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Original Research Article

Stress Induced Transcriptional Activation of *hif-1α* and *hsp-70* Genes in Air-Breathing Singhi Catfish, *Heteropneustes fossilis*

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Abstract: The molecular process that facilitates the adaptation ability of a oxyconformer like *Heteropneustes fossilis* remains largely unexplored. Air breathing Singhi catfish, *H. fossilis* generally inhabits the aquatic bodies characterized by low dissolved oxygen levels, a situation of hypoxia. Therefore, this study mainly focuses on the adaptation mechanism of such aquatic dwellers to acute hypoxic stressed condition through the molecular arrangement of the differentially expressed hypoxia inducible factor (*hif-1α*)and heat shock protein (*hsp-70)* genes. Logical reasoning to the facts behind the observed data justifies the underlying evidence of the study. In hypoxic conditions,2.8±0.1 mg/L D.O. and 0.98mg/L D.O. at time intervals of 8 and 12 hours; the transcriptional level of *hif-1α* and *hsp-70* genes in *H fossilis* were found to increase several folds when compared to concurrent normoxic controlled fish, studied by the relative expression of qPCR . Simultaneously, the respiratory organ, gills are seen to have significantly affected as a result of hypoxic conditions where 4.02 folds increase in *hif-1α* and 1.73 folds of increased expression in *hsp-70* genes after 12 hours (T2) of hypoxia were noticed. The purpose of this study is not only to offer intuitions to the adaptation in cellular functions of the hypoxia stressed fish, but also to find out the underlying phenomenon of such aquatic life form to dwell in areas of low dissolved oxygen levels where transcriptional factors *hif-1α* and *hsp-70* are prime supporters in their survival.
Key words: Adaptation, dissolved oxygen (D.O.), normoxia, hypoxia, qPCR.

Introduction

Hypoxia, an environmental stressor, refers to large temporal and spatial variations in oxygen content, an essential phenomenon in the Hydrosphere. It is considered to be amongst the major global problems with ecological consequences to the survival necessities of the aquatic lives (Wilhelm-Filho *et al.,* 2005).Oxidative stress have direct impact on damage of important macromolecules like DNA, proteins and lipids, which indirectly hamper the other important physiological and pathological changes in the organism (Miller *et al.,* 1993; Welker *et al.*, 2013; Wu 2002). In addition to the natural environment, hypoxic events are important factors in aquaculture industries for healthy growth of fishes (Fitzgibbon *et al.*, 2007). Dissolved Oxygen (D.O.), a key factor indicating water quality, greatly influence the survival ability of fishes with high fluctuations in densely stocked shallow ponds.

Mammals, including human suffer detrimental effects to hypoxic conditions, for their tissues cannot withstand lack of oxygen even for a short period (Vander *et al.*, 2005). However, fishes evolved to withstand hypoxia responses have survival strategy for long exposures to hypoxia with physiological and biochemical adaptations (Iwama *et al.*, 2005; Nikinmaa 2002). Specialized strategies by some fishes to tolerate

