGCAAGTGACATCACAGAA3'
P53	forward-5'CCCGGCGATCATGGATTTAGG3' reverse-5'CAACGTCCACCACCATTTGAAC3'
Bax	forward-5'GATACGGGCAGTGGCAATGA3' reverse-5'ACTCCGGGTCACTTCAGCAT3'
Bcl2	forward-5'CGAGTGTGTGGAGAAGGAGATG3' reverse-5'TGGTTGTCTAGGTAGACGGTCAT3'
β-actin	forward-5'CGAGCAGGAGATGGGAACC3' reverse-5'CAACGGAAACGCTCATTAC3'

β-actin was used as an internal control. A 25 µl sample containing 12.5 µl SYBER Green PCR Master Mix, 0.5 µl (10 µg/ml) of each forward and reverse primer, 5 µl diluted cDNA and 6.5 µl water was prepared for each sample. Reactions were carried out in a 96-well system. Thermal cycle was set at: 95ºC for 2 min, followed by 45 cycles of 95ºC for 20 sec, 60ºC for 30 sec and 72ºC for 30 sec, 95ºC for 1 min and 55ºC for 1 min, and, finally, 80 cycles of 55ºC for 10 sec (Bio-rad iCycler iQ Real time PCR Detection System, CA, USA).

Statistical Analysis

SPSS statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. RT-PCR data from 4 independent samples were analyzed using a Chemi-imager with AlphaEase™ software (Alpha Innotech Corp.). Fold change was calculated using the formula:

$$\text{Fold change} = 2^{-\Delta(\Delta C_T)}$$

...where:

$\Delta C_T = C_{T(\text{target})} - C_{T(\beta\text{-actin})}$ and

$\Delta(\Delta C_T) = \Delta C_{T(\text{stimulated})} - \Delta C_{T(\text{control})}$.

Statistical significance of the differences relative to controls was tested with Student's *t*-test ($p \leq 0.05$). One-way analysis of variance (ANOVA) was performed to test for significant differences in gene expression:

(a) in both sexes from hypoxic and normoxic groups at 2 months
(b) in different body regions (head and tail) of larvae at 36 hpf from hypoxic and normoxic groups
(c) of HIF-1α and the Bax/Bcl-2 ratio at different time points between the hypoxic and normoxic groups.

The Pearson correlation test was used to assess the relationships between the expression of selected genes and the Bax/Bcl-2 ratio.

Results

Temporal Change of Transcription Patterns

No significant change in HIF-1a mRNA expression was found in the first hpf, when embryos were exposed to hypoxia. HIF-1α transcription was significantly up-regulated (+53.8%; *t*-test, $p<0.001$) after 2-h exposure to hypoxia, and down-regulated afterwards. At 4, 10, and 120 hpf, expression of HIF-1α were only approximately 50% of the normoxic values (Fig. 1A).

VEGF transcription was significantly up-regulated by hypoxia at 24, 48, and 120 hpf, and EPO transcription was up-regulated at 10 and 40 dpf. VEGF mRNA expression significantly increased from 1.21±0.09 at 24 hpf (*t*-test, $p<0.01$) to 5.67±0.21 at 40 dpf (*t*-test, $p<0.001$), and the fold change of EPO mRNA increased from 1.37±0.32 at 10 dpf (*t*-test, $p<0.05$) to 5.00±0.23 at 40 dpf (*t*-test, $p<0.001$) (Fig. 1B).

The mRNA expression of p53 was significantly up-regulated by hypoxia at 48 hpf (fold change = 1.52±0.18, *t*-test, $p<0.01$), 10 dpf (fold change = 1.57±0.14, *t*-test, $p<0.001$) and 40 dpf (fold change = 1.64±0.29, *t*-test, $p<0.01$). Bax mRNA expression was also up-regulated by hypoxia except at 24 hpf, with changes ranging from 1.07±0.08-fold at 120 hpf (*t*-test, $p<0.05$) to 2.75±0.26-fold at 40 dpf (*t*-test, $p<0.001$). Bcl-2 mRNA expression was up-regulated by hypoxia at 48 hpf (fold change = 1.13±0.05, *t*-test, $p<0.01$) and 40 dpf (fold change = 1.33±0.16, *t*-test, $p<0.01$), but down-regulated at the other time points (Fig. 2).

