Marine Environmental Research 110 (2015) 142–151

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

HIFs genes expression and hematology indices responses to different oxygen treatments in an ovoviviparous teleost species *Sebastes schlegelii*

Weijie Mu [a, b], Haishen Wen [a, *], Jifang Li [a], Feng He [a]

[a] Fisheries College, Ocean University of China, Qingdao 266003, China
[b] College of Life Science and Technology, Harbin Normal University, Harbin 150025, China

ARTICLE INFO

Article history:
Received 18 January 2015
Received in revised form
16 April 2015
Accepted 26 April 2015
Available online 8 May 2015

Keywords:
Aquaculture
Hypoxia
Tissue analysis
mRNA expression
Real-time-PCR
Cortisol
Hematology indices

ABSTRACT

Hypoxia-inducible factors (HIFs) are transcription factors considered as a respond factor to oxygen tension. By using quantitative real-time PCR, expression files of HIF-1α and HIF-2α mRNA were detected in the Korean rockfish ovary, liver, gill and spleen after 30 min and 60 min acute hypoxia exposure. Meanwhile, the cortisol levels, white blood cells and several serum biochemical values of Korean rockfish under different oxygen concentration treatments were also detected. All the results might be helpful for further understanding of the potential effect of hypoxia in ovoviviparous fish.

1. Introduction

Owing to continuous spread of coastal eutrophication and global warming, hypoxia is becoming a global problem affecting the coastal waters (Vaquer-Sunyer and Duarte, 2008). For all kinds of organisms, hypoxia affects their complex cellular interaction networks and normal development. Some reports suggested that hypoxia was considered as a major force in shaping the physiological evolution of animals, by changing species abundances and altering species community composition (Dauer, 1993; Rytkönen et al., 2007; Weisberg et al., 2008).

Hypoxia-inducible factors (HIFs) are implicated to play essential roles in oxygen homeostasis and innate immune responses of various tissues (Choi et al., 2013; Semenza and Wang, 1992). They are heterodimers consisting of two subunits, HIF-α and HIF-β (also known as aryl-hydrocarbon receptor nuclear translocator, ARNT) which belong to the basic helix-loop-helix (bHLH)- Per-Arnt-Sim (PAS) family of transcription factors (Sogawa and Fujii-Kuriyama, 1997; Wang and Zhang, 1995; Wenger et al., 1997). So far, three types of HIF-αs have been characterized in mammals (HIF-1α, HIF-2α and HIF-3α) (Ratcliffe, 2007). Amongst these three types, HIF-1α is believed to be critical in the oxygen uptake or delivery of anaerobic respiration intermediates (Hu et al., 2003; Semenza, 2000), and HIF-2α is proposed to play important roles in angiogenesis (Takeda et al., 2004) and in erythropoiesis (Hu et al., 2006; Law et al., 2006). In fish, HIF-1α has been identified in several species, such as scale-less carp *Gymnocypris przewalskii* (Cao et al., 2005), asp *Aspius aspius* and sea bass *Dicentrarchus labrax* (Terova et al., 2008), HIF-2α has been identified in killifish *Fundulus heteroclitus* (Powell and Hahn, 2002), and HIF-3α has been found in Chinese sucker *Myxocyprinus asiaticus* (Chen et al., 2012). In addition, both of the HIF-1α and HIF-2α were found in zebrafish *Danio rerio* (Rojas et al., 2007), grass carp *Ctenopharyngodon idella* (Law et al., 2006), rainbow wotrout *Oncorhynchus mykiss* (Soitamo et al., 2001), Atlantic croaker *Micropogonias undulates* (Rahman and Thomas, 2007), wuchang bream *Megalobrama amblycephala* (Shen et al., 2010) and Chinese sucker *M. asiaticus* (Chen et al., 2012). The gene of HIF-4α was reported in grass carp *C. idella* (Law et al., 2006), which was supposed to be high homology with

* Corresponding author. Fisheries College, Ocean University of China, 5 Yushan Road, Qingdao 266003, China.
E-mail address: wenhaishen@ouc.edu.cn (H. Wen).

http://dx.doi.org/10.1016/j.marenvres.2015.04.008
0141-1136/

mammalians HIF (Law et al., 2006; Powell and Hahn, 2002). Interestingly, as the HIF-αs genes repertoire were duplicated in teleosts specific genome duplication, more copies of HIF-αs genes (HIF-1αA, HIF-1αB, HIF-2αA and HIF-2αB) have been reported in some cyprinid fish nowadays (Rytkönen et al., 2013).

When the external environment changes, there are a lot of factors in vivo varied but not limited to corstisol content and blood parameters varied. These factors become index to detect organism's adaption to environment. Blood parameters can be an indicator of fish health status (Harikrishnan et al., 2011). It has been well known that white blood cell count reflected certain diseases and injury in fish body, as rearing environment can affect a number of circulating leukocytes in fish (Luo and Shibuya, 2001). White blood cell concentration of fish usually increases to improve their immune ability and resist the injury continue, where insufficient oxygen supply occurred in water, causing by hypoxia (Chen et al., 1997). Glucose and cholesterol are products during metabolism in fish, this process are inhibited by lacking oxygen. However, study in rats showed that hypoxia caused an increase in glucose transporters in plasma, and Ca^{+2} play a role in stimulation of glucose transport (Cartee et al., 1991). Therefore, the changes of serum glucose, cholesterol and calcium need to be examined in a marine teleost species exposed to hypoxia. Cortisol is one of the most commonly used indicators of various stresses in teleost, which can induce secondary responses related mainly to energy requirements (Pickering et al., 1982; Rotllant and Tort, 1997; Van Raaij et al., 1996).

Korean rockfish (*Sebastes schlegeli*) is a kind of ovoviviparous fish, and it is one of the most intensively cultured and commercially important species in Korea, Japan and China (Mu et al., 2013; Lee et al., 1994). However, aquaculture production of many fish including this species has rapidly decreased during the last decade, because of overfishing and polluted water from coastal industries (Kim and Kang, 2004). Korean rockfish had a highly demand in oxygen, meanwhile, it had a large amount of brood, which embryos needed adequate supply of oxygen from female body. Thus, we used female Korean rockfish as an excellent marine species to investigate the molecular and physiological responses to hypoxia of fish. Apparently, there is a wide range of oxygen tolerance among different fishes. Common carp is less tolerant to lack of oxygen than the goldfish (van den Thillart and van Waarde, 1985), and the dissolved oxygen in cultured marine fish is approximately 6–9 mg/L.

So far, no work has been reported on expression patterns of HIF-αs in Korean rockfish, linking with the levels of serum cortisol, white blood cells, serum glucose, cholesterol and calcium. The aim of this study was to isolate and characterize transcripts of the oxygen-regulated HIF-1α and HIF-1α subunits from an ovoviviparous fish, Korean rockfish, and to describe the transcriptional responses of HIF-as genes to different oxygen concentration stress in the adult tissues.

2. Materials and methods

2.1. Experimental fish and holding conditions

Adult Korean rockfish were obtained from a commercial fisheries farm in coastal area of Shandong province, China. The adult fish with the length of 28–29 cm (635.15 ± 18.96 g) were maintained in the tanks for 3–5 days before experiments. Then, samples were random collected for the timing expression study. By preliminary experiment in our lab, the appropriate concentration of culturing Korean rockfish is around 7.5 mg/L, which is using for the control dissolved oxygen in this study. The DO concentration in the airtight hypoxic treatment experimental tanks was lowered to 7.5 mg/L of initial dissolved oxygen. Fish were sampled from the tanks after 0 min, 30 min and 1 h of exposure to hypoxic condition (N ≧ 4). Another experiment was conducted with different initial DO levels (8.5, 7.2, 4.5 mg/L) for 1 h (average temperature: 24 °C, average salinity 30‰). Fish in different groups were also cultured in airtight tanks, except the control group (cultured in the open tanks with 7.5 mg/L of dissolved oxygen) (N ≧ 4). The oxygen tension in each tank was regulated by the use of gas mixer combining air and nitrogen. Dissolved oxygen was measured using COD reactor (Hach, USA). Fish samples were anaesthetized in tricaine methanesulfonate MS-222 (100 mg/L, Sigma, St. Louis, MO). Various tissues including heart, liver, spleen, head kidney, ceaca, ovary, testis, fat, kidney, gill, brain and intestine were collected, immediately frozen in liquid nitrogen and stored at −80 °C until analyses. Animal care and experiments were performed according to Animal Management Rules of the Ministry of Health of the People's Republic of China.

2.2. Total RNA extraction and reverse transcription (RT)

Total RNA of different tissues were extracted with RNAiso reagent (Takara, Japan) according to the manufacturer's protocols. And RNA concentration was measured using a UV spectrophotometer (ChampGel 5000, China). First-strand cDNA was synthesized by reverse transcriptase kit (Takara, Japan) as follows: 1 μg of total RNA was reverse transcribed into cDNA in a volume of 10 μL, containing random primers and M-MLV reverse transcriptase.

