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Hypoxia-inducible Factor-1 Alpha (HIF-1α) and its Role in the Hypoxic Preconditioning and Gene Expression Patterns in Zebrafish (Danio rerio) Embryos

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Hypoxia-inducible factor-1 alpha (HIF-1α) and its role in the hypoxic preconditioning and gene expression patterns in zebrafish (*Danio rerio*) embryos

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Abstract

Preconditioning to lowered oxygen levels (hypoxia) may occur when an animal is subjected to non-lethal levels of hypoxia and then returned to normoxic conditions. During a subsequent exposure to hypoxia, the pre-exposed animals may exhibit increased tolerance compared to naïve animals. I have demonstrated, by analysis of critical PO_2's (Pcrit), that zebrafish embryos can be preconditioned to hypoxia. Preconditioned embryos display a lower Pcrit than controls, indicating a heightened ability to endure hypoxic conditions. The role of the hypoxia inducible factor-1 (HIF-1) in promoting hypoxic preconditioning was examined. To determine the role of HIF-1 in preconditioning, embryos deficient in HIF-1α (using antisense oligonucleotide morpholinos) were assessed under hypoxic conditions. No significant difference between preconditioning capacities of control- and HIF-1α deficient embryos was observed. In addition to assessing Pcrit, a suite of hypoxia responsive genes were analysed by real time PCR. IGFBP-2 showed a significant decrease in expression in the HIF-1α deficient embryos, and EPO showed a significant increase in HIF-1α deficient embryos. All other genes examined showed no significant change between treated and control embryos.

Résumé

Le préconditionnement à de bas niveaux d'oxygène (hypoxie) peut arriver quand un animal est sujet à de niveaux non-mortels d'hypoxie puis remis dans des conditions normoxiques. Lors d'une prochaine exposition à l'hypoxie, les animaux déjà soumis à l'hypoxie peuvent démontrer une tolérance accrue comparativement à des animaux naïfs. J'ai démontré, par l'analyse du PO2 critique (Pcrit), que les embryons de dard-perches peuvent être préconditionnés à l'hypoxie. Les embryons préconditionnés ont un Pcrit plus bas que les contrôles, ce qui indique une habilité accrue à endurer des conditions hypoxiques. Le rôle du facteur d'hypoxie inductible-1 (HIF-1) dans le préconditionnement à l'hypoxie a été étudié. Afin de déterminer le rôle de HIF-1 dans le préconditionnement, des embryons dépourvus de HIF-1α (en utilisant des morpholinos oligos antisenses) ont étés examinés sous conditions hypoxiques. Aucune différence significative n'a été observée entre les capacités de préconditionnement des embryons contrôles et ceux dépourvus de HIF-1α. En plus d'observer le Pcrit, une série de gènes répondant à l'hypoxie a été analysés avec la technique du PCR en temps réel. L'expression d'IGFBP-2 démontre une réduction significative dans les embryons dépourvus de HIF-1α et l'expression d'EPO démontre une augmentation significative dans les embryons dépourvues de HIF-1α. Tout les autres gènes examinés ne démontrent pas de différence significatives entre les embryons traités et les contrôles.

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Abbreviations used:

CT: Threshold cycle
dpf: Days post-fertilization
ENO: Enolase
EPO: Erythropoietin
HIF: Hypoxia Inducible Factor
hpf: Hours post-fertilization
HRE: Hypoxia response element
IGFBP: Insulin-like Growth Factor binding protein
LDH: Lactate dehydrogenase
P4H: Prolyl-4-hydroxylase
PGK: Phosphoglycerate kinase
RT-PCR: Reverse transcriptase polymerase chain reaction
VEGF: Vascular endothelial growth factor

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Introduction

Zebrafish

The zebrafish (*Danio rerio*) has emerged as a valuable model organism for research in vertebrate developmental biology. These freshwater fish originate in rivers in India (Briggs, 2002). They have a short generation time (approximately four months) and the number of eggs produced at each mating is high (in the hundreds). The eggs are fertilized externally, allowing all stages of development to be studied. The embryos hatch usually at around 48 hours post fertilization (hpf) (Briggs, 2002). Because the embryos and larvae are transparent until approximately five days post fertilization (dpf), it is possible to view developmental changes in the zebrafish under a microscope (Briggs, 2002).

The zebrafish has also become an excellent genetic model organism, in part because the full genome has been sequenced (www.ensembl.org). In 2002, Ton et al used cDNA microarray technology to study the gene expression patterns of zebrafish during development, as well as observing the genetic responses to hypoxia during development (Ton et al, 2002).

Because they live in aquatic environments, where oxygen levels may fluctuate daily, seasonally and spatially (Nikinmaa, 2002), fish are excellent subjects for hypoxia-related studies, and make these studies relevant from an ecological standpoint (Rees et al, 2001). Changes in temperature have a major effect on oxygen demand in fish. For every 10°C increase in temperature, there is more than twice the demand for oxygen (Nikinmaa, 2002).

The supply-demand relationships for oxygen and for CO_2 change much more during development of the embryo and larva than they do during the adult stage (Bagatto et al, 2001). Until approximately 14 dpf, zebrafish are not dependent on convective oxygen transport, as there is sufficient oxygen diffusing through the body surface to meet metabolic needs. (Schwerte et al, 2003).

Zebrafish embryos can survive up to twenty-four hours in anoxia by entering into a state of suspended animation, in which all metabolic processes are arrested (Padilla & Roth, 2001). Cardiac function, cell division and developmental progression are stopped until oxygen is restored. Furthermore, it was observed that the cell cycle was arrested in either S phase or G_2, meaning that no cells entered mitosis while in suspended animation. During normoxia, approximately 15% of cells at any given time were in mitosis. Being in suspended animation, cells maintained low membrane permeability (Padilla & Roth, 2001). As the embryos get older, they become more sensitive to hypoxic stress (Ton et al, 2002), although they are still capable of entering a state of suspended animation at any embryonic stage (Padilla & Roth, 2001). Numerous studies have shown that growth rates in fish embryos and larvae are severely retarded under hypoxic conditions (e.g. Drexel et al, 2002). There is evidence to show that this developmental retardation may be due, at least in part, to induction of IGFBP-1 during hypoxia. In zebrafish deficient in Insulin-like growth factor binding protein (IGFBP)-1, there was a significant lessening of retardation in hypoxia, whereas over-expression of IGFBP-1 caused growth and developmental retardation during normoxia (Kajimura et al, 2004).