Spatial Change of mRNA Expression Pattern in Head and Tail

After exposure to hypoxia for 36 h., HIF-1α mRNA expression was down-regulated in the head (fold-change = 0.75±0.12, *t*-test, $p<0.01$) and tail (fold-change = 0.81±0.07, *t*-test, $p<0.01$); while mRNA expressions of VEGF (fold-change = 1.61±0.24 in the head, *t*-test, $p<0.01$; and 1.36±0.18 in the tail, *t*-test, $p<0.01$), EPO (5.36±0.40 in the head, *t*-test, $p<0.001$; and 2.71±0.14 in the tail, *t*-test, $p<0.001$) and p53 (1.35±0.12 in the head, *t*-test, $p<0.01$; and 1.46±0.25 in the tail, *t*-test, $p<0.01$) were significantly up-regulated by hypoxia in both the head and tail (Fig. 3).

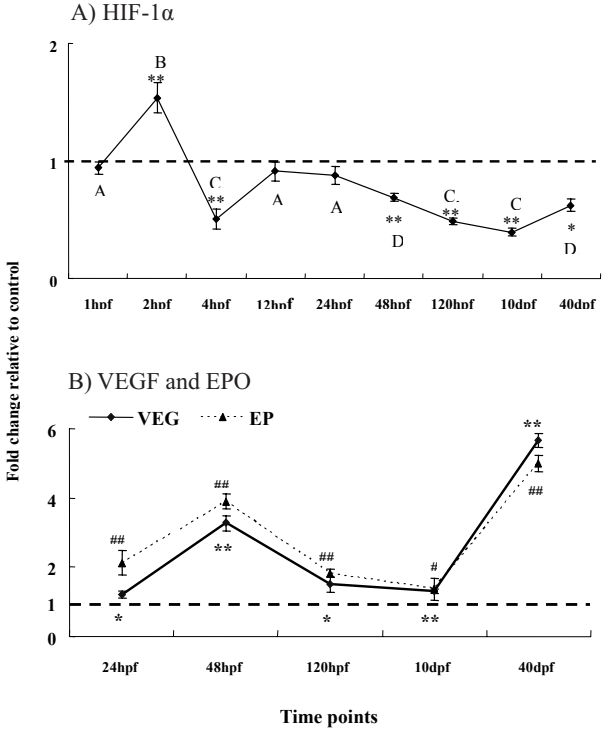


Fig. 1. Temporal change of (A) HIF-1α; (B) VEGF and EPO mRNA levels in zebrafish upon exposure to continuous hypoxia (0.8 mg $O_2 \cdot l^{-1}$). Data are presented as fold change relative to control group (1 on Y axis). * indicates mRNA expression of HIF-1α and VEGF significantly different from the normoxic control (*t*-test: **p<0.01; ***p< 0.001); # indicates mRNA expression of EPO significantly different from the normoxic control (*t*-test: #p<0.05; ###p< 0.001). Letters indicate data from the same/different group (one way ANOVA, p<0.05). N=4, Mean±SD.

Notably, EPO mRNA level was significantly higher in the head than in the tail (*t*-test, p<0.05). In the head region, up-regulation of Bax mRNA expression (fold-change = 1.14±0.09, *t*-test, p<0.01) coupled with down-regulation of Bcl-2 (fold-change = 0.72±0.10, *t*-test, p<0.01) was observed. In contrast, significant down-regulation of Bax mRNA expression (fold-change = 0.85±0.06, *t*-test, p<0.01) was found in the tail region (Fig. 3).

Change of mRNA Expression Pattern between Males and Females

At 60 dpf, no significant difference in transcription pattern was observed in HIF-1a, VEGF, and EPO between hypoxic males and hypoxic females. HIF-1a mRNA expression was significantly down-regulated by hypoxia in both genders (fold-change = 0.84±0.05 in female, *t*-test, p<0.1; and 0.60±0.11 in male; *t*-test, p<0.001). VEGF (fold-change = 2.17±0.21 in female, *t*-test, p<0.001; and 1.66±0.11 in male, *t*-test, p<0.001) and EPO (fold-change = 1.55±0.06 in female, *t*-test, p<0.001; and 2.41±0.21 in male, *t*-test, p<0.001) was over-expressed at transcriptional level in both males and females exposed to hypoxia when compared to the normoxic group (Fig. 4).

