Hif1α Mediated Ho-1 Expression in Fish Adipocytes: Role of Hypoxia In Ennore Estuary

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Abstract

Heme oxygenase-1 (HO-1), an inducible stress protein; is involved in the regulation of adipogenesis, cellular bioenergetics and cytoprotective mechanism against hypoxia induced oxidative stress. Pollutant aquatic environment leads to large oxygen fluctuations which may cause alteration in fish adaptation mechanism by HIF1α regulation. Environmental pollutants have a high tendency to accumulate, persist and bio-concentrate in adipocytes due to its lipophilic nature which ultimately causes adipocytes hypoxia that in turn affects metabolic energy balance. CYP1A2 and HO-1 are the chief detoxifying and cytoprotective enzymes involved in the oxidative metabolism of a wide variety of pollutants and its mediated cytotoxic products. The key role of hypoxia and its mediated signaling protein changes in fish adipocytes due to low dissolved oxygen in Ennore estuary was demonstrated in our previous work. Therefore, we analyzed the effect of pollutants induced hypoxia by assessing CD, HNE, TAC, G3PDH, ATP, HIF1α, HO-1 and CYP1A2 in fish adipocytes of control/unpolluted site and test/polluted site. Increase in CD, HNE, HIF1α, HO-1 along with a decrease in TAC, G3PDH, ATP and CYP1A2 observed in test adipocytes. From the results achieved, it shows that induction of HO-1 by HIF1α is crucial in maintaining the cell integrity to secure adipocytes and its maturation in the fish collected from the hypoxic environment. This study also highlights the role of HIF1α and HO-1 in regulating the expression of CYP1A2 during pollutants induced hypoxic condition.

Keywords: Adipocytes; Energy; Hypoxia; Metabolism; Pollutants

Introduction

Molecular mechanisms during depleted oxygen condition and strategy behind the adipocyte cell survival mechanism are poorly understood. In particular the responding regulatory mechanism is almost unknown in fish adipocytes. Estuarine ecosystems are more tender to hypoxic conditions in which fish exhibit a wide range of tactics to counteract aquatic hypoxia. In response to hypoxia, cells can sense oxygen depletion and adapt to the hypoxia environment through a series of distinctive biological processes [1]. Adipocytes are the principal cells involved in the regulation of energy homeostasis for sustaining cell survival in the hypoxic environment. In the present study, heme oxygenase-1 (HO-1, small heat shock protein, MW. 32kDa) is analyzed in the adipocytes of grey mullet, which is a better marker of the cellular response to a hypoxic insult [2]. Here the pollutants induced stress condition is described as pollutants induced hypoxia, as pollutants have a major role in triggering cellular hypoxia [3]. HO-1 is an inducible gene whose transcription is regulated in response to a wide variety of cellular stress stimuli, including oxidative stress, hypoxia and inflammatory cytokines [4]. HO-1, a metabolic enzyme utilizes electrons supplied by NADPH CYP 450 reductase (CPR) and oxygen to break the heme moiety to liberate biliverdin (BV), carbon monoxide (CO) and iron. Though heme acts as a prime part of numerous metabolic enzymes as a core catalytic unit and as a cofactor in enzymatic processes (e.g., cytochrome p450, INOS and guanylate cyclase) [5], its accumulation during oxidative stress (OS) may cause extensive toxicity and free radical generation. Hence it is degraded by the cytoprotective enzyme HO-1, which is the only catalytic process that enables removal of toxic heme.

More free radicals are likely to be produced during cellular hypoxia. Accumulation of free radicals in turn leads to the production of ROS and RNS which ultimately results in the generation of cellular antioxidants in an attempt to counteract the possible damage [6]. Present work encompasses two different areas of toxicological research by analyzing the oxidants such as conjugated diene (CD), hydroxynonenal (HNE) and antioxidant status by total antioxidant capacity (TAC). Peroxidation of lipids leads to the formation of primary product conjugated dienes (CDs) and CD on further oxidation leads to the formation of HNE, which serves as the end product [7,8]. Analyzing OS marker is crucial as it diffuse within the cell directly by reacting with cell surface proteins, thereby causing secondary alterations in the structure and functions of cells [9]. Thus it causes cellular redox signal transduction, which in turn propagates their noxious action [10]. The measure of total antioxidant capacity (TAC) is considered to be vital, as it measures the cumulative action of all the antioxidants during stress condition [11].