hypoxia results in behavioural responses like surface breathing, reduced activity, and increased ventilation rate (Abele and Puntarulo 2004; Camargo and Martinez 2007; Chung-Ying Li *et al*., 2000). This process of adaptation is physiological and the metabolic changes in organisms caused by hypoxic stress are mediated by Hypoxia Inducible Factor (*hif*) (Ortiz-Barahona *et al*., 2010). *hif-* regulated genes get activated when these *hif* factors bind to the regulatory regions of hypoxia inducible genes (Mohindra *et al*., 2013). *hif-1α* and *hif-2α*, these two alpha subunits have unique and complementary roles in adaptive response to tissue hypoxia (Rahman and Thomas 2007) and the distinct mechanism for hypoxia tolerance is provided by them. Another *hif-3α*, have also been reported to be a subunit of *hif* functioning in accordance to stress response. When low oxygen availability is experienced, this transcription factors control cell division, gene expressionand DNA replication for the survival of the cells (Huang 2013)' by forming a heterodimeric DNA-binding complex with *hif-1β* by any of the *hif-α* subunits. This interacts with the hypoxia response element (HRE), 5′-RCGTG-3′ on the promoter region of target genes (Wang and Semenza 1993) and thus accelerates transcription of genes leading to a series of biochemical physiological responses to survive under hypoxic condition. Also, during environmental stresses, all vertebrates including the stressed fishes exhibit a generalized stress response. During cellular stress response and protein integrity, another factor *hsp* plays an important role (Feder and Hofmann 1999; Iwama *et al.,* 2004; Keller *et al*., 2008; Multhoff *et al*., 2007). Amongst the evolutionarily conserved protein, *hsp-70* are widely distributed and plays a central role in cellular homeostasis (Morano 2007; Padmini and Rani 2008). Variety of stressors including hyperthermia, hypoxia, osmotic stress, heavy metal exposure, oxidative stress, etc. induces elevated expression of *hsp-70* genes (Mu *et al*., 2013). During cellular damage, *hsp*-70 is rapidly synthesized and thus beneficial for maintaining the normal physiological function during cellular stress (Padmini and Meenakshi 2016; Taleb *et al.,* 2008). Recent developments in studies have detected the transcriptional expressions yet only a few published data on gene expression patterns of *hif-1α* and *hsp-70* in hypoxic *H. fossilis*. are available (Mustafa *et al*., 2011; Soitamo *et al*., 2001). Also, studies on cellular stress have reported higher expression of *hsp-70* in tissues of stressed organisms (Kanika *et al*., 2014; Wen-Hua Li *et al.,* 2019; Ying-Li Han *et al*., 2016).

As the aquatic ecosystem experiences exposure to thermal and various other stressors, fishes are excellent model for investigation of the physiology and function, of *hsp* regulation and *hif* expression. Fishes which live in hypoxic water must implement specific adaptations to support the rate of oxygen uptake (Elliott *et al*., 2013). The Asian stinging catfish or fossil cat, *Heteropneustes fossilis* is a species of air sac catfish that generally inhabit stagnant water bodies, muddy ponds, ditches, swamps and marshy lands which have low DO levels and are well adapted for adverse ecological conditions fatal for other species of fish. Thus, to study the mechanism of hypoxia and expression of various genes responsible for stress, *H. fossilis* are used as the model organisms (Hossain *et al*., 2013). This study mainly focuses on the change in the relative expression of *hsp-70* and *hif-1α* gene by qPCR as these are the primary factors responsible for survival of fish in areas of low D.O.

Materials and methods

Experimental animals and their acclimatization

Live freshwater asian catfish, *Heteropneustes fossilis* of body weight 50±10 g were collected from Aquaculture and Biodiversity centre of Gauhati University. Fishes were kept in a glass aquarium with sufficient water at normoxia, (7.5±0.1 mg/L, D.O.), to acclimatize under normal laboratory conditions for 10 days with constant 12 hour light/dark cycle and temperature around 28 ± 2°C. Water was changed daily and the fishes were fed with artificial fish food (OPTIMUM highly nutritious food for all aquarium fishes). The dead and the diseased fishes were removed when noticed. No sex differentiation was done in the fishes during the study period. Feeding was stopped 48 hrs before the start of experiment (Mohindra *et al*., 2013).

Experimental design and tissue sampling

The acclimatized fishes were divided into two groups - control and treated . The control group of acclimatized fishes (10 Nos.) was maintained at normoxic water, the state at normal levels of oxygen (7.5±0.1 mg/L.D.O.) with normal aeration. The treated groups of fishes were transferred in the aquarium having length, width and depth of 30cms with an air tight lid. The aquarium was filled with water up to the brim. D.O. level of water was checked with D.O. meter 314 (Systronics) and was found to be 7.5±0.1mg/L D.O. The lid of the aquarium was sealed tightly for no access to air prior to the experiment. After 8 hours, when hypoxic level was attained, states of lower than normal level of oxygen (2.8±0.1 mg/L D.O.), a group of fishes (6 nos.) were separated as 'T1' group of fishes. The hypoxic treatment continued further, till DO level dropped to 0.98±0.1mg/L DO and the fishes were marked as 'T2' group which was in 12 hrs of hypoxic stress. The collected fishes in different groups were sacrificed by cervical dislocation and liver, muscle, brain and gills were collected and stored at -80°C until further analysis.