2.3. Isolation and PCR amplification of HIF-1α and HIF-2α cDNA

For amplification of the HIF-αs cDNA fragment from ovary, two pairs of degenerate primers (HIF-1αF/HIF-1αR, HIF-2αF/HIF-2αR, Table 1) were designed from highly conserved amino acid sequences among fish species using Code Hop online, which is a web-based primer design program (Rose et al., 1998). PCR reaction was carried out in a volume of 50 μL containing 2 μL of cDNA from ovarian following the manufacturer's protocols (Takara, Japan). PCR was performed using the following touchdown PCR cycling conditions: 5 min denaturation step at 94 °C, 10 cycles of 30 s at 94 °C, 30 s at a range of annealing temperature from 70 °C to 60 °C, decreasing 1 °C each cycle, and 35 s at 72 °C, then followed by additional 30 cycles of 30 s at 94 °C, 30 s at 62 °C for HIF-1αF/HIF-1αR or 30 s at 60 °C for HIF-2αF/HIF-2αR, and 35 s at 72 °C, finally ended with 10 min at 72 °C for extension. Amplified fragments were gel-purified on 1.5% agarose gel using the TIAN gel midi Purification Kit (Tiangen, China) and then cloned into the PGM-T vector (Tiangen, China), propagated in E. coli (Trans5α, Transgen, China). Clones were subjected to direct sequencing by the ABI3730XL sequencer (ABI, USA). The 5′ and 3′ RACE reactions were used the SMART™ RACE cDNA amplification kit (Clontech, USA), primers and nested primers were shown in Table 1. PCR was performed using the following PCR cycling conditions: 5 min denaturing step, at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 65 °C for HIF-1-5, or 30 s at 67 °C for HIF-1-5-N, HIF-1-3 and H1-1-3-N, or 30 s at 56 °C for HIF-2-5 and HIF-2-5-N, or 30 s at 57 °C for HIF-2-3 and HIF-2-3-N, then followed by additional step at 72 °C for 1 min, finally ended with 10 min at 72 °C for extension. The PCR products were gel-purified and sequenced as above (see 2.3).

2.4. Phylogenetic analysis and sequence analysis

The amino acid sequences of Korean rockfish HIF-1α and -2α cDNAs were predicted using a translator program which is available at the open reading frame finder (NCBI, http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Multiple protein sequence alignments of HIF-1α and -2αwere conducted using ClustalX version 1.81 (Thompson et al., 1997). Phylogenetic analyses were conducted using MEGA version

Table 1
Primers used for genes cDNA RACE, RT-PCR and real-time PCR.

Primers	Sequence (5′-3′)	Position	Usage
HIF-1α			
HIF–1F	CGGCGGAAGGAGAAGTCCmgngaygcngc	1382–1409	Degenerate primer
HIF-1R	GCCTCGGGCTCCTCCttnarytgrtc	2286–2312	Degenerate primer
HIF-1-5	GGAAGCATCCCATCATTGTATAGAGG	1485–1507	5′-RACE primer
HIF-1-5-N	TGAAGTCCAGGAAGATGATTGCGTCT	1435–1461	Nested 5′-RACE primer
HIF-1-3	ACACAGGCAATGGAAGACTTAGATCTGG	1767–1795	3′-RACE primer
HIF-1-3-N	GTCTTGTGGACCAATCAAATTCCTCG	1865–1891	Nested 3′-RACE primer
HIF-1-e-F	ATTGTCACTCCCTATCCAAG	2445–2465	RT-PCR and qPCR primer
HIF-1-e-R	CAAAACCCAGATGCTGCTAT	2579–2599	RT-PCR and qPCR primer
HIF-2α			
HIF–2F	GAGGATGAAATGCACGGTGAC	777–798	Degenerate primer
HIF-2R	TGAGCTGTAGTAATCACCTGGG	1736–1758	Degenerate primer
HIF-2-5	GGTTCGCACATCAGGACAG	928–947	5′-RACE primer
HIF-2-5-N	GCTGACTTGAGGTTGACGGT	810–830	Nested 5′-RACE primer
HIF-2-3	TTCACCGTGCAGCAGAATC	1666–1685	3′-RACE primer
HIF-2-3-N	CAGCAGCCCAGGTGATTACT	1731–1751	Nested 3′-RACE primer
HIF-2-e-F	CGATGAAAACGGACAGATGG	507–527	RT-PCR and qPCR primer
HIF-2-e-R	TGTTGTGCTCGGACAGGAAT	590–610	RT-PCR and qPCR primer
18SF	CCTGAGAAACGGCTACCATC	____	reference primer
18SR	CCAATTACAGGGCCTCGAAAG	____	reference primer

4.0 (Tamura et al., 2007). A rooted phylogenetic tree was constructed by means of the neighbor-joining algorithm (Saitou and Nei, 1987), and the data was re-sampled via 1000 bootstrapping replicates.

2.5. Tissue expression of the HIF-αs genes

To examine the expression of HIF-1α and HIF-2α transcripts in various tissues by RT-PCR, total RNA was extracted from ovary, liver, kidney, head kidney, brain, heart, spleen, caeca, stomach, fat, gills, intestinal, pituitary of a female fish in late-vitellogenic stage and only testis of male one in spermiated stage using Trizol reagent. Extracted RNA was treated with RNA DNase I before reverse transcription, avoiding genomic contamination. And RNA was reverse transcribed using M-MLV RT (Promega, USA), following the instruction. 18S rRNA (Table 1) was used as an internal control, and PCR cycling conditions were as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s, 55.3 °C (HIF-1-e-F and HIF-1-e-R) or 58.6 °C (HIF-2-e-F and HIF-2-e-R) for 30s, 72 °C for 30 s, and finally 72 °C for 10 min. Six microliters of each reaction product was resolved on a 1.5% agarose gel containing ethidium bromide (EB) and visualized on a Gel system (Tanon).

2.6. Quantitative real-time PCR

Expression patterns of the Korean rockfish HIF-1α and HIF-2α genes were analyzed using quantitative real-time PCR (qRT-PCR). PCR analyses were performed using Eppendorf iCycler iQ multicolor real-time PCR detection system (Eppendorf, Hamburg, USA) and the iQ™ SYBR Green Supermix (Takara, Japan) according to the manufacturer's protocol. To prevent the genomic DNA amplification, the mRNA was treated using DNase I (Takara, Japan) and ribonuclease inhibitor (Takara, Japan). PCR was carried out in triplicates, and the PCR reaction was performed as following: incubation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 20 s and 72 °C for 30 s. The relative quantification of the target and reference genes was evaluated using standard curves. 18S rRNA was selected as an internal control based on expression stable, and primer sequences are showed in Table 1. The cycle threshold (Ct) values were obtained from the exponential phase of qRT-PCR amplification, and results were analyzed using the comparative Ct method. The HIF-αs expression levels were normalized against 18S rRNA expression level to generate a ΔCt value (ΔCt = target gene Ct − reference gene Ct), and the relative expression of HIF-αs/18S was analyzed according to the expression $2^{-\Delta\Delta\ Ct}$ (Livak and Schmittgen, 2001). The control group samples were used as calibrator for comparative relative qRT-PCR.

2.7. Steroid radioimmunoassay

Serum level of cortisol was measured using Iodine [^{125}I]. Radioimmunoassay Kits (Tianjin Nine Tripods Medical & Bioengi-neering Co., Ltd., Sino-US joint-venture enterprise) under different dissolved oxygen levels fish. The binding rate is highly specific with an extremely low cross reactivity to other naturally occurring steroids, which was less than 0.1% to most circulating steroids. The coefficients of intra-assay and inter-assay variation were 7.3–11.6% for the cortisol assay. Any samples with coefficient of variation higher than 30% were not included in the analyses. The assay sensitivity reached to 0.21 μg/dL for cortisol by the kit protocol, respectively.

2.8. Hematology and biochemical indices

Blood samples with different oxygen concentration treatments were collected from the caudal vein with use of ethylenediaminetetraacetic acid (EDTA) K2. After collection, the samples were immediately stored at a temperature of 4 °C. Within 10 min, they were centrifuged at 3000 g and 4 °C for 10 min. Aliquots of the plasma were stored at −80 °C. Red blood cells counts (× 10^{12} μL) (RBC), serum glucose, cholesterol and serum calcium levels were determined from the venous blood samples by conventional methods using BS-1800 Auto Hematology Analyzer (ShenZhen Mindry Bio-Medical Electronicsco., LTD, Guangzhou, China).

2.9. Statistics

The relevant values in this study were analyzed by analysis of variance (ANOVA) followed by Duncan's multiple range tests. Statistical significance was considered as $P < 0.05$.