Metabolism is the foundation of biological system which allows the organisms to respond and adapt to any intracellular and extracellular changes during stress. Lipid metabolism plays a vital role, which helps to maintain a milieu conductive to the production and storage of energy and to cellular growth [12]. Triacylglycerol (TG) serves as the major energy storage form in adipocytes. Fatty acids (namely, long chain acyl-CoA) and glycerol 3-phosphate (G3P) serves as the direct precursors for TG synthesis in adipose tissue. Glycerol 3-phosphate dehydrogenase (GPDH) is a key enzyme providing glycerol 3-phosphate for TG synthesis in adipose tissue and is regarded as a marker for adipocyte differentiation. GPDH is the target gene regulated by peroxisome

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proliferator activated receptor γ (PPARγ) which is a crucial activator of adipogenesis [13].

Environmental hypoxia often evokes ATP alteration in the stressed cells, which is highly detrimental in the cellular bioenergetics [14]. High-energy phosphate in the form of ATP is required for virtually all synthetic and degradative processes within the cell, including membrane transport, protein synthesis and lipogenesis [15]. ATP, the energy store of cells, is produced mainly by oxidative phosphorylation in the electron transport system [16] or by glycolytic pathway [17]. The major causes of ATP depletion are reduced supply of oxygen, nutrients, mitochondrial damage and the actions of toxins [18].

Hypoxia-inducible factor 1 (HIF1) is considered to be the most critical transcription factor and a master regulator that plays a key role in regulation of metabolic adaptation during hypoxia. It is a heterodimer composed of α and β subunits in which HIF1α is sensitive to oxygen tension, whereas HIF1β (also referred to as aryl hydrocarbon receptor nuclear translocator, ARNT) is a constitutively expressed and oxygen-insensitive subunit [19]. It activates a broad range of genes which are protecting cells against hypoxia [20]. Under normoxia, HIF-1α has an extremely short half-life of less than five minutes, being continually synthesized and degraded, mediated by the hydroxylation of two prolyl residues (402 and 564) in the oxygen-dependent degradation domain (ODD) by the specific prolyl hydroxylases and by preventing it from associating with ARNT. Under hypoxic conditions, the hydroxylation of prolyl residues is inhibited, leading to stabilization of HIF-1α and which dimerizes with ARNT, that evades the proteasomal destruction leading to its accumulation [21,22] which in turn regulate the adaptation mechanism for cell survival [23].

CYP450 are known as mixed function oxidases involved in the oxidative metabolism of xenobiotes [24] and it is regulated by HO-1 and HIF1α [21,25]. Typically, the substrates for these enzymes are highly lipophilic, so they tend to escape from the liver detoxification system and accumulate in fat tissue where they are metabolized by the P450s to produce a more water-soluble product, facilitating their disposition and clearance. Similar to HO-1, the P450 enzymes use molecular oxygen and electrons provided by CPR to insert an oxygen atom into a substrate molecule [25]. Xenobiotics bind with the AHR (aryl hydrocarbon receptor), inducing the formation of nuclear AHR-ARNT heterodimers, which up regulates genes encoding xenobiotics metabolizing enzymes [26]. Minghua et al. [27] reported that ARNT has differential potential to form heterodimers with either the AHR or ARNT heterodimers, which up regulates genes encoding xenobiotics metabolism [28].

The molecular mechanism by which cells respond to hypoxia and the process of metabolic reprogramming is unknown. Understanding the molecular changes in fish exposed to hypoxic stress could reveal novel mechanisms of stress tolerance that may shed new light on adipocyte biology and remodeling during a natural environmental stress condition. Therefore, here we outline the key components of the cellular hypoxia signaling pathway mediated metabolic reprogramming in fish adipocytes which necessitates the rapid knowledge of adaptive changes in metabolic organization.

Materials and Methods

Study site and study animal sampling

*Mugil cephalus* (M.cephalus) commonly known as grey mullet with an average length of 30-32 cm were collected from Kovalam (control) and Ennore (test) estuaries using baited minnow traps, which were situated on the east coast of India. Contamination of this estuary by heavy metals and the difference in physical, chemical and biological characteristics has already been confirmed by previous studies [29-33]. Water quality was assessed by analyzing the dissolved oxygen level of both Kovalam and Ennore estuary: The dissolved oxygen level was estimated by CHEMLINE portable dissolved oxygen meter CL-930 and it is expressed as ppm. Simultaneously fish were collected from both estuaries and placed immediately into insulated containers filled with aerated estuarine water at ambient temperature (25-30°C) and salinity (24-29 ppt). Fish were maintained in the above specified conditions for 4-5 hrs until the start of the experimental procedure for the isolation of adipocytes. The experiments were divided into 3 batches with minimum 5 samples at each time.