Differential mRNA expressions of p53 and Bcl-2 were clearly evident in males and females. In hypoxic females, both p53 and Bcl-2 mRNA expressions were up-regulated (fold-changes = 1.46±0.22, *t*-test, p<0.01 and 1.48±0.10,

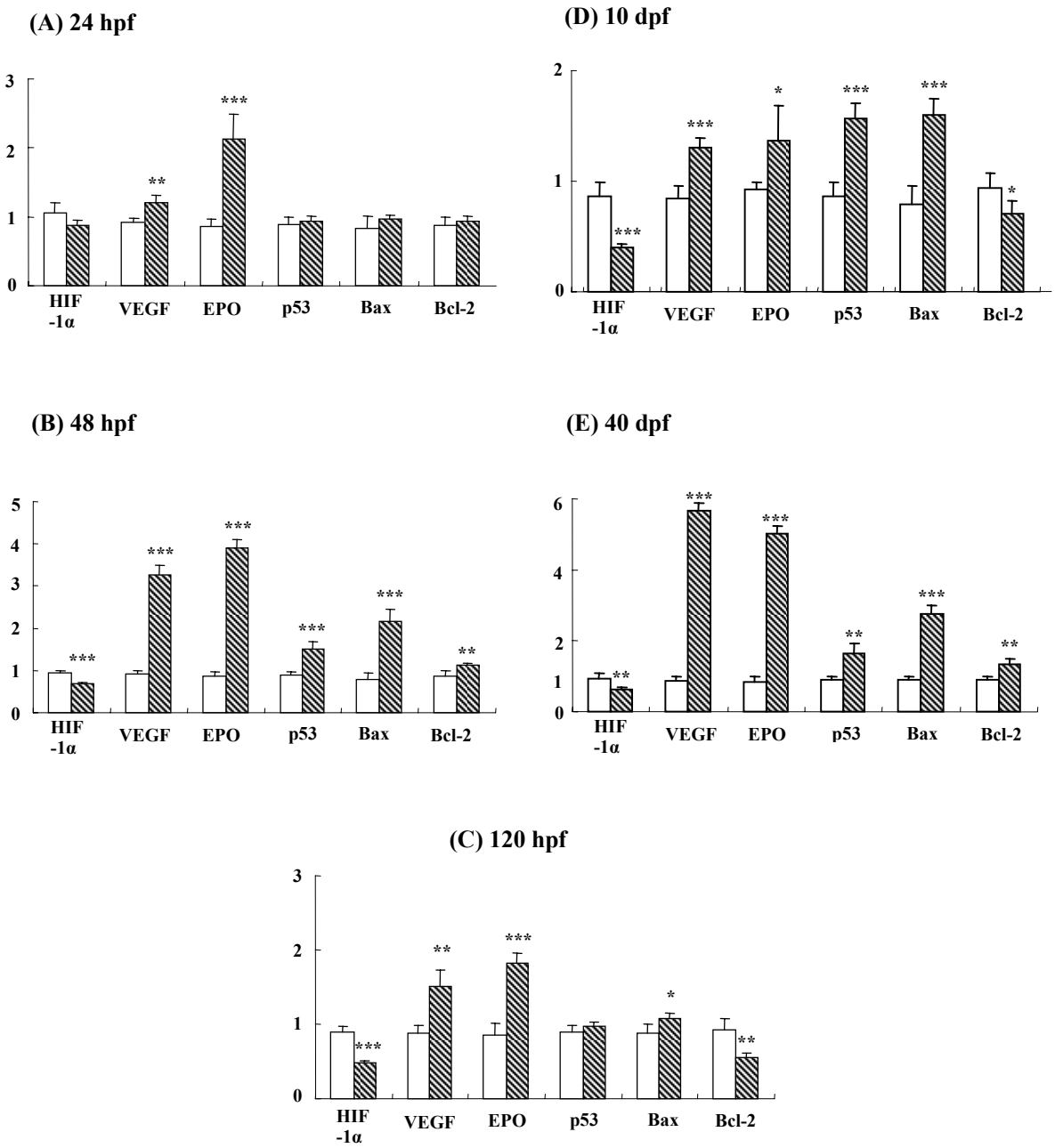


Fig. 2. mRNA expression of HIF-1α, VEGF, EPO, p53, Bax, and Bcl-2 in zebrafish at (A) 24 hpf, (B) 48 hpf, (C) 120 hpf, (D) 10 dpf, and (E) 40 dpf upon exposure to normoxia (5.8 mg $O_2 \cdot l^{-1}$) and continuous hypoxia (0.8 mg $O_2 \cdot l^{-1}$). Values significantly different from the normoxic control are indicated by asterisks (*t*-test: **p<0.01; ***p<0.001). N=4, Mean±SD.

Table 2. Correlation coefficients (r) between the mRNA expressions of various hypoxia-inducible genes, apoptosis control genes, and Bax/Bcl-2.

Genes	Correlation coefficient (r)						
	HIF-1α	VEGF	EPO	P53	Bax	Bcl-2	Bax/Bcl-2
HIF-1a	1	0.086	0.308	-0.359	-0.081	0.510	-0.884*
VEGF	0.086	1	0.962**	0.649	0.932*	0.854	0.325
EPO	0.308	0.962**	1	0.547	0.879*	0.923*	0.133