Preconditioning

Rees et al (2001) demonstrated that it is possible to acclimatize adult zebrafish to hypoxia by first subjecting them to non-lethal levels of hypoxia. Following this exposure, the fish are more likely to survive more severe levels of hypoxia than fish that were not pre-conditioned (Rees et al, 2001). In their experiments, Rees et al (2001) found that acclimation depended on several factors, including the sex of the fish, and the season during which experiments were conducted. This indicates that a genetic factor is probably involved in pre-conditioning. It is likely that pre-conditioning arises from a combination of behavioral, environmental, genetic and biochemical factors (Rees et al, 2001). It has also been shown that preconditioning to hypoxia occurs in mammals. In rats that were subjected to 8% oxygen for 3 hours and subsequently exposed to hypoxia after a 24 hour recovery period, there was a significant level of protection against cerebral infarction caused by hypoxia (Semenza, 2001a). This preconditioning also induced hypoxia-inducible-factor-1α (HIF–1α) expression in the brain. HIF-1 is a transcription factor that regulates many of the genes involved in the hypoxic response. Several genes, both HIF-1 dependent and independent, were shown to increase in expression during hypoxic preconditioning in rat brains. Many of the HIF-1 dependent genes examined responded to hypoxic treatment in the same manner in both neonatal and adult rat brain, although expression levels of proteins were not examined (Tang et al, 2005). Research is examining HIF-1 and the angiogenic factor, carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM-1) as a mechanism of hypoxia-induced cardioprotection (Chen et al, 2005).

Similar to the phenomenon of preconditioning, training in hypoxia results in an upregulation of HIF-1α, and an increase in the expression levels of myoglobin, vascular

endothelial growth factor (VEGF), and glycolytic enzyme mRNA (Hoppeler & Vogt, 2001). In zebrafish, cardiac activity becomes responsive to environmental hypoxia at around the time of hatching (48 hpf). Exposure to chronic hypoxia elevated the heart rate of the embryos at 4.5 and 6 dpf (Jacob et al, 2002).

Hypoxic response

All nucleated cells in the human body sense changes in oxygen concentration, and respond accordingly (Semenza, 2000a). Although the mechanism of oxygen sensing is still being studied, it appears as though the molecule responsible for oxygen sensing is a protein that contains heme, and binds to oxygen (Chi & Karliner, 2004). Some theories as to the actual mechanism of oxygen sensing include oxygen-dependent regulatory enzymes, and the generation of reactive oxygen species. It is likely that the mitochondria play some role in oxygen sensing (Cummins & Taylor, 2005). Mitochondria may act by increasing reactive oxygen species generation during hypoxia, and thereby initiate a signaling pathway leading to the adaptation to hypoxia via HIF-1 induction (Bardos & Ashcroft, 2005). There is also evidence to show that prolyl-4-hydroxylases may play a role in oxygen sensing (Asikainen et al, 2005). Several changes occur when an animal is exposed to hypoxia. Overall, these changes include increased ventilation rate, increased anaerobic respiration, and in some cases, a decrease in metabolic rate (Powell & Hahn, 2002). There are also cases in which hypoxic conditions occur due to hypoxemia, where oxygen demand exceeds delivery to the tissue. Hypoxemia would occur, for example, in cases of increased metabolic activity. In such cases, anaerobic glycolysis is not induced, and aerobic respiration continues. Decreasing the capacity of the hemoglobin to transport oxygen can therefore stimulate a hypoxic response (Drexel et al, 2002).

The hypoxic response depends partially on the duration of exposure to hypoxia. In an acute exposure, lasting several seconds to several minutes, there is a post-translational change in the proteins that already exist within the cell, for example by phosphorylation. In cases of chronic hypoxia, lasting several minutes to hours or days, additional changes are elicited by altered levels of gene expression (Semenza, 2000a). Sustained hypoxia has been shown to damage the muscle structure of the animal (Hoppeler & Vogt, 2001), to modify cardiac activity (Pelster, 2002), and to alter differentiation and growth during the embryonic and larval development of zebrafish (Drexel et al, 2002).

Hypoxia induces many changes in the animal at both the physiological and at the cellular level. At the physiological level, hypoxia induces reflex hyperventilation, increased red-blood-cell production, new blood vessel formation, and other changes that can lead to increased oxygen delivery to the tissues. At the cellular level, adaptive responses to hypoxia include a switch from oxidative phosphorylation to anaerobic glycolysis, increased glucose uptake and an increased expression of stress proteins (Chun et al, 2002). Cell adhesion is reduced, resulting in the migration of cells (Greijer et al, 2005). Under hypoxic conditions, various genes are regulated through transactivation, signal transduction and oxygen sensing (Huang et al, 1996). There are numerous genes that are expressed as a result of anaerobic energy production following hypoxia. Some of these are transcribed in specific cells, and their products are then secreted as hormones that induce erythropoesis and vascularization, while others produce proteins that are expressed in a much wider range of cells and that play a role in basic cell metabolism (Behrooz & Ismail-Beigi, 1999). Many patterns of gene expression resulting from hypoxia are tissue-specific, and are associated with the role of a particular tissue in

metabolism under hypoxic conditions (Gracey et al, 2001). Some of the genes induced by hypoxia encode proteins that play roles in glycolysis, gluconeogenesis, iron/heme catabolism, amino acid synthesis, and inhibition of cell growth and division (Powell & Hahn, 2002). Microarray studies applied to the longjaw mudsucker, *Gillichthys mirabilis*, have shown over 120 hypoxia-related genes (Gracey et al, 2001).