Adipocytes isolation

Subcutaneous adipose tissue was carefully removed from each fish (n=20), then it was washed with distilled water separately and the adipocytes were isolated by the method of Rodbell (1964) [34] with some minor modifications (at temperature 18°C). Briefly, adipose tissue was cut into small pieces and incubated in polypropylene tubes with isosmotic Krebs' buffer (pH 7.4, 280 mM) containing collagenase type II (0.3 mg/ml) and 1% BSA without glucose for 60 min in a water bath under gentle shaking at 18°C. The cell suspension was filtered through a 100 µm filter to remove large undigested tissue particles and centrifuged at 700 g for 10 min. Then the pellet was washed by floation. Finally, floating cells were carefully removed as it contains mature adipocytes [35]. The isolated adipocytes from each fish (control and test fish) were separately used for following experiments.

Cell viability assay

The viability of adipocytes was determined by trypan blue staining [36]. This dye exclusion test is used to determine the number of viable cells present in a cell suspension and is based on the principle that live cells possess intact cell membrane that exclude dyes such as trypan blue, whereas dead cells do not exclude dyes. In brief, suspension cells were harvested by centrifugation. An equal volume of 0.4% (w/v) trypan blue was added to a cell suspension at a concentration of approximately 1 x 10^6 per mL. The cells were then incubated for 3 min and loaded into a hemacytometer. Nonviable, deep blue cells as well as viable, clear cells were counted in three separate fields using bright field microscopy. The viability percentage was calculated by dividing the number of viable cells by the number of total cells and multiplying it by 100.

Estimation of protein

To estimate the level of protein, adipocytes were harvested in cell suspension buffer (Kreb's buffer pH 7.4), centrifuged (15 minutes at 2000 rpm), and resuspended in cell lysis buffer (20 mM Tris pH 7.5, 1% Triton X- 100, 1 mM ethylenediamine tetraacetate acid (EDTA), 1 mM ethylene glycol-bis-(2-aminoethyl)-tetraacetic acid (EGTA), 1 mM phenylmethyl sulphonylfluoride (PMSF), 5 mM sodium pyrophosphate, 2 mM sodium orthovanadate and protease inhibitor). The cell suspension was incubated for 30 minutes at 4°C with occasional shaking and centrifuged at 16,000 xg for 10 minutes in 4°C to remove the cellular debris. The supernatant was the cell lysate, whose protein concentration was expressed as mg protein/g of fish tissue.
fresh weight of adipose tissue. Based on the requirement of the sample for each experiment, sample was diluted to acquire 100 μg of protein.

**Estimation of stress markers**

Conjugated diene was measured by the method of Recknagel and Glende [38]. HNE was assessed by ELISA kit MBS161454 (My Biosource) following the manufacturer's instructions.

**Estimation of TAC**

TAC was evaluated by the method described by Prieto et al. [39] and expressed as Trolox equivalent in mmol /L.

**Assay of GPDH**

GPDH activity was measured by following Sottile and Seuwen [40] with slight modifications. Cells were washed with PBS and the assay mixture was added to the tube (0.1 M triethanolamine, 2.5 mM EDTA, 0.1 mM b-mercaptoethanol, and 334 mM NADH, pH 7.7) and tubes were incubated for 10 min at 30°C. The reaction was started by adding 4 mM dihydroxyacetone phosphate. GPDH activity was measured spectrophotometrically at 340 nm. GPDH served as the standard for the assay and results were expressed as mU/mg protein (1 U=1 mmol NADH/min).

**Estimation of ATP/ADP ratio**

The ratio of ATP to ADP was determined by HPLC according to the method of Anderson and Murphy [41].

**Quantification of HIF1α, CYP1A2 and HO-1 using ELISA**

HIF1α, CYP1A2, and HO-1 were quantified using HIF1α ELISA kit (CSB-E12112H, CUSA BIOTECh, China, 96 T), CYP1A2 ELISA kit (E93294Hu, Prolab marketing PVT ltd), HO-1 (ADI-EKS-800, Biogeniux) according to the manufacturer's instructions.