P53	-0.359	0.649	0.547	1	0.874	0.549	0.715
Bax	-0.081	0.932*	0.879*	0.874	1	0.812	0.521
Bcl-2	0.510	0.854	0.923*	0.549	0.812	1	-0.074
Bax/Bcl-2	-0.884*	0.325	0.133	0.715	0.521	-0.074	1

*p<0.05; **p<0.01

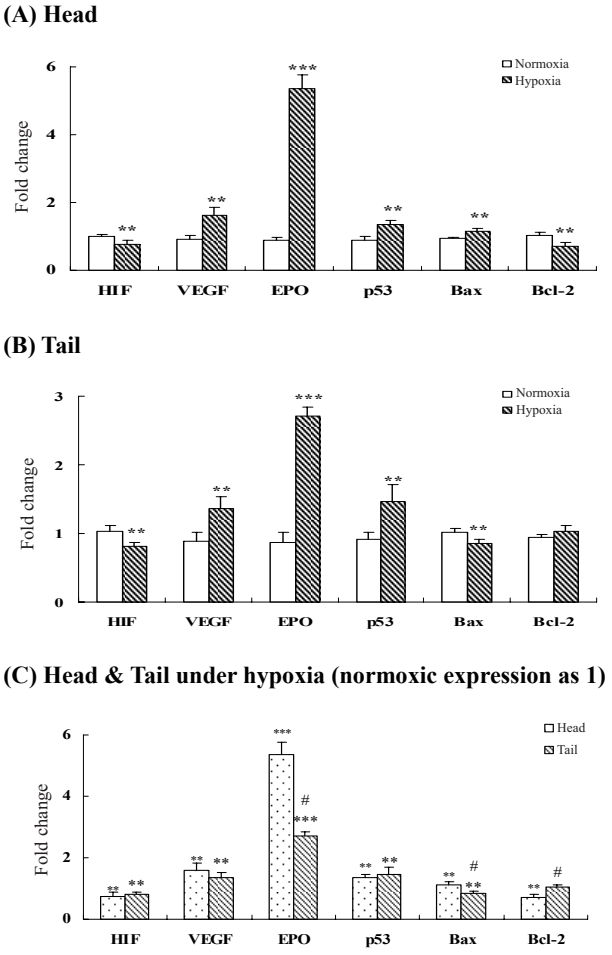


Fig. 3. mRNA expressions of HIF-1α, VEGF, EPO, p53, Bax, and Bcl-2 in head and tail of zebrafish (36 hpf) upon exposure to normoxia (5.8 mg $O_2 \cdot l^{-1}$) and continuous hypoxia (0.8 mg $O_2 \cdot l^{-1}$). (A) Head, (B) Tail, and (C) Relative expression in head and tail with corresponding normoxic levels as 1. Values significantly different from the normoxic control are indicated by asterisks (*t*-test: **p<0.01; ***p<0.001), # indicates significant difference in mRNA expression of a specific gene between head and tail, as revealed by one way ANOVA (p<0.05). N=4, Mean±SD.

t-test, p<0.001, respectively), while down-regulations of these two genes were observed in hypoxic males (fold-changes = 0.92±0.07 and 0.73±0.14, respectively; *t*-test, p<0.05) (Fig. 4).