HIF-1

HIF-1 (hypoxia inducible factor-1) is responsible for the regulation of many of the genes associated with the hypoxic response. The binding of HIF-1 to the regulated gene at the hypoxia response element, HRE, induces transcription of the gene (Chun et al, 2002). HIF-1 was originally discovered in 1992 as being responsible for the expression of erythropoietin (EPO) in hypoxic conditions (Dery et al, 2005). Besides HIF-1, mammals and zebrafish also have HIF-2 and HIF-3 (Powell & Hahn, 2002). While HIF-1α plays a general role in regulating transcription of hypoxia-induced genes in the nucleus of all cells, HIF-2α and HIF-3α play more of a limited role in oxygen homeostasis (Semenza, 2000b). Histological analysis of HIF-1α-deficient mouse embryos showed defects in the cephalic mesenchyme, the blood vessels, and the presumptive myocardium, as well as failure of neural tube closure, suggesting that HIF-1α is required for the normal development of major tissues and organs (Tyer et al, 1998).

HIF-1 is a heterodimeric protein made up of two basic helix-loop-helix proteins. The two subunits of HIF-1 are HIF-1α and HIF-1ß, both being members of the Per-ARNT-Sim (PAS) family (Chun et al, 2002; Elson et al, 2001; Huang et al, 1996). HIF-1ß is a previously identified protein, the aryl hydrocarbon nuclear receptor translocator,

ARNT, which is dimerized with the aryl hydrocarbon receptor (Chun et al, 2002; Powell & Hahn, 2002). The size of the HIF-1β subunit is between 91 and 94 kDa (Huang et al, 1996). HIF-1β also dimerizes with other transcription factors. (Soitamo et al, 2001), whereas HIF-1α is a protein that is exclusively associated with the transcription of genes induced or repressed during hypoxia (Chun et al, 2002). The mammalian HIF-1α subunit is an 826 amino acid protein (Chun et al, 2002) of 120 kDa (Huang et al, 1996). The N-terminal of the HIF-1α peptide is made up of a basic domain, a helix-loop-helix domain, and a PAS domain. These domains of the N-terminal are responsible for dimerization of the HIF-1α peptide with the HIF-1β peptide, and also for binding of the complex to the HRE recognition sequence of the target gene (Nikinmaa, 2002). The C-terminal of HIF-1α contains the nuclear localization signal (NLS) domain, which is required for transporting the HIF-1 complex to the nucleus once it has been activated. There are two transactivation domains of the HIF-1α peptide (C-TAD and N-TAD), which are located at the C-terminal (Chun et al, 2002). It was in a 2001 study by Soitamo et al that a hypoxia inducible factor was first identified in fish, the rainbow trout (Soitamo et al, 2001). In mammals, there are at least three different forms of the hypoxia inducible factor-α: HIF-1α, HIF-2α and HIF-3α (Powell & Hahn, 2002).

It is the stability of the HIF-1α subunit that regulates the activity of HIF-1 (Huang et al, 1996). HIF-1α is regulated at many levels, including mRNA expression, protein expression, nuclear localization, and transactivation. The best understood of these is regulation via protein expression levels (Semenza, 2000b). Although mRNA levels of HIF-1α are expressed at relatively constant levels, protein levels are much higher during hypoxia than normoxia (Soitamo et al, 2001). The HIF-1α gene is constitutively

expressed through the action of the Sp1 transcription factors, and the HIF-1α promoter region also contains binding sites for transcription factors such as AP-1 & 2, NF-1 and NF-KB (Dery et al, 2005). During hypoxia, protein translation of HIF-1α is maintained by the presence of an internal ribosome entry site (IRES) which is found in the 5' untranslated region (UTR) of the HIF-1α gene (Lang et al, 2002).

Under normoxic conditions, the HIF-1α peptide is unstable, with a half-life of less than 5 minutes (Chun et al, 2002). The domain of the protein that is responsible for degradation during normoxia is termed the oxygen-dependent degradation domain, ODDD (Chun et al, 2002). The ODDD contains a hypoxia response element (that responds to hypoxia) and thereby prevents the peptide from being ubiquitinated and degraded. This HRE is identical in human, *Xenopus* and rainbow trout HIF-1α proteins, suggesting a high degree of evolutionary conservation in mechanisms of degradation (Soitamo et al, 2001). Within the ODDD of human HIF-1α, there are proline residues at positions 402 and 564, which are hydroxylated during normoxic conditions. The hydroxylated proline residues are recognized by the von Hippel-Lindau tumour suppressor protein (VHL). VHL then binds to HIF-1α and links it to the ubiquitination machinery, causing the protein to be degraded (Nikinmaa, 2002) (**Fig. 1a**). It has recently been noted, however, that prolonged hypoxia causes an induction of VHL expression, leading to a negative feedback loop by which HIF-1α is partially degraded in hypoxia (Karhausen et al, 2005).

In an environment of limited oxygen, hydroxylation of the proline residues does not occur and the peptide is stabilized (**Fig. 1b**). Hydroxylation of proline 564 may be enhanced by Cyclosporin A, resulting in an increased association with VHL, in which

case stabilization of HIF-1α, even in hypoxia, will not occur (D'Angelo et al, 2003). On the other hand, acidosis may trigger nucleolar sequestering of VHL, which leads to the stabilization of HIF-1α even in normoxia (Mekhail et al, 2004).