**Statistical Analysis**

Data were analyzed using statistical software package version 7.0. Student's t-test was used to ascertain the significance of variations between control and test fish adipocytes. All data were presented as mean ± SD. Differences were considered significant at p<0.05, p<0.01 and p<0.001.

**Results**

**Dissolved oxygen level**

The dissolved oxygen level is represented by Figure 1 which depicts the decreased DO level (29%; p<0.01) in Ennore estuary water when compared to Kovalam estuary water.

**Cell viability**

Figure 2 depicts the cell viability. Decrease in the cell viability (17%; p<0.05) observed in test adipocytes when compared to control adipocytes.

**Level of protein**

Decreased protein concentration (71%, p<0.001) observed in test adipocytes than control adipocytes (Figure 3).

**Oxidant status**

Figures 4 and 5 displays the OS markers (CD & HNE). It depicts an increase in CD (30%, p<0.01) and HNE level (55%, p<0.001) in test adipocytes than control adipocytes.

**Antioxidant status**

Decrease in the TAC (75%, p<0.001) observed in test adipocytes than control adipocytes (Figure 6).
Level of G3PDH and ATP/ADP ratio

Figures 7 and 8 demonstrates the level of G3PDH and ATP/ADP ratio. In test adipocytes G3PDH and ATP/ADP ratio decreased by 27% (p<0.01) and 56% (p<0.001) than control adipocytes.

Expression of HIF1α

Figure 9 depicts the increase in the expression of HIF1α by 38% (p<0.001) in test adipocytes than control adipocytes.

Expression of HO-1

Expression of HO-1 is presented in Figure 10. Increase in the expression of HO-1 by 44% (p<0.001) observed in test adipocytes than control adipocytes.

Expression of CYP1A2

Decrease in the expression of CYP1A2 by 28% (p<0.01) observed in test adipocytes than control adipocytes (Figure 11).

Discussion

Estuarine hypoxia is a key causative factor of environmental
proteins impression involved in the adaptive strategy of fish adipocytes
regulations, current research is directed towards finding out signaling
alterations in the adipose tissue which results in stress associated
endogenous ROS, for the maintenance of metabolic reprogramming
induced hypoxic condition. From the result, it is confirmed that HO-1
observed diminished G3PDH level in fish adipocytes under pollutants
inducer of HO-1 to provide cytoprotective function which was similar
to the study reported by Iles and his colleagues [49]. Terry and his
coauthors [50] reported that TNFα induces HO-1 to render protection
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to the study reported by Iles and his colleagues [49].

The fundamental biochemical abnormality in hypoxic cells is cell
injury with reduced intracellular generation of ATP [44]. As described
above, loss of ATP leads to the failure of many energy-dependent
cellular systems. Semenza (2007) [45] described that hypoxia induces
the expression of HIF-1α which results in the inhibition of the electron
transport chain and ATP production, limit the overproduction of
reactive oxygen species during hypoxia and slows the rate of oxygen
depletion. The coordinated regulation of metabolic demand and supply
is required to maintain metabolic adaptation and cellular homeostasis
during pollutants induced hypoxia and its mediated stressed condition.
Cytotoxic effect under pollutants exposure is confirmed by the cell
viability and the protein level, which were found to be declining in
the test fish adipocytes. The present study reveals that pollutants
serve as the key factor in the development of considerable distress in
fish adipocytes. It was depicted by the level of CD and HNE acitivity
significantly with the diminished TAC level in test fish adipocytes.

Zhang and his colleagues [42] reported that accumulation of intracellular peroxide products in adipocytes/adipose tissues
contributes to lipolytic activation. Coherently G3PDH level was found
to be decreased in test fish adipocytes when compared to control fish
adipocytes. Loss of G3PDH in adipose tissue may lead to loss of fat mass
[43] which depicts the existence of dysregulation in the TG synthesis
and adipogenesis as noted in our previous work [32].

Fish may adopt acute hypoxic stress by changes in behavior,
physical and morphological nature, whereas in chronic hypoxic
condition fish adapt at molecular level. The major molecular level
stress adaptation includes the activation of the HIF1α [46,33] which is
correlated to the present study. It was confirmed by the variation in
the expression of HIF1α in fish adipocytes from pollutants induced
hypoxic condition. It also emphasized that HIF1α protein is a candidate
biomarker for determining exposure of fish to hypoxic events [22].