RNA extraction and cDNA synthesis

Tissues weighing 100 mg were used for RNA extraction and purification was done using TRIzol (TRI Reagent) following a standardized protocol (Rio *et al*, 2010). From the extracted RNA, cDNA was synthesized using TaKaRa Prime Script ™ RT-PCR Kit after quantifying the total RNA in a Qubit 3.0 fluorometer (Thermo Fisher Scientific). Further, the cDNA samples were diluted 50 times with sterile miliQ water for qPCR studies.

Designing of Primers and qPCR analysis

The relative expression of *hif-1α* and *hsp-70* genes in different tissues was determined by quantitative PCR analysis. The specific primers for the genes *hif-1α* and *hsp-70* were designed using its species-specific cDNA sequences available in the NCBI database (Accession no- Table- 1) with the help of bioinformatics tool namely primer-BLAST (NCBI) and Oligo Calculator. The primers were checked for its specificity by running the PCR amplicons on 1.2% agarose gel .

qPCR analysis was performed using SYBR GREEN qPCR Master Mix (BIOradiantiTaq™UniversalSYBR® GREEN supermix) with 2.5µL of prepared c-DNA. The change in the expression of both *hif-1α* and *hsp-70* genes in the treated tissues were determined as fold changes with respect to the tissues of control fishes, using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Expressed mRNA levels of each sample were compared to the control group normalised against housekeeping gene, *β-actin* mRNA.

Statistical analysis

Experiments were conducted in triplicates and all values were presented as mean ± SEM. The significance was calculated using one-way ANOVA at 5% significance level, using ORIGIN 6.0 software.

Results

hif-1α gene expression analyses using qPCR

Upon exposure to hypoxic conditions at different DO levels, the expression of *hif-1α* in the tissue samples, as estimated by qPCR, are presented in Figure 1. According to the data, exposure of T1 group to 2.8±0.1 mg/L of D.O., after 8 hours of hypoxic stress; induced significant expression of *hif-1α* in the tissue samples showed a marked increase in the gills of treated by 3.60 times higher than liver, brain and muscle with 1.67, 1.56 and 1.21 times respectively as compared to normoxia group. On exposure to 0.98±0.1 mg/L of D.O. after 12 hours

Table 1. Table showing primers of reference gene and gene of interest with name and accession number

Gene	Forward primer (5'→ 3')	Reverse primer (3'→ 5')	Accession Number
β-actin	5'-CAGCTGAGCGTCAAATCGTG-3'	3'-TCCAGAGAGGATGAGGAGGC-5'	FJ 409641
hif-1α	5'-AACAGACCGTGCCTGAGA-3'	3'-CACAGAGCATGACAGGTCGT-5'	MH938568
hsp-70	5'-GAATTCATCAGCCTCCGCCA-3'	3'-CAAGATCGCATCCAAGC-5'	J N 120311

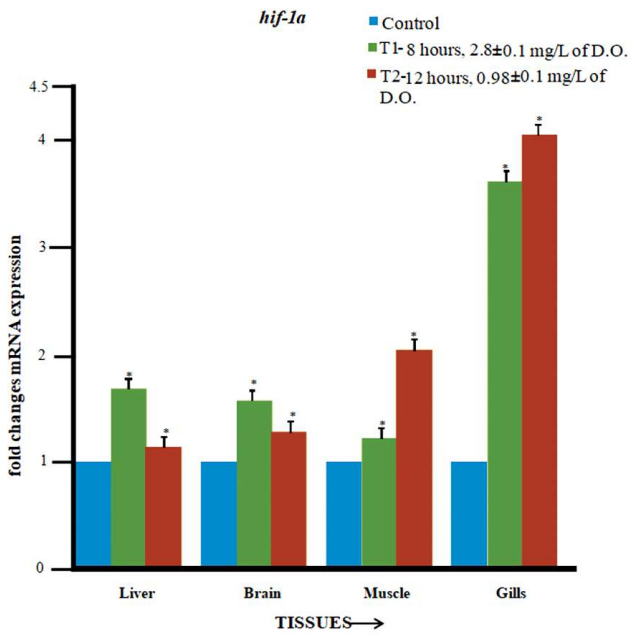

Fig. 1. *hif1-α* mRNA expression in various tissue samples in response to normoxia (control) and hypoxia (T1&T2) (DO 2.8±0.1 mg/L & DO 0.98±0.1 mg/L) after 8 hours and 12 hours as determined by real-time quantitative PCR in *Heteropneustes fossilis*. Levels of *hfhif 1-α* transcripts in exposed groups are expressed as fold changes of mRNA expression after being normalized against *hfactβ* standard. Values are mean ± SEM (n=3) of relative concentrations ($2^{-\Delta\Delta CT}$); *P values significant at 0.05 levels compared to control by One Way ANOVA analysis.