3. Results

3.1. Isolation and characterization of HIF-1α and HIF-2α cDNA

Our results revealed that the full-length HIF-1α cDNA of Korean

rockfish (GenBank: KC429679) consisted of 2996 bp, encoded a polypeptide of 852 amino acid residues. We also isolated Korean rockfish HIF-2α cDNA (GenBank: HQ432955) from ovary which was 3230 bp in length, encoded a polypeptide of 782 amino acid residues. It was identified that these two proteins contained 6 typical motifs: the β asichelix-loop-helix (bHLH) domain, Per-ARNT-Sim (PAS) -A and - B domains, PAS associated C-terminal (PAC) domain, oxygen -dependent degradation (ODD) domain, and N-terminal transactivation (TAD-N) domain (five of them showing in Fig. 1A and B). Phylogenetic analysis revealed that Hif-1α and Hif-2α Korean rockfish proteins cluster respectively with HIF-1α, HIF-2α, HIF-3α and HIF-4α of other vertebrate species (Fig. 2). The deduced amino acid sequence of Korean rockfish HIF-1α showed high identities with that of *M. undulates* (84%, Fig. 2). HIF-2α shared highest amino acid identity with *M. undulates* (84%, Fig. 2).

3.2. Tissue specific expression of HIF-1α and HIF-2α isoforms

The RT-PCR results showed that the HIF-1α and HIF-2α showed different spatial expression patterns. The HIF-1α was detected in brain, ovary, testis and head kidney, meanwhile, HIF-2α was found in all of 13 tissues we analysis in this study (Fig. 3).

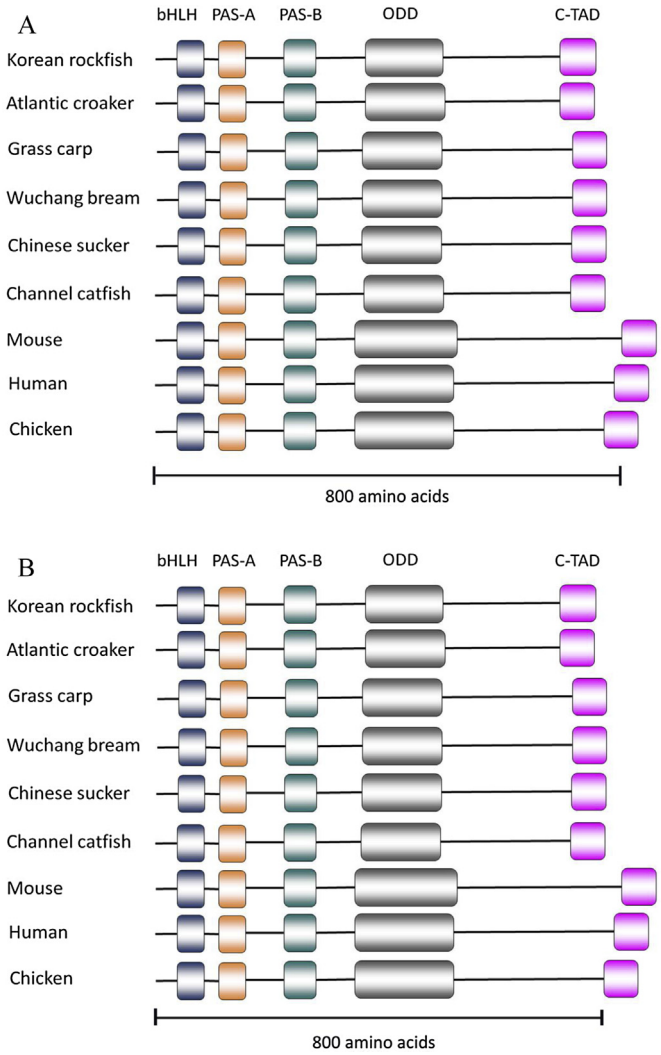

Fig. 1. (A and B). Alignment of Korean rockfish HIF-1α and HIF-2α deduced amino acid with Altantic croaker, grass carp, Wuchang bream, Chinese sucker, channel catfish, mouse, human, chicken. Blocks represented the bHLH, PAS-A, PAS-B, ODD and C-TAD domains.

3.3. HIF-αs expression after different oxygen treatments

In fish, liver, spleen and gills are very important tissues in understanding teleosts innate immunology (Tort et al., 2003), ovary is a vital organ to studying female brood eggs during hypoxia condition. The 18S rRNA was used as the reference gene for this assay. In the timing experiment, the HIF-1α mRNA level in ovary significantly increased at 30 min under hypoxia, and the level decreased at 1 h ($P < 0.05$). However, there was no significant difference in liver and gill. Moreover, HIF-1α mRNA level in spleen down-regulated at 30 min then up-regulated at 1 h ($P < 0.05$, Fig. 4). The transcription of HIF-2α in ovary and liver were quite different with HIF-1α. They were sharply decreased under hypoxia. But the levels in gill and spleen increased at first 30 min, and then dropped at 1 h ($P < 0.05$, Fig. 5).

The HIF-1α and HIF-2α expression levels in various oxygen concentrations were also detected by qRT-PCR. The result showed that was an increasing trend of HIF-1α transcripts in ovary after 1 h hypoxia (4.5 mg/L DO) treatment ($P < 0.05$, Fig. 6). However, HIF-2α mRNA levels decreased sharply in ovary and liver. In gill, HIF-2α transcripts were significant increased in the 4.5 mg/L DO treatment group compared to controls after 1 h exposure to hypoxic conditions ($P < 0.05$). In addition, the increased pattern of HIF-1α mRNA levels in spleen was found in 8.5 mg/L DO treatment group and HIF-2α was found in 7.2 mg/L DO treatment group, respectively ($P < 0.05$, Fig. 7).

3.4. Steroid radioimmunoassay (RIA)

After statistical analysis, though no significant difference is found in the results, changes in the serum cortisol under different oxygen treatments are shown in Fig. 8. The average of serum cortisol level under serve hypoxia was in timing expression analysis during 0 min was 4.88 ± 0.32 μg dl^{-1}, and decreased to 4.68 ± 0.19 μg dl^{-1} at 30 min, finally increased to 4.88 ± 0.23 μg dl^{-1} at 1 h ($P < 0.05$). Meanwhile, the amounts of serum cortisol levels in different hypoxia treatments after 1 h were shown that the highest level was in 8.5 mg/L DO group (4.95 ± 0.06 μg dl^{-1}), slightly higher than control group. After 1 h hypoxic treatment, the serum cortisol levels were 4.93 ± 0.05 μg dl^{-1} (4.5 mg/L DO), 4.47 ± 0.04 μg dl^{-1} (7.2 mg/L DO) and 4.88 ± 0.23 μg dl^{-1} (8.5 mg/L DO).

3.5. Hematology indices

Changes of WBCs and Serum biochemical values (glucose, cholesterol and calcium) under different oxygen concentration treatments were also detected in the present work (Table 2). The WBC level of fish initially increased from a control value of 115.87 ± 3.05 to 126.57 ± 1.85 on the 30 min, and the level decreased to 114.03 ± 3.27 on the 1 h. In different oxygen treatment groups, the highest level was in 7.2 mg/L (122.77 ± 2.79), but not significantly. The serum glucose and cholesterol levels in treated fish came down from the control value of 0.85 ± 0.70 and 14.70 ± 1.84 to 0.28 ± 0.08 and 4.11 ± 0.50 on 1 h, respectively. The highest levels of serum glucose and cholesterol were in control group. The serum glucose and cholesterol were shown lowest in 4.5 mg/L group (4.11 ± 0.50) and the 7.2 mg/L group (0.11 ± 0.01), respectively. On the other hand, the serum calcium level in the treated fish slightly increased from 0 min to 30 min (3.60 ± 0.20 to 3.95 ± 0.25), then significantly decreased to 2.71 ± 0.09 at 1 h. After 1 h treatment, the highest serum calcium level was in control group and the lowest serum calcium level was in 4.5 mg/L group.