Induction of HIF1α markedly enhances the level of HO-1 [47].
During hypoxia mediated oxidative stress, cellular heme is released
and contributes to the growing “free heme pool.” Free heme is a potent
pro-oxidant and increases ROS levels [48]. Elevated levels of heme are
toxic and catalyze oxidative damage to cells, tissues, and organs. Hence
the role of HO-1 is vital which acts as the crucial enzyme catalyzing
the heme degradation. Khitan and his colleagues [48] stated that the
protective effect of HO-1 is ascribed to its role in decreasing the cellular
heme content as they are highly toxic and leading to the generation of
ROS and RNS. Similarly, in the present study increased HO-1 expression
was observed in test adipocytes suggesting that HNE acts as a potent
inducer of HO-1 to provide cytoprotective function which was similar
to the study reported by Iles and his colleagues [49]. Terry and his
coauthors [50] reported that TNFα induces HO-1 to render protection
against oxidant stress, which is similar to our previous study where
we observed increased TNFa [32]. TNFα is also known to regulate
G3PDH and its mediated lipogenesis process [51]. Coherently we have
observed diminished G3PDH level in fish adipocytes under pollutants
induced hypoxic condition. From the result, it is confirmed that HO-1
is responsible for the regulation of adipocyte function by decreasing
endogenous ROS, for the maintenance of metabolic reprogramming
process in fish adipocytes.

In addition to oxidative stress markers, the major drug
metabolizing enzyme-antioxidant CYP1A2 was assessed in the present
cytotoxic effect under pollutants exposure is confirmed by the cell
viability and the protein level, which were found to be declining in
the test fish adipocytes. The present study reveals that pollutants
serve as the key factor in the development of considerable distress in
fish adipocytes. It was depicted by the level of CD and HNE activity
significantly with the diminished TAC level in test fish adipocytes.

Pollutants, evidenced by low dissolved oxygen in Ennore estuary
when compared to Kovalam estuary. Hypoxia triggers the metabolic
alterations in the adipose tissue which results in stress associated
tissue dysfunction. Due to the importance of adipocytes in energy
regulations, current research is directed towards finding out signaling
proteins impression involved in the adaptive strategy of fish adipocytes

Figure 9: Level of HIF1α in control and test adipocytes of M. cephalus. Values are expressed as means ± SD (n=20 fish per site).

Figure 10: Level of HO-1 in control and test adipocytes of M. cephalus. Values are expressed as means ± SD (n=20 fish per site).

Figure 11: Level of CYP1A2 in control and test adipocytes of M. cephalus. Values are expressed as means ± SD (n=20 fish per site).
study to demonstrate its effect in adipocytes during pollutants induced hypoxia. Fradette and Souich [52] have reported the reduction in the expression of CYP1A2 in hypoxia induced animal models. Both HIF1α and CYP1A2 are biomarkers of environmental exposure to hypoxia and xenobiotics, act through the same aryl hydrocarbon receptor [21]. Similarly, present work observed the decreased expression of CYP1A2 in test fish adipocytes compared to control fish adipocytes depicting the role of environmental pollutants in causing O2 deprivation which in turn results in diminished CYP1A2, but enhanced HIF1α. Morel and Barouki [53] have reported that ROS were shown to down-regulate CYP1A mRNA level in rat hepatocytes which is concomitant to our data. Reed and his colleagues (2011) [25] suggested that induction of HO-1 leads to the diminished level of CYP1A2 as they were sharing the same redox partner NADPH CYP 450 reductase for electrons to insert an oxygen atom into a substrate molecule. Diminished CYP1A2 may also be due to HIF1α dimerization with ARNT as CYP1A2 requires the activation and the association of AHR-ARNT complex [27]. Hence it is suggesting that elevation of HIF1α and HO-1 is crucial for regulating the adaptive mechanism rather than CYP1A2.

Current study clearly emphasized the effect of stress induced signaling protein changes in energy balance adapted by fish adipocytes during pollutants induced hypoxia. The primary key finding of this study includes the adaptive mechanism rendered by HIF1α in HO-1 induction and its subsequent effect on metabolic reprogramming. Decreased CYP1A2 in test fish adipocytes hints that adipocytes acts as a prime depot for toxic compounds due to the lipophilic nature of the toxic elements, thereby limiting the systemic toxicity by decreasing the availability of toxic chemicals to other cells. Hence the present study concludes that the differential expression of signaling and its associated metabolic reprogramming may serve as an adaptive mechanism for the cells to survive during environmental hypoxic condition.

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