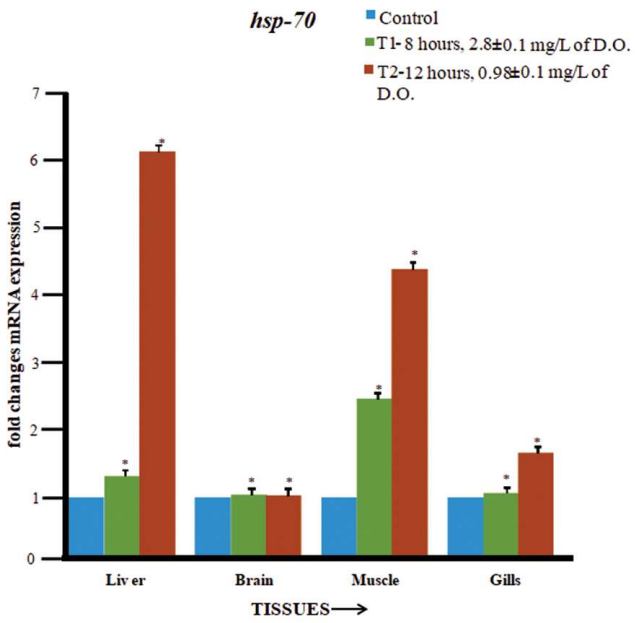

Fig. 2. *hsp 70* mRNA expression in various tissue samples in response to normoxia (control) and hypoxia (T1&T2) (DO 2.8±0.1 mg/L & DO 0.98±0.1 mg/L) after 8 hours and 12 hours as determined by real-time quantitative PCR in *Heteropneustes fossilis*. Levels of *hfhsp70* transcripts in exposed groups are expressed as fold changes of mRNA expression after being normalized against *hfactβ* standard. Values are mean ± SEM (n=3) of relative concentrations ($2^{-\Delta\Delta CT}$); *P values significant at 0.05 levels compared to control by One Way ANOVA analysis.

of induced hypoxic stress, expression in gills showed a higher level with 4.03 times more than that of muscle, brain and liver with 2.03, 1.27 and 1.13 respectively.

*hsp-70*gene expression analysis using qPCR

The expression of *hsp-70* gene in the tissue samples are presented in figure 2. The data demonstrated that *hsp-70* gene expression is significantly up-regulated in tissue sample when T1 group was exposed to 2.8±0.1 mg/L of D.O. after 8 hours of stress; marked an increase of 2.61 times higher in muscle, followed by liver, gills and brain with 1.34, 1.06 and 1.04 respectively. When T2 group was exposed to chronic hypoxic condition at 12 hours with 0.98±0.1 mg/L of D.O., the liver and muscle tissue sample showed significant up-regulated *hsp-70* gene expression with a higher expression of 6.68 and 4.75. Brain and gill tissue samples showed less significant manifestation with a level of 1.03 and 1.73 times lower in compared to liver and muscle data.

Discussion

Throughout the world, aquatic environments have faced low levels of dissolved oxygen, often for extended periods of time which make aquatic breathing insufficient or too costly for extraction of the required oxygen leading to hypoxic environment. According to Ecological Society of America, hypoxia occurs when the level of DO drops below survival levels, commonly thought to be less than 2-3mg/L. Many organisms have developed adaptational feature to overcome the adverse effects of loss of oxygen. Studies of hypoxial effects on the growth, food intake and physiological status of fishes (Jobling and Baardvik 1994; Mustafa *et al.*, 2011) are prevalent, but molecular responses to chronic hypoxia in fishes are less

studied and are the least in case of air- breathing fish, *H. fossilis.* Only limited number of studies on *hif-1α* and *hsp-70* regulation in fish is being reported (Rytkönen *et al.*, 2007; Terova *et al.*, 2008). This study has been adopted to enlighten on the scenario in *Heteropneustes fossilis* which are highly adapted to very low oxygen content in water, for determining the expression of the *hif-1α* and *hsp-70* genes, and study the changes in the structural pattern of gills that helps in its adaptation.