Recent studies have shown that there is also transcription-dependent HIF-1α degradation in hypoxia. In this case, HIF-1α accumulates, and transcriptionally activates its own degradation, independent of VHL. In the presence of transcription inhibitors, there was a super-induction of HIF-1α in hypoxia, but there was no induction in normoxia, suggesting that transcription-dependent degradation acts to prevent unnecessarily high levels of HIF-1α during hypoxia. Removing transcription inhibitors restored depletion and normal levels of HIF-1α (Demidenko et al, 2005).
Cells transfected with HIF-1α cDNA in which there is a mutation or deletion of the ODDD, constitutively express the HIF-1α peptide, making expression of the protein possible under normoxic conditions (Elson et al, 2001). It has been shown that HIF-1α can be over-expressed in mice, thereby causing hypervascularity and increasing expression of VEGF mRNA and protein. This leads to the possibility of using HIF-1α over-expression as a means of treatment for tissue ischemia (Elson et al, 2001).

It appears as though HIF-1α is not only regulated by oxygen, but also by several other factors such as transition metals and nitric oxide (Chun et al, 2002). Transition metals such as cobalt and nickel can stabilize HIF-1α by replacing the iron in the heme moiety of the oxygen sensor protein, causing HIF-1α activity to be induced and hypoxia-inducible genes to be expressed during normoxia (Chun et al, 2002). Nitric oxide (NO_2) at high concentrations can block stabilization of HIF-1α during hypoxia, and reduce the induction of HIF-1 DNA binding, consequently reducing the transcriptional activity of

HIF (Chun et al, 2002). HIF activity is also regulated by CO and NO, both of which can inhibit DNA-binding of HIF in hypoxic cells. DNA-binding inhibition by CO and NO does not affect HIF-1 protein expression (Semenza, 1999).

There are several non-hypoxic factors that have recently been discovered to induce HIF-1, such as growth factors, cytokines, vascular hormones and viral proteins. Unlike hypoxia, non-hypoxic mechanisms for activating HIF-1 seem to be cell-type specific (Dery et al, 2005).

HIF-1α activation involves redox-dependent stabilization of the protein (Huang et al, 1996). Redox stabilization may occur in the transactivation domain, and may involve the modification of cysteine residues (Nikinmaa, 2002). Soitamo et al (2001) demonstrated that rainbow trout HIF-1α has four cysteine residues in or near the area which, in mammalian HIF-1α, has been identified as the transactivation domain. It is therefore possible that fish HIF-1α is more readily regulated via redox-dependent stabilization than mammalian HIF-1α (Soitamo et al, 2001).

During hypoxia, HIF-1α protein levels increase exponentially as oxygen levels decrease (Semenza, 1999). The stabilization of HIF-1α leads to its transportation from the cytoplasm to the nucleus, where it forms a complex with HIF-1β (ARNT). Dimerization leads to activation of HIF-1, and it then binds to the HRE of the target gene (Nikinmaa, 2002; Powell & Hahn, 2002). The HRE to which the HIF-1 complex binds is a specific DNA consensus sequence, 5'RCGTG 3' (Chen et al, 2001). The sequence is located in the promoters of many hypoxia-inducible genes but may also be found within 5' flanking, 3' flanking or intervening sequences of the target gene (Semenza, 2000b). Once bound to the target gene, HIF-1 interacts with other transcription factors and

accessory proteins to enhance rates of gene expression (Rees et al, 2001). HIF-1α interacts specifically with its co-activator p300/CBP (CREB-binding protein) (Huang et al, 1996). Interaction with co-activators, such as p300, Ref-1, Jab1, SCR-1, and TIF2, is mediated by the C-terminal half of HIF-1α, which contains two transactivation domains, the C-transactivation domain and the N-transactivation domain (C-TAD and N-TAD) (Bardos & Ashcroft, 2005). Hydroxylation of an asparagine residue in the C-TAD of HIF-1α prevents its interaction with its coactivators. Factor-inhibiting-HIF-1 (FIH-1) is a protein that hydroxylates asparagine 803, preventing coactivator p300 from binding (Lando et al, 2002; Bardos & Ashcroft, 2005). Various studies have shown that by targeting the interaction between HIF-1α and p300/CBP, tumour growth can be significantly alleviated *in vivo* (Chau et al, 2005).

HIF-1 target genes

HIF-1α regulates expression of many of the genes induced by hypoxia. These include genes encoding glucose transporters and glycolytic enzymes, and genes enhancing tissue perfusion (Elson et al, 2001). HIF-1α regulated genes may be involved in either cellular or systemic responses to hypoxia, and may lead to augmented anaerobic metabolism, red blood cell formation, vascularization, and other factors leading to enhanced oxygen delivery (Gracey et al, 2000).

Some of the genes targeted by HIF-1 during hypoxia include genes encoding for several glycolytic enzymes; Vascular endothelial growth factor, VEGF; insulin-like growth factor I (IGF-I), and IGF binding protein 1; erythropoietin (EPO), and glucose transporter 1 (Glut 1) (Semenza, 2000b).

Glycolysis:

Adaptation to hypoxia requires the harmonized increase in expression of the genes encoding glycolytic enzymes (Kim & Dang, 2005) (**Fig. 2**).

ALDOLASE: Aldolase A is constitutively expressed, and is a housekeeping enzyme in the glycolytic pathway (Esposito et al, 2004).

PHOSPHOGLYCERATE KINASE: Phosphoglycerate kinase I is a cytoplasmic enzyme, generating ATP in the glycolytic pathway. It is also secreted by tumour cells where it acts as a disulfide reductase, stimulating the increase of angiostatin, a tumour blood vessel inhibitor (Myre and O'Day, 2004). Along with enolase, PGK is a key component of the host factor for transcription of the Sendai virus. Through an interaction with Tubulin, ENO and PGK integrate into an active transcription initiation complex and enhance elongation of the viral genome (Ogino et al, 2001).

LACTATE DEHYDROGENASE: Lactate dehydrogenase is a cytoplasmic enzyme that catalyzes the final step in the glycolytic pathway, and has been shown to be an important enzyme in embryonic development of zebrafish (Robles et al, 2004). It also has a non-glycolytic role in transcriptional regulation (Kim & Dang, 2005). It is a tetramer comprised of A and B subunits, and has been identified as a single-stranded DNA-binding protein in several cell types and organisms (Kim & Dang, 2005).