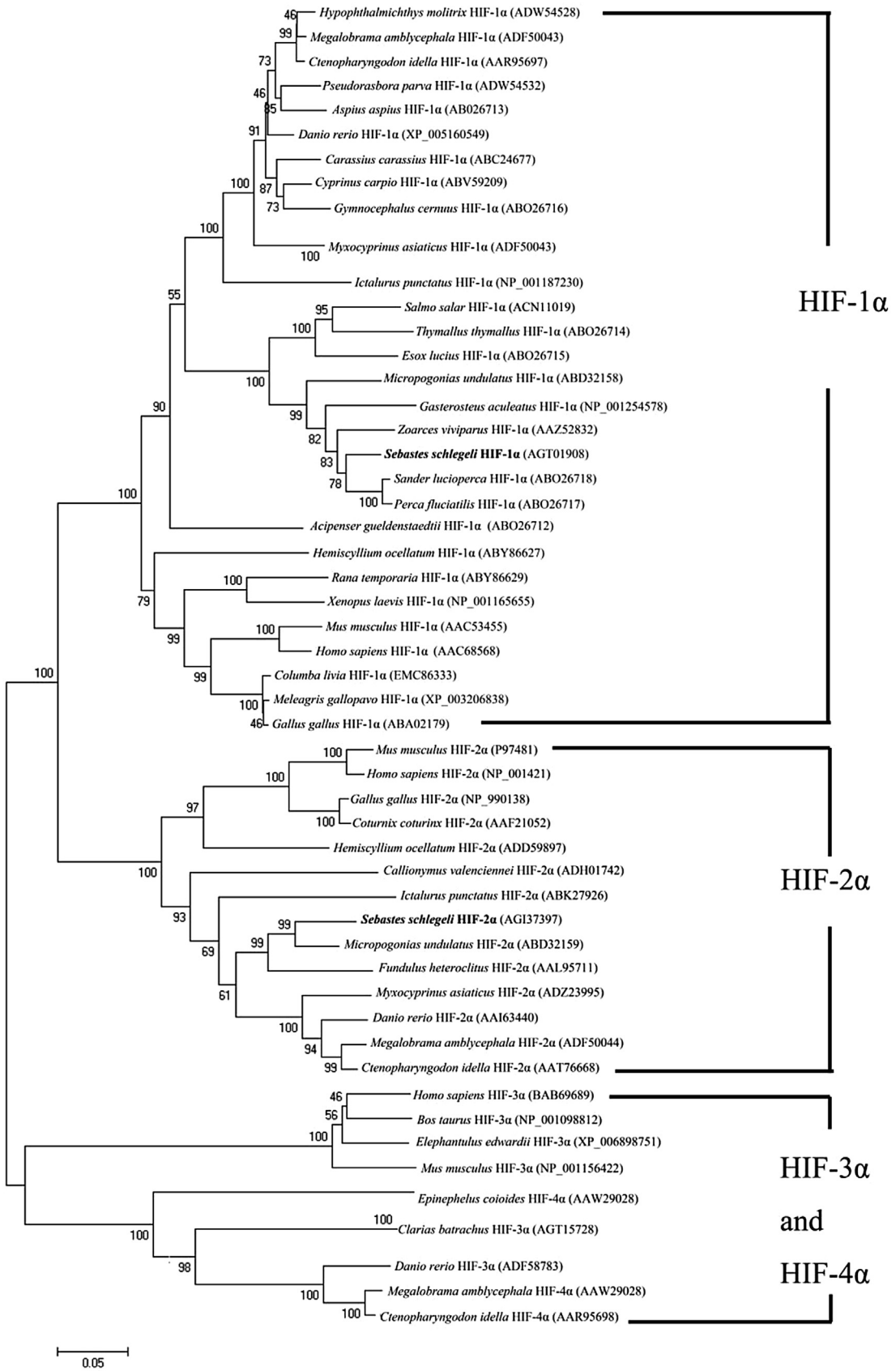

Fig. 2. Phylogenetic tree of Korean rockfish HIF-1α and HIF-2α proteins. The tree was generated by MEGA 4.0 software using the neighbor-joining method, following Clustal X. The bootstrap support for each branch (1000 replications) was shown. Asterisks represented the Chinese sucker HIF-α proteins.

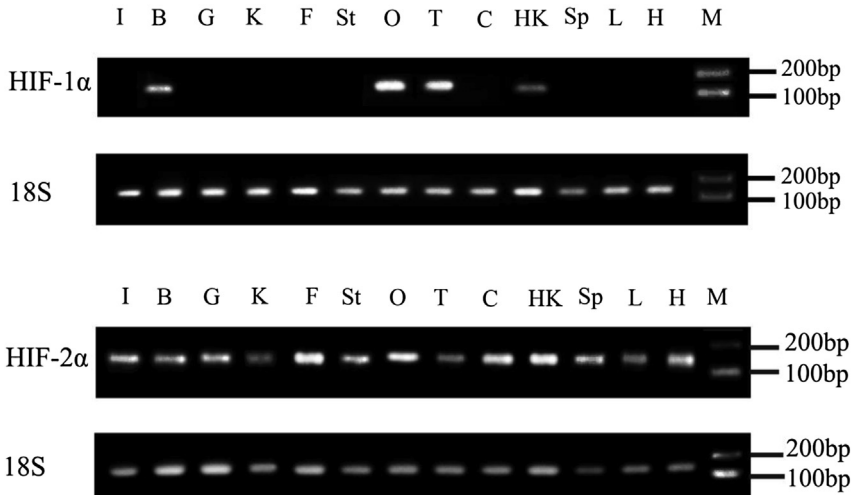

Fig. 3. Expression patterns of HIF-1α and HIF-2α in different adult tissues detected by RT-PCR without treatments in Korean rockfish. The 18S rRNA expression was used as internal control (lower panel). Ma: marker; H: heart; L: liver; SP: spleen; HK: head kidney; C: caecus; T: testis; O: ovary; St: stomach; F: fat; K: kidney; G: gill; B: brain; I: intestine.

4. Discussion

For the first time, our present study reported cloning and characterization of HIF-1α and HIF-2α cDNAs from Korean rockfish, ovoviviparous teleosts. The HIF-1α and HIF-2α of Korean rockfish contained all of the characteristic motifs of vertebrate HIF-αs proteins, indicating that they had similar functions in adaptation to hypoxia as in other vertebrate species (Rahman and Thomas, 2007). In Korean rockfish, the hypoxia response elements (HRE) of the core ODD domains of HIF-αs had high sequence identity to other vertebrates HIF-1α and -2α proteins, the result of which was also found in Chinese sucker and Atlantic croaker (Chen et al., 2012; Rahman and Thomas, 2007), suggesting a high degree of evolutionary conservation in degradation of HIF-αs proteins (Rahman and Thomas, 2007). Comparing the length of fish HIF-1α sequences with those of mammals, it was found that fish HIF-1α protein sequences were shorter than air-breathing vertebrates, suggesting that mammals may have more complex hypoxia regulation mechanism than fish (Powell and Hahn, 2002). Functionally important sequence motifs which are supposed to mediate HIF-α sub-cellular translocation and stability, and commonly described in other teleosts HIF-α protein, were also found in the KrHIF-1α and KrHIF-2α. Fig. 3 of phylogenetic tree showed that Korean rockfish HIF-1α and -2α proteins homologues of other vertebrate species, suggesting the

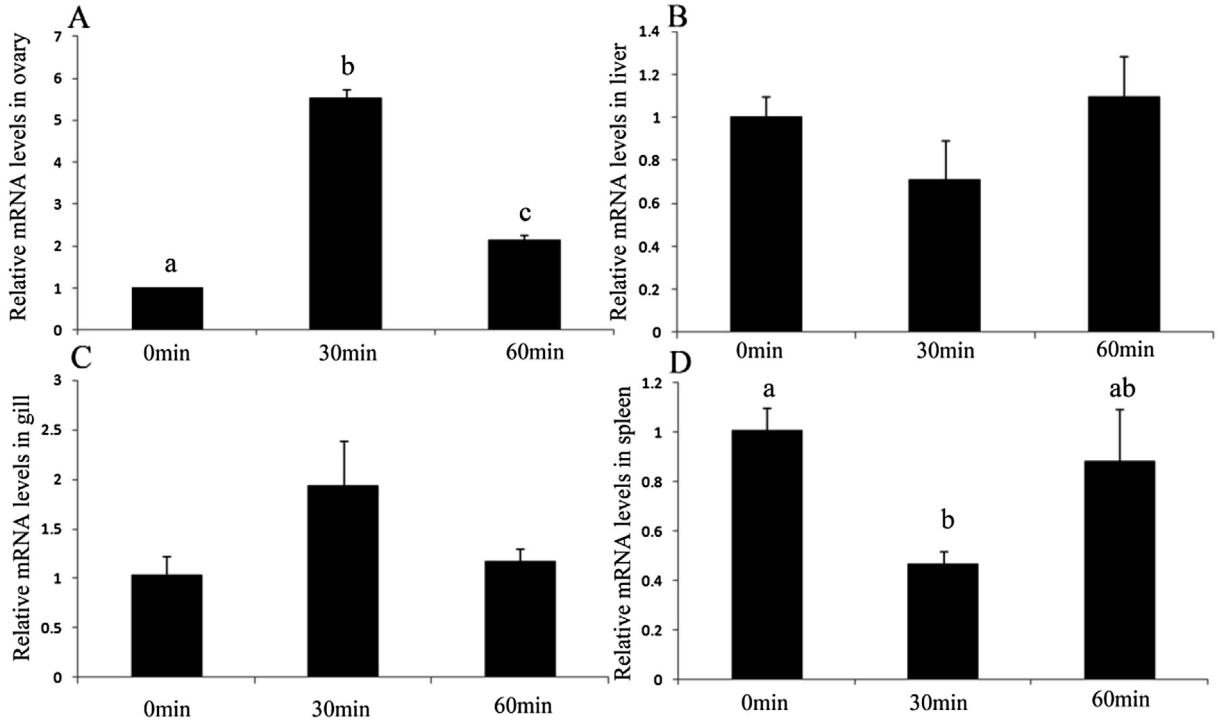

Fig. 4. Relative HIF-1α mRNA expression levels in ovary (A), liver (B), gill (C) and spleen (D) of female Korean rockfish during 1 h of treatments with hypoxia. Samples analyzed by relative quantitative real-time PCR. Expression of 18S rRNA was used as an internal control for real-time PCR. Values are expressed as mean ± standard error of mean. Different letters above the error bar showing statistical differences ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