During normoxic condition, *hif-1α* is rapidly degraded inspite of being continuosly synthesized (Bruick 2003).Under hypoxic stress, *hif-1α* heterodimerizes with *hif- 1β* in the nucleus and binds to the HRE in the enhancer region of target genes involved in specific response (Lando *et al.,* 2002). In this context, active participation of *hif- 1α* in adaptational process to the hypoxic environment were observed in all the tissue samples with the highest expressional level in gills of both T1 and T2 hypoxic conditions by 3.61 and 4.02 times respectively; followed by muscle, liver and brain simultaneously. Also, studies suggest that down regulation of *hif-1α*, the regulated subunit of *hif* transcription factor controls abnormal growth of cells, their metabolism, survival and proliferation (Xinqun *et al.,* 2008), which corresponds to significant low expression of *hif-1α* in liver and gillsas a result of low exposure to oxygen used as a survival strategy by *H fossilis* in hypoxic and stressed conditions. The amplified *β*-actin products showed consistency in tissues of both the treated and control fishes.

Studies reveal that amount of *hsp-70* production is positively correlated with hypoxia to induce tolerance to any stressed conditions, by rapidly synthesizing *hsp-70* response (Wen-Hua Li *et al.* 2019). In resemblance to other fishes and mammals which expressed significant upregulated *hsp-70* expression during stressed phase (Kanika *et al.*, 2014; Wang *et al.*, 2006), the data in this study also showed a similar pattern of increased upregulation in liver by 6.68 fold in T2 and with an increase of 2.61 fold in T1 of the muscle tissue that suggests the stress induced accumulation of misfolded, denatured proteins leading to enhanced *hsp-70* synthesis. This help us infer that *hsp-70* is directly correlated to the increased stressed response. In *H. fossilis*, tissue type, duration and type of exposure to stress may interfere in hsp-70 induction. Reports suggest that down regulation of *hsp-70* inhibits apoptosis as over expression of *hsp-70* production is positively correlated with hypoxia to induce tolerance ability by rapid synthesizing *hsp-70* in heat shock response (Chung-Ying Li *et al.,* 2000; Camargo and Martinez 2007; Abele and Puntarulo 2004). In contrast to liver and muscles, expression of *hsp-70* in the brain and gills were found to decrease in T1 with the expressional level of 1.01 and 1.06 times respectively, which may hinder apoptosis of the cells in brain and gills. Researches prove that due to stress, a significant reduction can also occur in *hsp-70* mRNA. Chronic stress inhibits *hsp-70* activity when studied in rat intestinal layer when exposed to heat stress (Ying-Li Han *et al.*, 2016). On the other, over expression of *hsp-70* may enhance a different Fas-mediated apoptotic cell death pathway (Liossis *et al.*, 1997).

Thus, data of the present study showed that the adaptation to hypoxic environment by *Heteropneustes fossilis* involved both *hsp-70* and *hif- 1α* gene expression. South-east Asia is the prime habitat of *H fossilis* and different environmental challenges like environmental ammonia, hypoxic and desiccation stresses have been noticed prevailing in this region. This fish being a euryhaline fish inhabiting fresh, brackish water and muddy marshes (Saha *et al.*, 1998, 2007); they face wide ranges of external osmolarity variation ranging from 100-350 mOsmol.l^{-1} (Sen 1985), that creates a problem when lived in the same habitat during different seasons of the year. In summer when the ponds and lakes are dried up, they are bound to migrate inside the mud peat to avoid total dehydration but this behavior is changed during the monsoons. Thus, they have an enormous capacity of adaptation that attracted for this study. The results of the present study suggest that the biomarkers chosen for the study are affected by hypoxia, and established that though low levels of oxygen content in water affects the fish to a great extent but its adaptation mechanism involving *hsp-70* and *hif- 1α* enables help in hypoxic stressed condition. This might be useful for further studies to elucidate different mechanism and interplay

of *hsp-70* and *hif- 1α* in stressed environment. Idea for development of such adaptive response in other species may also be obtained, either in their natural habitat or in cultured condition.

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