ENOLASE: Enolase is an enzyme in the glycolytic pathway which catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate. Like LDH, it also has a non-glycolytic function in transcriptional regulation (Kim and Dang, 2005). It is known to be down-regulated in lung cancer (Kim & Dang, 2005).

TRANSFERRIN: Transferrin is a protein that binds iron and transports it via the transferrin receptor (Aisen et al, 2001). It delivers iron to the bone marrow, where it is incorporated into the hemoglobin (Tyer et al, 1998).

IGFBPs: IGF-I and IGF binding proteins are cell survival proteins that promote anaerobic ATP synthesis (Chun et al, 2002). IGFBP-1 and 2 are the secreted binding proteins for the Insulin-like growth factor. IGFBP-1 is induced by HIF-1, although other IGFs and IGFBPs, including IGFBP-2 are not. (Kajimura et al, 2005). IGF-1 stimulates the accumulation of HIF-1α, as well as its nuclear translocation and its expression through post-transcriptional mechanisms (Treins et al, 2005).

VEGF: One of the crucial genes involved in vasculogenesis and angiogenesis is the vascular endothelial growth factor (VEGF). VEGF is expressed in almost all human cells as a response to hypoxia, and this expression is regulated at multiple levels, including but not limited to transcription, RNA stability and translation (Semenza, 2003a). VEGF is a protein that increases vascularization, thereby increasing oxygen delivery to tissues (Semenza, 2000a; Goishi and Klagsbrun, 2004). It is one of the first genes to be induced following hypoxia sensing, due to the fact that it allows for an increase in immediate availability of oxygen from capillaries by increased vascular permeability (Ryan et al, 1998). In a study examining the effect of hypoxic preconditioning in the brain of adult mouse, VEGF was found to be induced sometime between 1 and 6 hours of hypoxia (Tang et al, 2005). The introduction of constitutively active HIF-1α can affect the induction of VEGF mRNA, as well as the resulting phenotype of increased angiogenesis, even in the absence of hypoxia (Elson et al, 2001). Ryan et al (1998) showed that the response of VEGF to hypoxia was only partially eliminated in the absence of HIF-1α, and that the remaining induction was likely due to hypoxia-induced stabilization of the

VEGF transcript. This stabilization is mediated independently of HIF-1α, through an element at the 3' end (Ryan et al, 1998).

PROLYL-4-HYDROXYLASE: To date, three prolyl-4-hydroxylases (P4H 1-3) have been characterized (Asikainen et al, 2005). These enzymes belong to the iron- and 2-oxoglutarate-dependent deoxygenase superfamily (Treins et al, 2005, Schofield & Ratcliffe, 2004). P4H catalyzes the hydroxylation of two specific prolyl residues in the degradation domain of HIF-1, thereby regulating the Oxygen-dependent degradation of the protein (Selak et al, 2005). P4H-2 mRNA is induced by hypoxia through a HIF-1 dependent signaling pathway (Treins et al, 2005). Cellular knockdown of PHD-2 leads to the stabilization of HIF-1, and its complex formation in normoxia (Berra et al, 2003). HIF-Prolyl-4-hydroxylases differ from other P4Hs in mammals, which are active predominantly in the modeling of procollagen. HIF-P4H requires oxygen as a substrate, and iron and ascorbate as cofactors for enzyme activity (Asikainen et al, 2005).

EPO: Erythropoietin is a glycoprotein that increases RBC formation (Chun et al, 2002). It is produced primarily by the kidney (Krantz, 1991), but is also present in the liver (Jelkmann, 1992). EPO RNA levels increase under hypoxic conditions, thus increasing red blood cell formation, whereas during hyperoxia EPO RNA levels decrease (Krantz, 1991). EPO is specific to production of RBCs, and has little or no effect on other cell types (Krantz, 1991).

Embryonic stem cells that did not express the HIF-1α gene showed a marked decrease of VEGF induction, as well as that of several glucose transporters and glycolytic enzymes such as Ald, ENO, LDH, and PGK, as a result of hypoxia, indicating that the presence of an active HIF-1α subunit is crucial for the full response of these genes to

hypoxia (Tyer et al, 1998). Ryan et al also noted by Northern blot that Ald, PGK and LDH were not induced by hypoxia when HIF-1α was absent (Ryan et al, 1998). HIF-1 contributes to cellular processes that normally occur under normoxia, such as tissue development, regulation of cell death and survival, immune responses and adaptation to mechanical stress (Chun et al, 2002).

Although many hypoxia-responsive genes are regulated by HIF-1, there are also several which are independent of HIF-1. In a study by Greijer et al, 89% of genes shown to be up-regulated by hypoxia were regulated by HIF-1, while only 18% of genes shown to be down-regulated by hypoxia were HIF-1-responsive. Among the genes that were up-regulated by HIF-1 were LDH (3.4 fold), aldolase (3.2 fold), PGK (3.2 fold) and enolase (2.3 fold). Several P4Ds were also HIF-1 up-regulated. There were significantly more genes down- than up-regulated by hypoxia (Greijer et al, 2005).

Morpholino oligonucleotides

Morpholino oligonucleotides (morpholinos) are chemically modified oligonucleotides with a morpholine backbone. Because the backbone of the oligonucleotide is more rigid than that of either DNA or RNA, it is much more stable and does not readily degrade in the organism. The use of antisense morpholinos has been demonstrated as an effective tool to specifically inhibit translation in zebrafish (Nasevicius & Ekker, 2000). They can be designed to bind to the translational start site of the transcript of interest, thereby blocking initiation of translation and effectively knocking down the gene function. Multiple gene knockdowns are also possible using this technology. Morpholinos can be injected into the yolk of the zebrafish embryo

immediately following fertilization, and the targeted gene will be completely suppressed throughout the first two days of development (Nasevicius & Ekker, 2000).