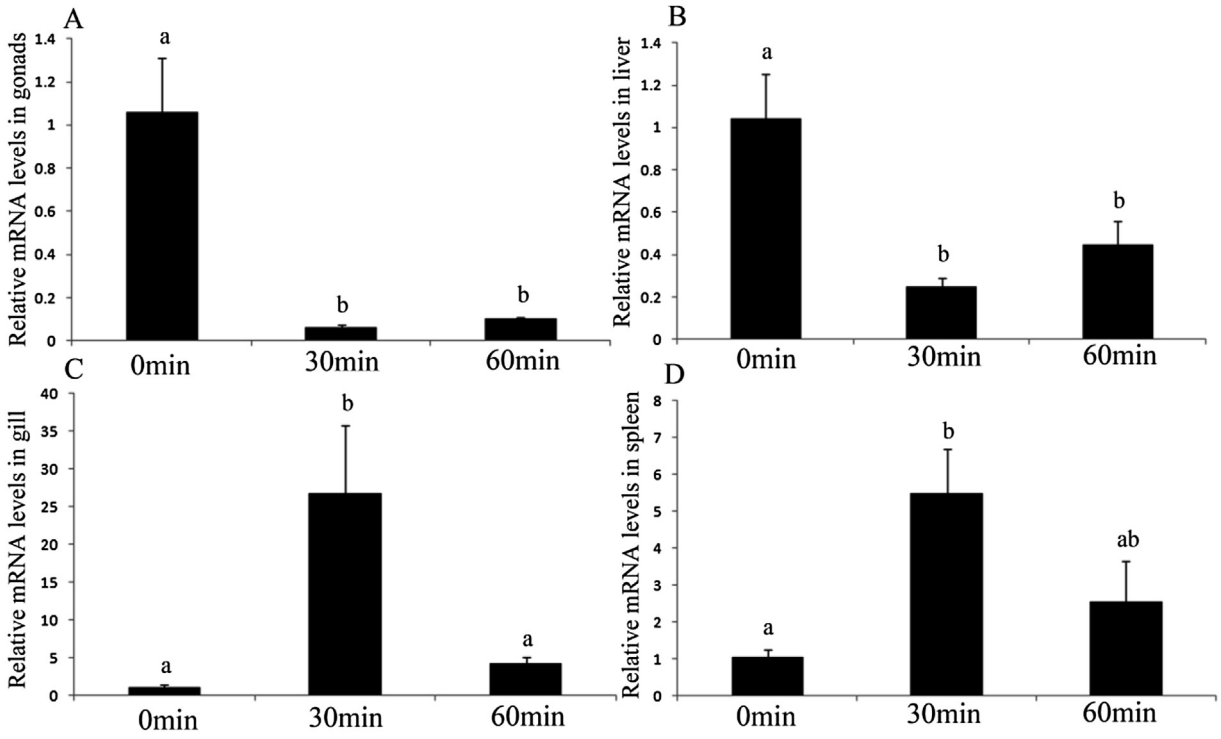

Fig. 5. Relative HIF-2α mRNA expression levels in ovary (A), liver (B), gill (C) and spleen (D) of female Korean rockfish during 1 h of treatments with hypoxia. Samples analyzed by relative quantitative real-time PCR. Expression of 18S rRNA was used as an internal control for real-time PCR. Values are expressed as mean ± standard error of mean. Different letters above the error bar showing statistical differences ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

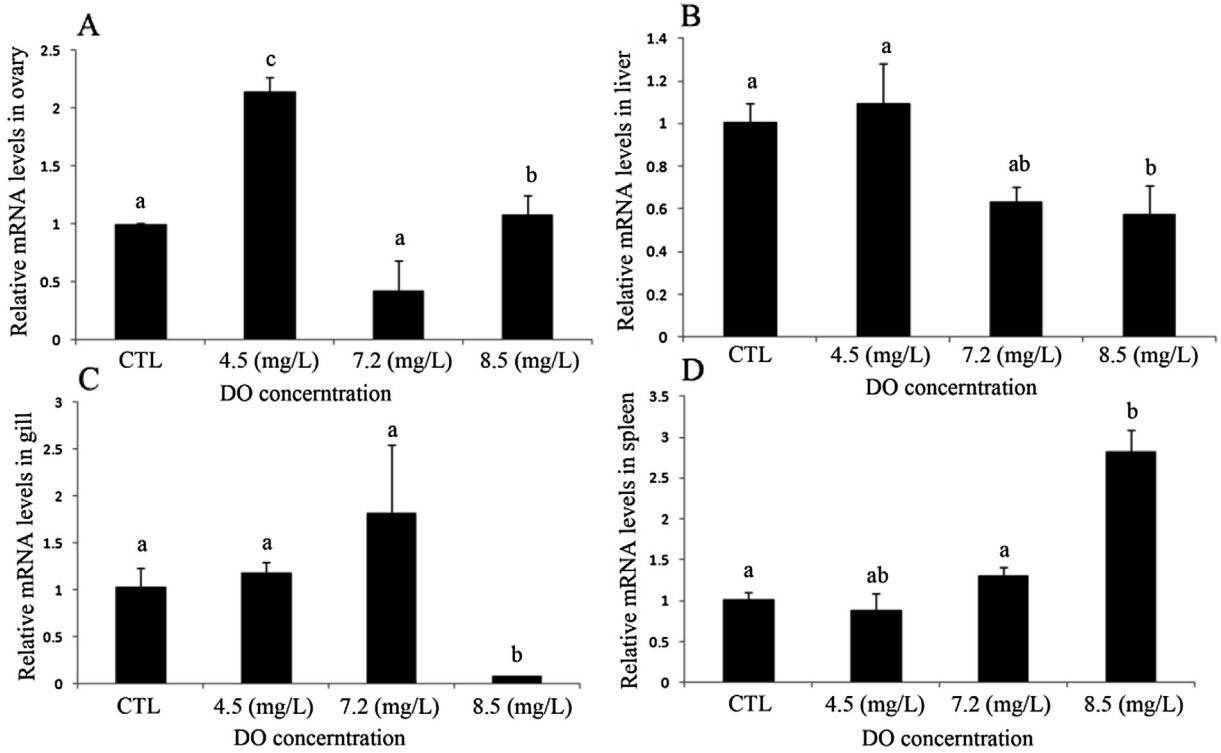

Fig. 6. Relative HIF-1α mRNA expression levels in ovary (A), liver (B), gill (C) and spleen (D) of female Korean rockfish after 1 h different oxygen concentration treatments. Samples analyzed by relative quantitative real-time PCR. Expression of 18S rRNA was used as an internal control for real-time PCR. Data presented as the mean normalized gene expression (MNE) levels ± standard error of the mean of triplicate samples. Different letters above the error bar showing statistical differences ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