Bax/Bcl2 Ratio

Bax/Bcl-2 was about 2.0 (ranging from 1.93±0.23 to 2.30±0.24) within the first 40 days of development in the hypoxic treatments (except the value was 1.04±0.09 at 24 hpf), while the ratio was about 1.0 (ranging from 0.84±0.06 to 1.02±0.07) in the normoxic control at each time point (t-test, p<0.001) (Fig. 5A). At 36 hpf, the Bax/Bcl-2 ratio was significantly higher in the head (1.61±0.21) than in the tail (0.82±0.05; t-test, p<0.001) (Fig. 5B). The mRNA expression ratio was significantly higher in males (1.09±0.27) than in females (0.55±0.03) after 60 days of development (t-test, p<0.01) (Fig. 5C).

Correlation between Transcription Patterns of Various Genes

Table 2 shows Pearson correlation coefficients between mRNA expressions of various genes in hypoxic fish during the first 40 days of development. The results demonstrated that:

(I) Significant positive correlations were found among VEGF, EPO, and Bax (p<0.05), and the correlation between VEGF and EPO was highly significant (p<0.01)

(II) Significant positive correlation was found between EPO and Bcl-2 (p<0.05)

(III) Significant negative correlation was found between HIF-1a and Bax/Bcl-2 ratio (p<0.05).

Discussion

In this study, mRNA expression of HIF-1α mRNA in hypoxic fish was significantly up-regulated at 2 hpf, then down-regulated at all subsequent time points (i.e. 4, 12, 24,

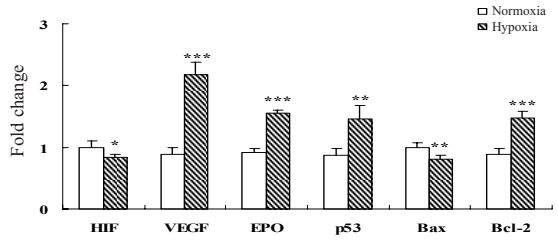
48, and 120 hpf; and 10, 40, and 60 dpf) upon continuous exposure to hypoxia. This is consistent with previous *in vitro* studies in carcinoma cells, which demonstrated that HIF-1α mRNA expression was transient and where up-regulation was only observed within the first several hours of exposure to hypoxia [23]. The up-regulation of HIF-1α within the first 2 h of exposure may serve to up-regulate VEGF, EPO, and other hypoxia-related genes.

In this study, significantly higher mRNA levels of VEGF were found in hypoxic males and females at each time point, with the highest fold-change (5.7±0.2 fold) recorded at 40 dpf. Given that even modest VEGF over-expression from its endogenous locus in mice resulted in severe abnormalities in heart development and embryonic lethality [24], the significant up-regulation of VEGF mRNA expression observed during the embryonic development of zebrafish may lead to a higher mortality in the hypoxic groups [25].

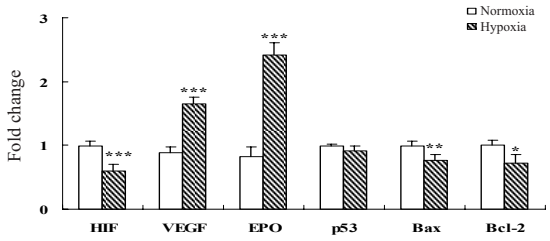
In metazoan organisms, EPO is up-regulated under hypoxia as an adaptive strategy to enhance the production of red blood cells and oxygen delivery [26]. The same adaptive strategy is also adopted by the zebrafish to cope with hypoxia during its embryonic development. This study revealed that EPO mRNA expression was significantly up-regulated by hypoxia at each time point (except 10 dpf), and a 5-fold increase was recorded at 40 dpf. Ueba et al. [27] demonstrated that an over-expression of EPO can enhance the activity of Akt, which in turn inhibits the cardiomyocyte apoptosis, indicating the possibility that the up-regulation of EPO mRNA expression during zebrafish development is related to the apoptosis altered by hypoxia.