Significance

Ischemia is a major cause of tissue damage and associated conditions such as heart attack and stroke. As well as using the direct approach of delivering angiogenic factors to increase angiogenesis of ischemic tissue, other, less direct approaches have also been studied. The administration of mutant, constitutively expressed HIF-1α has been examined as a potential therapy for ischemic damage (Semenza, 2001b). The advantage of using HIF-1α as a therapeutic agent rather than specific angiogenic proteins is that HIF-1α also works to induce several other angiogenic factors as well as other genes that are necessary for the adaptations that cells undergo during hypoxia. Evidence of the advantage of using HIF-1α treatment rather than VEGF treatment was emphasized in a 2001 study that used VEGF as a proangiogenic therapy. The vessels resulting from this therapy were leaky and nonfunctional, whereas therapy with HIF-1α led to intact and functional vessels (Elson et al, 2002).

It has been well established that hypoxia is present in many forms of mammalian tumours. Clinical studies have provided evidence that low oxygen levels within tumours can lead to a prognosis of poor outcome, and can be positively correlated with the risk of developing metastases that are distant from the primary site of tumour growth, independent of therapeutic treatment (Hockel et al, 1996, 1999). In human malignancies and metastases, HIF-1α is often over-expressed, which may allow HIF-1α to be used as an early indicator of cancer cells (Semenza, 1999).

The primary method of ATP production used by hypoxic cancer cells is glycolysis. Many of the enzymes in the glycolytic pathway are under the transcriptional regulation of HIF-1α (Harris, 2002).

Most of the drugs in clinical trials do not directly target HIF-1, but act in an indirect manner by inhibiting HIF-1 regulators, by blocking HIF-1 expression, or by inducing expression of HIF-1 inhibitors. Currently, there are a large number of drugs in clinical trials as anticancer agents that are based on their anti-angiogenic properties. There are, however, concerns that perhaps inhibition of angiogenesis may lead to the selection of cancer cells that are adapted to hypoxia (Semenza, 2003b). It is therefore important to view anti-angiogenic therapy not as a stand-alone treatment for cancer, but rather as one component of a combination of therapeutic agents.

Hypothesis

Preliminary experiments showed that zebrafish embryos, as well as adults, can be preconditioned to hypoxia, and that more severe levels of hypoxic preconditioning led to an increased tolerance to subsequent bouts of hypoxia. Previous studies with rats have demonstrated an induction of HIF-1α that coincided with preconditioning. Several genes, both dependent and independent of HIF-1α, were also induced during preconditioning. These findings suggest that HIF-1α may play a role in the phenomenon of preconditioning in mammals, either directly, or through the action of HIF-1α target genes.

This leads to my hypothesis, that HIF-1α plays a significant role in preconditioning of zebrafish embryos to hypoxia. If the hypothesis is correct, then

preconditioning will not occur in the absence of HIF-1α, as in the morpholino-injected (morphant) embryos, and critical pO_2 in hypoxia-treated morphant embryos is expected to be higher than in hypoxia-treated control embryos. Critical pO_2 is the threshold partial pressure of oxygen at which an organism must compensate metabolically for the reduced oxygen availability. To determine if the action of HIF-1α in preconditioning is direct or indirect, it is necessary to determine the extent to which HIF-1 regulates hypoxia-induced genes in zebrafish embryos. Genes that are highly dependent on HIF-1 are expected to show lower expression levels in hypoxia-treated morphant embryos than in hypoxia-treated controls due to lack of HIF-1-regulated induction, while HIF-1-independent genes will show no difference between morphant and control groups. Any genes that demonstrate significant HIF-1 regulation through relative expression experiments would be good candidate genes for knockdown and further preconditioning experiments.

Figure 1: Oxygen-dependent regulation of HIF-1α peptide (adapted from Semenza, 2003). Under normoxic conditions (a) HIF-1α is hydroxylated at various amino acids and binds to the Von Hippel Lindau protein, targeting the HIF-1α subunit for degradation via the ubiquitination pathway. Under hypoxic conditions (b) oxygen is limited and hydroxylation cannot occur. VHL does not signal HIF-1α for ubiquitination, and co-factors bind, leading to transcriptional activation of HIF-1α.