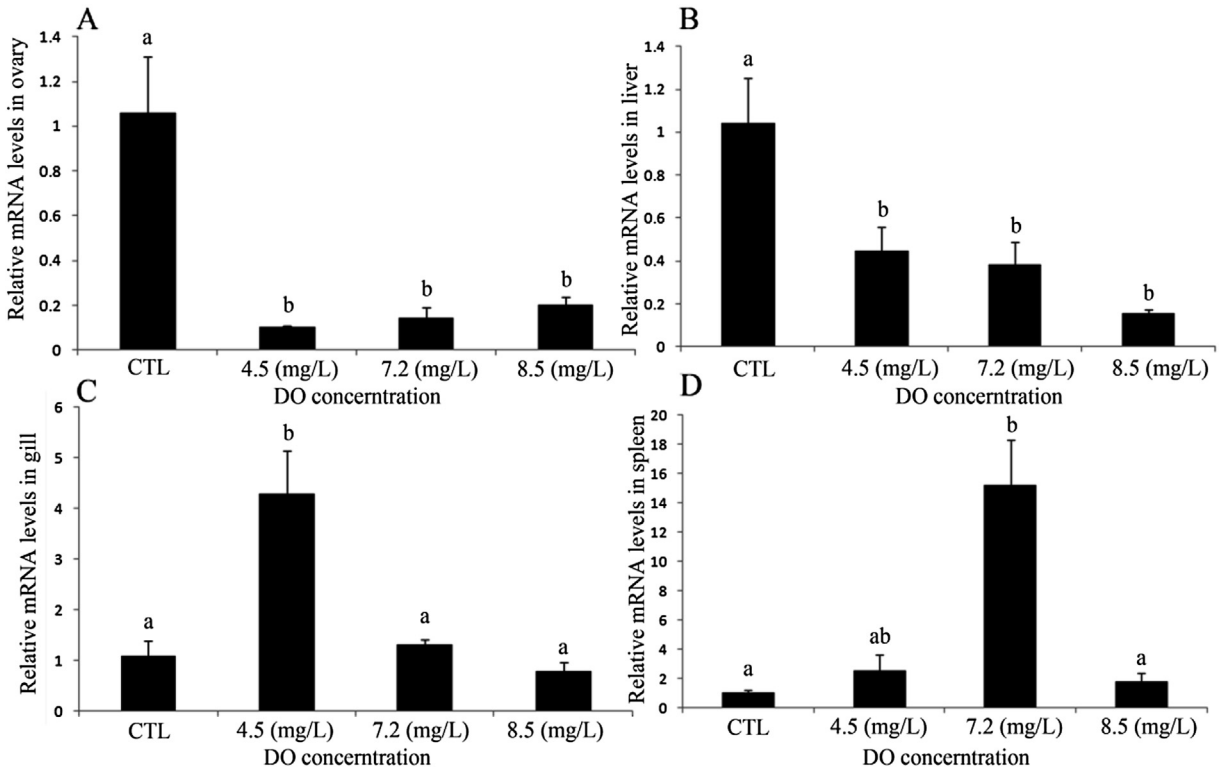

Fig. 7. Relative HIF-2α mRNA expression levels in ovary (A), liver (B), gill (C) and spleen (D) of female Korean rockfish after 1 h different oxygen concentration treatments. Samples analyzed by relative quantitative real-time PCR. Expression of 18S rRNA was used as an internal control for real-time PCR. Data presented as the mean normalized gene expression (MNE) levels ± standard error of the mean of triplicate samples. Different letters above the error bar showing statistical differences ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

high evolutionary conservation in their coding sequences. Moreover, study in eastern oysters reviewed that the genetic diversification of HIF-αs isoforms by phylogenetic analysis, indicating functional diversification and specialization of the oxygen-sensing pathways in vertebrates, which parallels situation observed for many other important genes (Piontkivska et al., 2011).In our study, the HIF-1α and -2α mRNAs expressions in various tissues were detected under normoxia conditions by semi RT-PCR, which showed the expression levels between two genes were quite different. The HIF-1α was transcribed in the ovary, testis, head kidney and brain, which was consisted with previous study in Chinese sucker (Powell and Hahn, 2002), whereas the HIF-2α was abundantly expressed in most of the Korean rockfish tissues. It is suggested that the distinctive expression pattern of the HIF-1α and -2α mRNAs in adult tissues implied their possible involvements in different physiological functions (Rahman and Thomas, 2007). In addition, the mRNA transcript of HIF-1α in liver was highest under normoxia conditions in European sea bass and Wuchang bream (Cao et al., 2005; Rahman and Thomas, 2007). However in grass carp, HIF-1α mRNAs was highly detected in eyes and kidney (Hu et al., 2006). In addition, the HIF-1α and -2α mRNAs levels were highly in gill, ovary and brain in zebrafish (Chen et al., 2012). It was suggested that various responsive mechanisms on HIF-αs might be evolved in different fish species to accommodate themselves to scarce oxygen environments (Rytkonen et al., 2007), and probably these mechanisms are correlated to the species-specific oxygen demands (Rimoldi et al., 2012).

In the present study, mRNA level of HIF-1αincreased in Korean

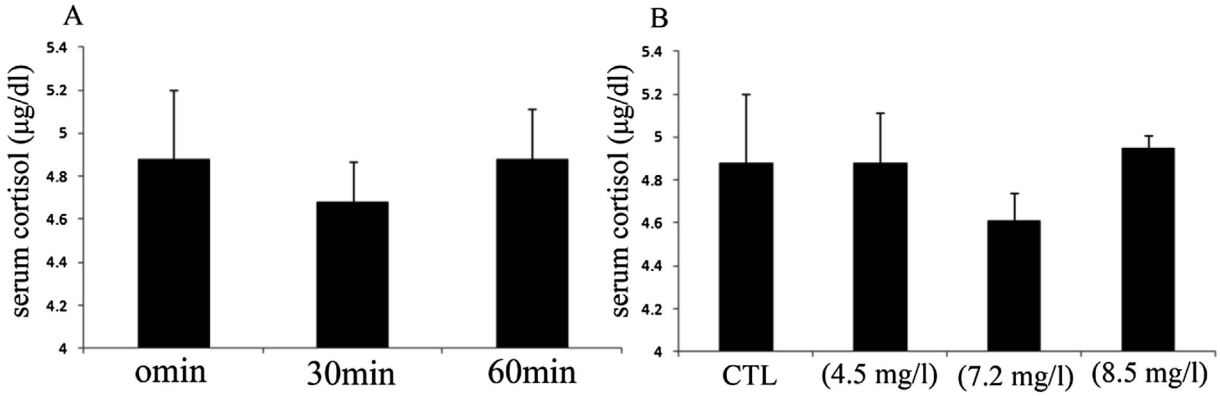

Fig. 8. Serum cortisol levels of female Korean rockfish, during 1 h of treatments with hypoxia (A) and after 1 h different oxygen concentration treatments (B). Values are expressed as mean standard error of mean. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's multiple).

Table 2
Changes of WBCs, Serum biochemical values (glucose, cholesterol and calcium) under different oxygen concentration treatments. Data presented as the mean normalized gene expression (MNE) levels ± standard error of the mean. Different letters indicate significant difference ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

Hypoxia	WBCs	Glucose	Cholesterol	Calcium
0 min	115.87 ± 3.05^{a}	0.85 ± 0.70^{a}	14.70 ± 1.84^{a}	3.60 ± 0.20^{a}
30 min	126.57 ± 1.85^{b}	0.73 ± 0.28^{a}	7.92 ± 0.23^{b}	3.95 ± 0.25^{a}
60 min	114.03 ± 3.27^{a}	0.28 ± 0.08^{b}	4.11 ± 0.50^{c}	2.71 ± 0.09^{b}
Control group	115.87 ± 3.05^{a}	0.85 ± 0.70^{a}	14.70 ± 1.84^{a}	3.60 ± 0.20^{a}
4.5(mg/L)	114.03 ± 3.27^{a}	0.28 ± 0.08^{b}	4.11 ± 0.50^{c}	2.71 ± 0.09^{b}
7.2(mg/L)	122.77 ± 2.79^{a}	0.11 ± 0.01^{b}	7.92 ± 0.94^{c}	3.10 ± 0.17^{c}
8.5(mg/L)	120.35 ± 3.05^{a}	0.21 ± 0.03^{b}	5.94 ± 0.38^{bc}	3.00 ± 0.33^{c}

rockfish ovary after 30 min acute hypoxia exposure, whereas HIF-2α mRNA decreased significantly during 30 min hypoxia exposure. Similarly, up-regulation of HIF-1α, but not HIF-2α transcript levels was observed after acute exposure of grass carp to severe hypoxia (Law et al., 2006). This might due to HIF-2α was not be involved in the early phase of the adaptation response to hypoxia (Rahman and Thomas, 2007). Unexpectedly, HIF-1α mRNA levels were not significantly different in the liver and gill of Korean rockfish after hypoxic conditions, although HIF-2α transcript levels were significantly induced at this time in Korean rockfish gill collected from the hypoxic (Fig. 4). Moreover, no significant changes of HIF-1α mRNAs were found during in rainbow trout and Wuchang bream (Soitamo et al., 2001; Shen et al., 2010), but mRNA levels of HIF-2α in Wuchang bream were markedly induced in the liver (Shen et al., 2010) under hypoxia, which were the same with our study. Our results confirmed the differences in the timing and sensitivity of the HIF-1α and HIF-2α responses to hypoxia exposure (Kodama et al., 2012). However, in the Atlantic croaker, both of the HIF-1α and -2α mRNAs were highly expressed in the liver under hypoxic treatment (Rahman and Thomas, 2007), showing the specific expression pattern between different species. In the present study, changes in transcript levels of HIF-1α and HIF-2α were also investigated under hypoxic conditions in Korean rockfish spleen because spleen is a mainly immune organ in fish (L.Taysse et al., 1998), and is involved in erythropoietic process responses to hypoxia in fish (Lai et al., 2006). The spleen HIF-1α transcript levels in Korean rockfish, significantly increased after 30 min of hypoxia exposure, then decreased after 60 min treatment. However, a down-regulation of HIF-2α mRNA was evident in the spleen at 30 min, and a up-regulation was found at 60 min. Similar findings were observed in wuchang bream, whichHIF-1α mRNA levels in spleen were significantly elevated after hypoxia exposure, then were back to basal control levels, while HIF-2a mRNA levels displayed the contrast pattern (Shen et al., 2010). This expression pattern in spleen suggested that HIF-1α and -2α would be involved in different physiological functions against hypoxia (Compernolle et al., 2002; Hu et al., 2003). These findings from our studies suggested that HIF-1α and HIF-2a might have different function within the past 60 min hypoxia treatment. All the above results suggested that HIF-αs had different fish species responsive mechanisms to accommodate themselves to hypoxia environments (Shen et al., 2010).