In zebrafish, p53 also regulates apoptosis in a similar way as in mammalian cells. Apoptosis in zebrafish embryos induced by camptothecin and UV is solely mediated

(A) Female



(B) Male



(C) Female & Male under hypoxia (normoxic expression as 1)

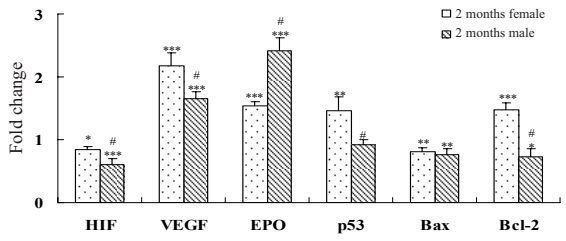
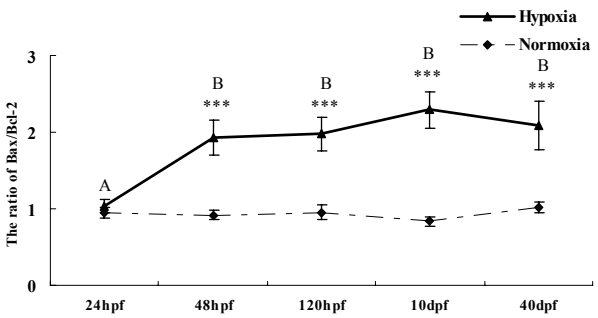
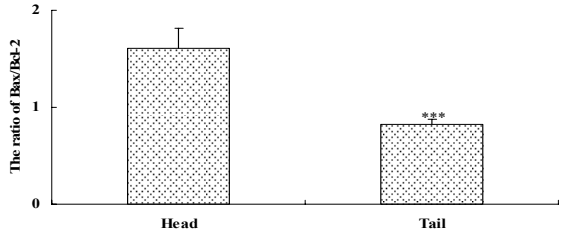


Fig. 4. mRNA expressions of HIF-1α, VEGF, EPO, p53, Bax, and Bcl-2 in females and males after 60 days of exposure to normoxia (5.8 mg $O_2 \cdot l^{-1}$) and continuous hypoxia (0.8 mg $O_2 \cdot l^{-1}$). (A) Female, (B) Male, and (C) Relative mRNA expression in female and male with corresponding normoxic levels as 1. Values significantly different from the normoxic control are indicated by asterisks (*t*-test: *p<0.05; **p<0.01; ***p<0.001); # indicates significant difference between males and females, as revealed by one way ANOVA (p<0.05). N=4, Mean±SD.

(A) At different time points during development



(B) Head and tail (ratio in normoxia as one)



(C) Female and male (ratio in normoxia as one)

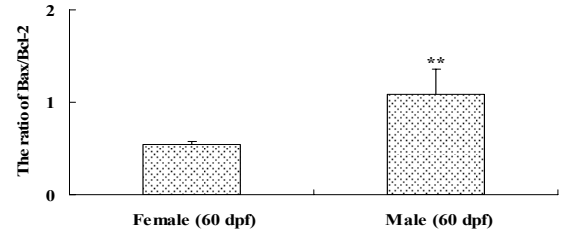


Fig. 5. Bax/Bcl-2 ratio of mRNA expression in zebrafish during development upon exposure to normoxia (5.8 mg $O_2 \cdot l^{-1}$) and continuous hypoxia (0.8 mg $O_2 \cdot l^{-1}$). (A) at different time points during development; (B) in head and tail regions of hypoxic fish at 36 hpf; (C) in males and females after 60 days of development under hypoxia. (B-C) with corresponding normoxic ratio as 1. Values significantly different from the normoxic control are indicated by asterisks (*t*-test: ***p<0.001). Different letters indicate significant differences at different time points as revealed by one way ANOVA (p<0.05). N=4, Mean±SD.

through an increase in p53 activity caused by DNA damage, and only a slight growth retardation and no increase in TUNEL- (terminal deoxynucleotide transferase-mediated dUTP-digoxygenin nick-end-labeling staining) positive cells was found in p53-deficient embryos upon exposure to apoptosis-inducing stressors [28]. This suggests that the up-regulation of p53 is responsible for the occurrence of apoptosis and indicates the conserved effects of p53 on apoptosis and cell cycle arrest in fish as well as in mammals. However, whether p53 in fish would have anti-teratogenic effects similar to those in mammals remains unknown.