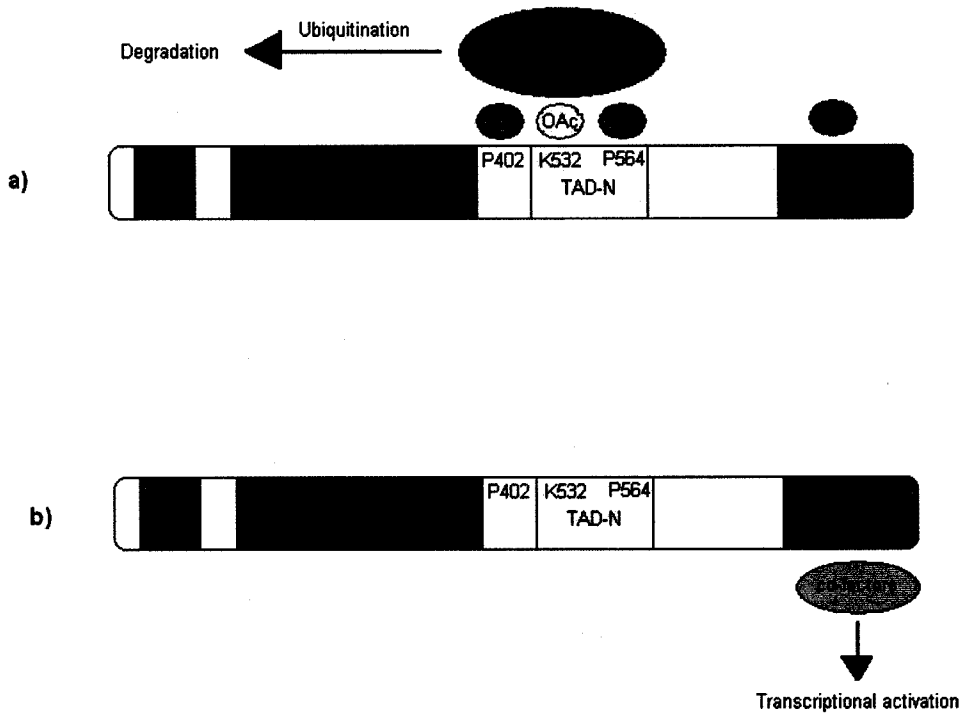
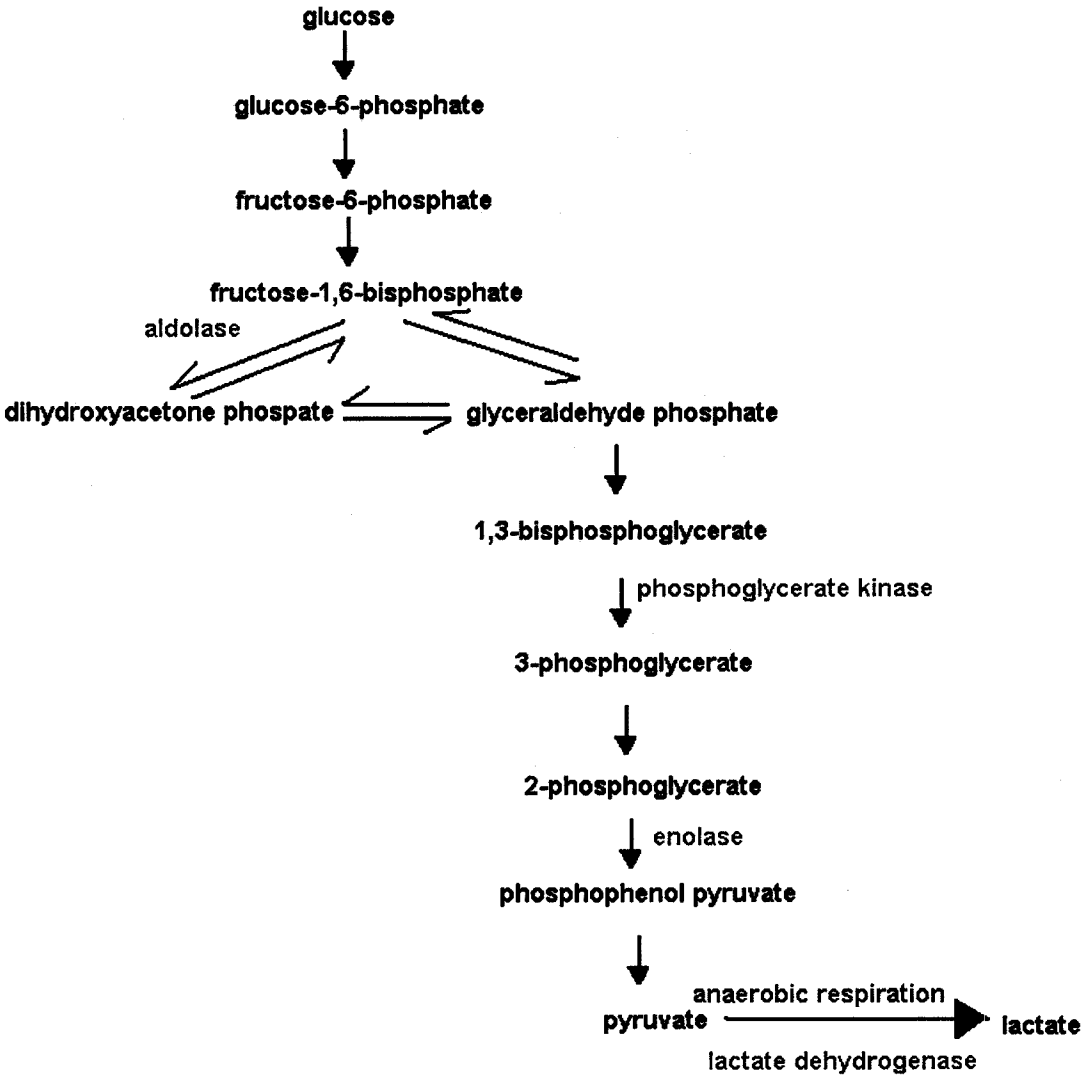


Figure 2: Glycolysis (Webster, 2003)



Materials and Methods

1. Experimental organism

Adult zebrafish were obtained from Mirdo Importations Canada in Montreal. Fish were maintained in a holding facility kept at 28°C. The room in which the fish were kept was on an automatic light cycle of 14:10 hours light:dark. Fish tanks (Aquatic Habitats) were connected to reservoirs keeping water at 28°C. Fish were fed once or twice daily with #1 Crumble (Aquatic Habitats).

Embryo-collection traps were placed inside each tank of 20-30 fish between 2-6pm and left overnight. Fish spawned at 9 AM when the lights came on, and embryos were collected from the traps shortly thereafter, at 0hpf.

2. Microinjections

Microinjections were performed on one- or two-cell-stage embryos (0-1.5 hpf) using a Narishige IM 300 microinjector system connected to nitrogen gas. Embryos were injected in the yolk unless the cell was readily accessible. Needles were made from glass filaments with an outer diameter of 1.0 mm and an inner diameter of 0.50 mm. Needles were pulled using a KOPF needle/pipette puller (model 730).

Morpholino

Morpholino was obtained from GeneTools, and was designed to target the translational start site of HIF-1α. The full HIF-1α sequence was sent to GeneTools, and the

morpholino was custom designed. The sequence of the morpholino was 5'GTGACAACTCCAGTATCCATTCCTG 3'. Stock morpholino mix was diluted 1/2x with 1x injection buffer. Injection solution was dyed with 0.2 µl of 2% phenol red per 20 µl injection solution (final concentration 0.02% phenol red) for visibility.