Cortisol is a main glucocorticoid in fish which undertakes important biological effects in response to stressors to maintain homeostasis (Salas-Leiton et al., 2012). Many reports suggested that exposure of fish to common stressors activated the hypothalamic-pituitary-interrenal axis leading to increased plasma cortisol (Swift, 1981; Bertotto et al., 2011). However, in our study, the cortisol level did not show significant, which result was the same with the study of Eurasian perch (Douxfils et al., 2012). But works in Siberian sturgeon (*Acipencer baeri*) and spotted wolf fish (*Anarhichas minor*) showed the significant cortisol elevation after a severe hypoxic stress (Lays et al., 2009; Maxime et al., 1995). The pattern of cortisol level in Korean rockfish decreased at 30 min, and increased to the same level of control group at 1 h. Studies of Eurasian perch has been demonstrated that cortisol level was peaked earlier than 1 h following an acute stressor (Jentoft et al., 2005; Milla et al., 2010; Acerete et al., 2004), but was not increased following 4 h hypoxia exposure (Douxfils et al., 2012). Thus, our results suggested that the cortisol decreased after 30 min duration of hypoxia, and returned to normal level at 1 h treatment. In addition, it showed that cortisol levels were similar to the controls in most treatment groups (DO = 4.5, 8.5), and became lower at DO = 7.2 group indicating a probably decrease in sensitivity of the HPI axis at this oxygen concentration.

In our study, the levels of serum cholesterol, serum glucose and serum calcium were also detected. Studies by Li et al. suggested that HIF-1 may impact on posttranscriptional regulation of SREBP-1, thus HIF-1 may affected the lipid metabolism induced by intermittent hypoxia (Li et al., 2006). In Korean rockfish liver, the expression pattern of HIF-1α and HIF-2α showed relevance with the cholesterol level during first 30 min hypoxia exposure, but not last 30 min. In human, patterns of increasing cholesterol levels under hypoxia were significantly prevented by the knockdown of HIF-2α expression (Cao et al., 2014), showing relevance of HIF and cholesterol. Our study also indicated that HIFs may affect hypoxia lipid homeostasis in Korean rockfish liver. In addition, the serum glucose and serum calcium were decreased during 1 h exposure. It suggested that the activation of the immune system and inflammation could be marked by WBCs levels (Vozarova et al., 2002). In our work, the increase of WBCs levels at 30 min showed the immune enhancement of Korean rockfish. However, the WBCs levels in 1 h were decreased, indicating the immune system of Korean rockfish may be damaged.

In conclusion, we identified and characterized two distinct HIF-α isoforms (HIF-1α and HIF-2α) in the Korean rockfish. To our knowledge, this is the first report concerning the HIF-αs cDNAs of ovoviviparous fish species. Comparison of HIF-αs of Korean rockfish with various other species indicated that the molecular structures of these proteins were highly conserved throughout evolution. Since the HIF-2α mRNA expressed more widely in tissues than that of HIF-1α, these proteins may play different roles in Korean rockfish. In addition, the expression patterns of HIF-αs in ovary under different oxygen concentrations were fairly different with other teleost species, suggesting that HIF-αs would be involved in different physiological function against stress stimulation.

Acknowledgment

This research was supported by The National Natural Science Funds (41476110) and (41176122).

References

Acerete, L., Balascha, J.C., Espinosab, E., Josab, A., Torta, L., 2004. Physiological responses in Eurasian perch (*Perca fluviatilis, L.*) subjected to stress by transport and handling. Aquaculture 237, 167–178.
Bertotto, D., Poltronieri, C., Negrato, E., Richard, J., Pascoli, F., Simontacchi, C., Radaelli, G., 2011. Whole body cortisol and expression of HSP70, IGF-I and MSTN in early development of sea bass subjected to heat shock. Gen. Comp. Endocrinol. 174, 44–50.
Cao, Y.B., Chen, X.Q., Du, J.Z., Wang, Y.X., 2005. Molecular cloning of hypoxia-inducible factor 1 alpha from brain of scale-less carp (*Gymnocypris przewalskii*) in Lake Qinghai of China. Comp. Biochem. Physiol. Part A. Mol. Integr. Physiol. 140, 257–273.
Cao, R., Zhao, X., Li, S., Zhou, H., Chen, W., Ren, L., Zhou, X., Zhang, H., Shi, R., 2014. Hypoxia induces dysregulation of lipid metabolism in HepG2 cells via activation of HIF-2α. Cell Physiol. Biochem. 2014 (34), 1427–1441.
Cartee, G.D., Douen, A.G., Ramlal, T., Klip, A., Holloszy, J.O., 1991. Stimulation of glucose transport in skeletal muscle by hypoxia. J. Appl. Physiol. 70, 1593–1600.
Chen, F.H., Chen, B.S., Yang, Y.Y., Luo, Q.C., 1997. Hematopathological studies in