In this study, p53 was up-regulated at transcriptional level by hypoxia at, 10, 40, and 48 hpf, but no change was found at other time points. The role that p53 played in hypoxia-induced apoptosis during zebrafish development remains unclear. Since 48 hpf is a critical period during the early embryonic development of zebrafish, the up-regulation of p53 at 48 hpf may be crucial in enabling the zebrafish to cope with hypoxia and ensure normal development by inhibiting the induction of "abnormal" apoptosis, although the precise role of p53 is not known.

It is well known that HIF-1α is the master regulator of many hypoxia-inducible genes [1-3]. The significant correlation observed in this study between VEGF and EPO transcription is therefore not surprising, since both genes are tightly up-regulated by HIF-1α [5, 18, 29]. The significant correlation between HIF-1a mRNA expression and Bax/Bcl-2 ratio found in this study may suggest that apoptotic genes such as Bax and Bcl-2 also are regulated by HIF-1α at the mRNA level, although further studies are required to confirm the result. Significant correlations were also found between mRNA expressions of EPO and Bax, and EPO and Bcl2, which suggests that there might be possible relationship between EPO and apoptosis control genes (at the mRNA level) during embryonic development of zebrafish. Over-expression of EPO may enhance the activity of Akt, which in turn inhibits apoptosis induced by hypoxia.

Our study showed that during the first 120 h of development, 48 hpf appeared to be the most sensitive window to hypoxia, at which time significant responses in all genes studied were clearly evident. Ton et al. [30] indicated that zebrafish embryos underwent adaptive changes in gene expression in response to hypoxia. Likewise, two zebrafish cardiac myosin genes that display chamber-specific expression patterns, myosin light chain 2 and ventricle myosin heavy chain, are also up-regulated at 48 hpf [31]. 48 hpf appears to be a critical period during the early embryonic development of zebrafish, especially the development of the cardiac system. The expression of specific genes at 48 hpf may be crucial in enabling the zebrafish to cope with prolonged hypoxia. 40 dpf, when zebrafish larvae develop into juveniles, appears to be another sensitive window to hypoxia; at this time, all hypoxia-inducible genes were significantly affected at mRNA level. 40 dpf also is a critical stage for zebrafish sexual differentiation, and at this time point, mRNA levels of all selected sex hormone control genes decreased in the hypoxic group, indicating a general down-regulation of steroidogenesis. The transcriptional down-regulation of these sex hormone control genes may account for the observed disturbed sex hormone balance at later developmental stages.

In the future, in-depth investigations should be carried out to determine whether HIF-1α mRNA levels correlate with their respective protein levels and the tissue specificity of HIF-1α expression in fish, it is well known that since HIF-protein level is controlled independently from mRNA level. Further studies are also required to elucidate the effects of hypoxia on the development of the vascular system in zebrafish, and to find other key factors that are also involved in this process. The expression pattern of EPO in zebrafish during development is needed to ascertain whether the EPO up-regulation is related to the altered apoptosis pattern caused by hypoxia.

Conclusions

This study indicated that up-regulation of HIF-1α mRNA expression occurred within the first 2 h of exposure to hypoxia, followed by up-regulation of other hypoxia-inducible genes controlled by HIF-1α, such as erythropoietin and vascular endothelial growth factor. 48 hpf and 40 dpf were the two sensitive windows to hypoxia, at which time significant changes in mRNA expression of all selected hypoxia-inducible genes were clearly evident. A higher ratio of pro-apoptotic gene (Bax) vs. anti-apoptotic gene (Bcl-2) expressions appeared in the head as compared with the tail under hypoxia. A higher Bax/Bcl-2 ratio was observed in hypoxic males than in hypoxic females, suggesting that hypoxia potentially favors the formation of testes by inducing apoptosis in ovaries during the hermaphroditism in zebrafish, which in turn cause a male-biased sex ratio.

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