proliferative kidney disease of red grouper. Top. Oceanol. 16, 49–53 (Chinese paper with English abstract).
Chen, N., Chen, L.P., Zhang, J., Chen, C., Wei, X.L., Gul, Y., Wang, W.M., Wang, H.L., 2012. Molecular characterization and expression analysis of three hypoxia-inducible factor alpha subunits, HIF-1α/2 α/3 α of the hypoxia-sensitive freshwater species, Chinese sucker. Gene 498, 81–90.
Choi, S.H., Jee, B.Y., Lee, S.J., Cho, M.Y., Lee, S.J., Kim, J.W., Jeong, H.D., Kim, K.H., 2013. Effects of RNA interference-mediated knock-down of hypoxia-inducible factor-α on respiratory burst activity of the Pacific oyster Crassostrea gigas hemocytes. Fish Shellfish Immunol. 35, 476–479.
Compernolle, V., Brusselmans, K., Acker, T., Hoet, P., Tjwa, M., Beck, H., Plaisance, S., Dor, Y., Keshet, E., Lupu, F., Nemery, B., Dewerchin, M., Van Veldhoven, P., Plate, K., Moons, L., Collen, D., Carmeliet, P., 2002. Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat. Med. 8, 702–710.
Dauer, D.M., 1993. Biological criteria, environmental health and estuarine macrobenthic community structure. Mar. Pollut. Bull. 26, 249–257.
Douxfils, J., Deprez, M., Mandiki, S.N.M., Milla, S., Henrotte, E., Mathieu, C., Silvestre, F., Vandecan, M., Rougeot, C., Mélard, C., Dieu, M., Raes, M., Kestemont, P., 2012. Physiological and proteomic responses to single and repeated hypoxia in juvenile Eurasian perch under domestication – Clues to physiological acclimation and humoral immune modulations. Fish Shellfish Immunol. 33, 1112–1122.
Harikrishnan, R., Kim, M.C., Kim, J.S., Balasundaram, C., Heo, M.S., 2011. Protective effect of herbal and probiotics enriched diet on haematological and immunity status of Oplegnathus fasciatus (Temminck & Schlegel) against Edwardsiella tarda. Fish Shellfish Immunol. 30, 886–893.
Hu, C.J., Wang, L.Y., Chodosh, L.A., Keith, B., Simon, M.C., 2003. Differential roles of hypoxia-inducible factor 1 α (HIF-1α) and HIF-2α in hypoxic gene regulation. Mol. Cell. Biol. 23, 9361–9374.
Hu, C.J., Iyer, S., Sataur, A., Covello, K.L., Chodosh, L.A., Simon, M.C., 2006. Differential regulation of the transcriptional activities of hypoxia-inducible factor 1 Alpha (HIF-1α) and HIF- 2α in stem cells. Mol. Cell. Biol. 26, 3514–3526.
Jentoft, S., Aastveit, A.H., Torjesen, P.A., Andersen, O., 2005. Effects of stress on growth, cortisol and glucose levels in non-domesticated Eurasian perch (Perca fluviatilis) and domesticated rainbow trout (Oncorhynchus mykiss). Comp. Biochem. Physiol. Part A. Mol. Integr. Physiol. 2005 (141), 353–358.
Kim, S.G., Kang, J.C., 2004. Effect of dietary copper exposure on accumulation, growth and hematological parameters of the juvenile rockfish, *Sebastes schlegeli*. Mar. Environ. Res. 58, 65–82.
Kodama, K., Rahman, M.S., Horiguchi, T., Thomas, P., 2012. Upregulation of hypoxia-inducible factor (HIF)-1α and HIF-2α mRNA levels in dragonet *Callionymus valenciennei* exposed to environmental hypoxia in Tokyo Bay. Mar. Pollut. Bull. 64, 1339–1347.
Lai, J.C.C., Kakuta, L., Mok, H.O., Rummer, J.L., Randall, D., 2006. Effects of moderate and substantial hypoxia on erythropoietin levels in rainbow trout kidney and spleen. J. Exp. Biol. 209, 2734–2738.
Law, S.H.W., Wu, R.S.S., Ng, P.K.S., Yu, R.M.K., Kong, R.Y.C., 2006. Cloning and expression analysis of two distinct HIF-alpha isoforms—gcHIF-1alpha and gcHIF-4alpha-from the hypoxia-tolerant grass carp. *Ctenopharyngodon idellus*. BMC Mol. Biol. 7, 1–15.
Lays, N., Iversen, M.M.T., Frantzen, M., Jorgensen, E.H., 2009. Physiological stress responses in spotted wolfish (*Anarhichas minor*) subjected to acute disturbance and progressive hypoxia. Aquaculture 295, 126–133.
Lee, S.M., Lee, J.Y., Hur, S.B., 1994. Essentiality of dietary eicosapentaenoic acid and docosahexaenoic acid in Korean rockfish, *Sebastes schlegeli*. Bull. Korean Chem. Soc. 27, 712–726.
Li, J.G., Bosch-Marce, M., Nanayakkara, A., Savransky, V., Fried, S.K., Semenza, G.L., Polotsky, V.Y., 2006. Altered metabolic responses to intermittent hypoxia in mice with partial deficiency of hypoxia-inducible factor-1α. Physiol. Genomics 25, 450–457.
Luo, J., Shibuya, M., 2001. A variant of nuclear localization signal of bipartite-type is required for the nuclear translocation of hypoxia inducible factors (1 alpha, 2 alpha, and 3 alpha). Oncogene 20, 1435–1444.
Maxime, V., Nonnotte, G., Peyraud, C., Williot, P., Truchot, J.P., 1995. Circulatory and respiratory effects of an hypoxic stress in the Siberian sturgeon. Respir. Physiol. 100, 203–212.
Milla, S., Mathieu, C., Wang, N., Lambert, S., Nadzialek, S., Massart, S., Henrotte, E., Douxfils, J., Mélard, C., Mandiki, S.N., Kestemont, P., 2010. Spleen immune status is affected after acute handling stress but not regulated by cortisol in Eurasian perch, Perca fluviatilis. Fish Shellfish Immunol. 28, 931–941.
Mu, W.J., Wen, H.S., Li, J.F., He, F., 2013. Cloning and expression analysis of a HSP70 gene from Korean rockfish (*Sebastes schlegeli*). Fish Shellfish Immunol. 35, 1111–1121.
Pickering, A.D., Pottinger, T.G., Christie, P., 1982. Recovery of the brown trout, Salmo trutta L., from acute handling stress: a time-course study. J. Fish. Biol. 20, 229–244.
Piontkivska, H., Chung, J.S., Ivanina, A.V., Sokolov, E.P., Techa, S., Sokolova, I.M., 2011. Molecular characterization and mRNA expression of two key enzymes of hypoxia-sensing pathways in eastern oysters *Crassostrea virginica* (Gmelin): hypoxia-inducible factor α (HIF-α) and HIF-prolylhydroxylase (PHD). Comp. Biochem. Physiol. Part D Genomics Proteomics 6, 103–114.
Powell, W.H., Hahn, M.E., 2002. Identification and functional characterization of hypoxia-inducible factor 2α from the estuarine teleost, Fundulus heteroclitus: interaction of HIF-2α with two ARNT2 splice variants. J. Exp. Zool. A. Ecol. Genet. Physiol. 294, 17–29.
Rahman, M.S., Thomas, P., 2007. Molecular cloning, characterization and expression of two hypoxia inducible factor alpha subunits, HIF-1a and HIF-2a, in a hypoxia tolerant marine teleost, Atlantic croaker (*Micropogonias undulates*). Gene 396, 273–282.
Rimoldi, S., Terova, G., Ceccuzzi, P., Marelli, S., Antoini, M., Saroglia, M., 2012. HIF-1α mRNA levels in Eurasian perch (*Perca fluviatilis*) exposed to acute and chronic hypoxia. Mol. Biol. Rep. 39, 4009–4015.
Rojas, D.A., Perez-Munizaga, D.A., Centanin, L., Antonelli, M., Wappner, P., Allende, M.L., Reyes, A.E., 2007. Cloning of hif-1α and hif-2α and mRNA expression pattern during development in zebrafish. Gene Exp. Patterns 7, 339–345.
Rose, T.M., Schultz, E.R., Henikoff, J.G., Pietrokovski, S., McCallum, C.M., Steven, Henikoff, 1998. Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. Nucleic Acids Res. 26, 1628–1635.
Rotllant, J., Tort, L., 1997. Cortisol and glucose responses after acute stress by net handling in the sparid red porgy previously subjected to crowding stress. J. Fish. Biol. 51, 21–28.
Rytkonen, K.T., Vuori, K.A.M., Primmer, C.R., Nikinmaa, M., 2007. Comparison of hypoxia-inducible factor-1 alpha in hypoxia sensitive and hypoxia tolerant fish species. Comp. Biochem. Physiol. Part D Genomics Proteomics 2, 177–186.
Rytkönen, K.T., Vuori, K.A., Primmer, C.R., Nikinmaa, M., 2007. Comparison of hypoxia-inducible factor-1 alpha in hypoxia-sensitive and hypoxia-tolerant fish species. Comp. Biochem. Physiol. Part D Genomics Proteomics 2, 177–186.
Rytkönen, K.T., Akbarzadeh, A., Miandare, H.K., Kamei, H., Duan, C., Leder, E.H., Williams, T.A., Nikinmaa, M., 2013. Subfunctionalization of cyprinid hypoxia-inducible factors for roles in development and oxygen-sensing. Evolution 67, 873–882.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Salas-Leiton, E., Coste, O., Asensio, E., Infante, C., Cañavate, J.P., Manchado, M., 2012. Dexamethasone modulates expression of genes involved in the innate immune system, growth and stress and increases susceptibility to bacterial disease in Senegalese sole (*Solea senegalensis* Kaup, 1858). Fish shellfish Immunol. 32, 769–778.
Semenza, G.L., 2000. HIF-1: mediator of physiological and pathophysiologic al responses to hypoxia. J. Appl. Physiol. 88, 1474–1480.
Semenza, G.L., Wang, G.L., 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol. Cell. Biol. 12, 5447–5454.
Shen, R.J., Jiang, X.Y., Pu, J.W., Zou, S.M., 2010. HIF-1α and -2α genes in a hypoxia-sensitive teleost species *Megalobrama amblycephala*: cDNA cloning, expression and different responses to hypoxia. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 157, 273–280.
Sogawa, K., Fujii-Kuriyama, Y., 1997. Ah receptor, a novel ligand-activated transcription factor. J. Biochem. 122, 1075–1079.
Soitamo, A.J., Rabergh, C.M.I., Gassmann, M., Sistonen, L., Nikinmaa, M., 2001. Characterization of a hypoxia-inducible factor (HIF-1alpha) from rainbow trout. J. Biol. Chem. 276, 19699–19705.
Swift, D.J., 1981. Changes in selected blood component concentrations of rainbow trout, Salmo gairdnerz Richardson, exposed to hypoxia or sublethal concentrations of phenol or ammonia. J. Fish. Biol. 19, 45–61.
Takeda, N., Maemura, K., Imai, Y., Harada, T., Kawanami, D., Nojiri, T., Manabe, I., Nagai, R., 2004. Endothelial PAS domain protein 1 gene promotes angiogenesis through the transactivation of both vascularendothelial growth factor and its receptor, Flt-1. Circ. Res. 95, 146–153.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) soft-ware version 4.0. Mol. Biol. Evol. 24, 1596–1599.
Terova, G., Rimoldi, S., Cora, S., Bernardini, G., Gornati, R., Saroglia, M., 2008. Acute and chronic hypoxia affects HIF-1α mRNA levels in sea bass (*Dicentrarchus labrax*). Aquaculture 279, 150–159.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
Tort, L., Balasch, J.C., Mackenzie, S., 2003. Fish immune system. A crossroads between innate and adaptive responses. Inmunología 22, 277–286.
Van Raaij, M.T.M., Pit, D.S.S., Balm, P.H.M., Steffens, A.B., Vanden Thillart, G.E., 1996. Behavioral strategy and the physiological stress response in rainbow trout exposed to severe hypoxia. Horm. Behav. 30, 85–92.
Vaquer-Sunyer, R., Duarte, C.M., 2008. Thresholds of hypoxia for marine biodiversity. PNAS 105, 15452–15457.
Vozarova, B., Weyer, C., Lindsay, R.S., Pratley, R.E., Bogardus, C., Tataranni, P.A., 2002. High white blood cell count is associated with a worsening of insulin sensitivity and predicts the development of type 2 diabetes. Diabetes 51, 455–461.
Wang, C.D., Zhang, F.S., 1995. Effect of enviromental oxygen deficiency on embryos and larvae of bay scallop Argopecten irradians irradians. Chin. J. Oceanol. Limnol. 13, 362–369.
Weisberg, S.B., Thompson, B., Ranasinghe, J.A., Montagne, D.E., Cadien, D.B., Dauer, D.M., Diener, D., Oliver, J., Reish, D.J., Velarde, R.G., Word, J.Q., 2008. The level of agreement among experts applying best professional judgment to assess the condition of benthic infaunal communities. Ecol. Indic. 8, 389–394.
Wenger, R.H., Kvietikova, I., Rolfs, A., Gassmann, M., Marti, H.H., 1997. Hypoxia-inducible factor-1 alpha is regulated at the post-mRNA level. Kidney Int. 51, 560–563.