Over-expression

A mutant form of human HIF-1α was obtained from the lab of Dr. Bill Wilmore at Carleton University. The mutation is in the degradation domain and prevents the degradation of the HIF-1α protein during normoxia. The mutant HIF-1α was amplified using a forward primer containing a T3 promoter. Forward primer sequence: 5'GAATTAACCCTCACTAAAGGGACCCAT 3' (T_m 66.6°C). Reverse primer sequence: 5'TTCCTGCGTTATCCCCTGAT 3' (T_m 57.0°C). PCR product was run on a 0.8% agarose gel and the band was purified using a Sigma Gen-elute kit. The purified PCR product was used as a template to synthesize mRNA.

To synthesize mRNA, purified PCR product was mixed with 0.5 mM rNTP mix, 1x buffer, 10 mM DTT, 50 units RNase inhibitor and 40 units T3 RNA polymerase. The reaction was incubated at 37°C for 2 hours and then stopped by adding 200mM EDTA and 400mM LiCl. mRNA was precipitated overnight in 99% EtOH at -80°C and then resuspended in 50 µl DEPC-treated water. mRNA was dyed with 2% phenol red for a final concentration of 0.02% phenol red, and injected into the yolk of one-or two-cell-stage embryos.

3. Respirometry

Embryo collection and treatment

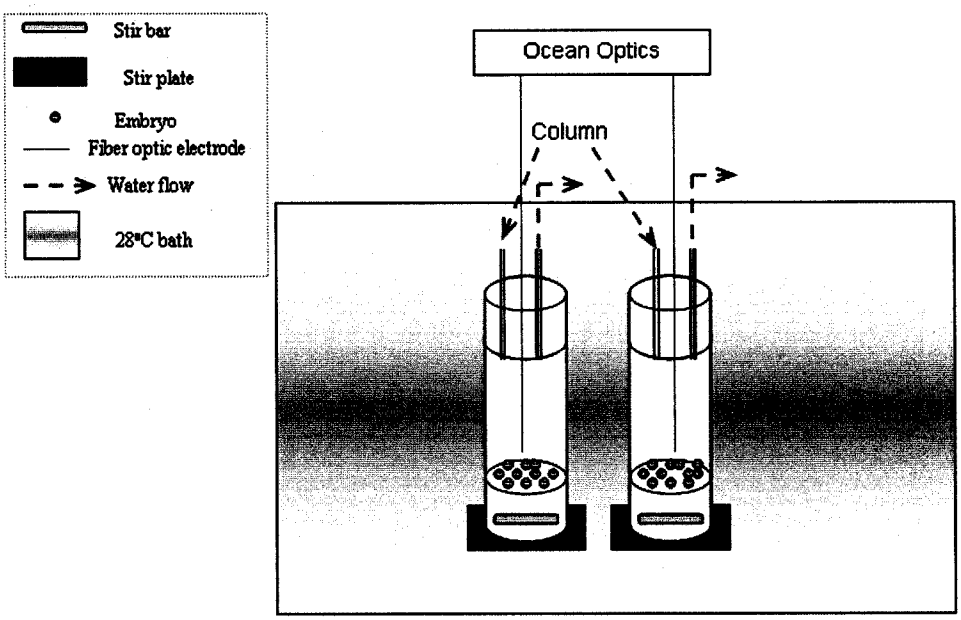
Embryos were collected immediately following fertilization, and injected (control embryos were injected with injection buffer). Following injection, the embryos were incubated for 8 hours at 28°C and then put into hypoxia (35 Torr) overnight (approximately 16 hours).

Respirometer set-up

A closed-system dual respirometer was purchased from Loligo Systems ApS in Denmark. The system comprises a thermo- regulated water bath (28°C) and two glass chambers (**fig. 3**). Chambers were submerged in the water bath and were individually connected to an external water source. Each chamber is 3 mL in volume, and contains a stir bar, which is separated from the embryos by a screen. Forty embryos were inserted into each chamber and allowed to acclimatize for one hour prior to experimentation. Fiber optic oxygen electrodes (Ocean Optics) were calibrated immediately prior to each experiment using 2% sodium sulfite solution as the lower end (0 Torr), and air-saturated water as the upper end (153 Torr). The upper end was calibrated in the respirometry chamber immediately preceding an experimental run. Fiber optic oxygen electrodes (Ocean Optics FOXY AL-300) were used to monitor oxygen partial pressure around the embryos for the duration of the experiment. The water occupied by the embryos was stirred constantly. Electrode readings were allowed to stabilize for 10 minutes after their insertion into the chambers, and the fresh water supply was then cut off from the embryos. Embryos were allowed to consume oxygen until the PO_2 in the chamber fell

below 10 Torr. The decrease in PO_2 was recorded in a log file using Ocean Optics acquisition software.

Figure 3: Closed system dual respirometer



Analysis of respirometry results

Respirometry results were analyzed according to Barrionuevo & Burggren (1999). Ocean Optics text files were exported to Excel and the data were divided into 8 intervals of 15 Torr (130, 115, 100, 85, 70, 55, 40, 25 Torr). Each interval was plotted separately in SigmaPlot as PO_2 versus time elapsed (**fig. 4**). The slope of the eight best-fit lines were calculated and plotted as slope (signifying O_2 consumption rate) versus starting PO_2. From the resulting graph, a critical PO_2 (p_{crit}) was determined using two least-square linear regressions (**fig. 5**). The critical PO_2 is the point at which embryos can no longer sustain their metabolic rate, and oxygen consumption rates therefore decrease.

Statistics

A two-tailed T-test was performed to determine if the Pcrit of experimental groups was significantly different than that of control groups. Significance was accepted at $P<0.05$.

Figure 4: Partial pressure of oxygen (pO2) as a function of time as zebrafish embryos consume oxygen. Representation of full respirometry run (Nov. 27/05, hypoxic group).

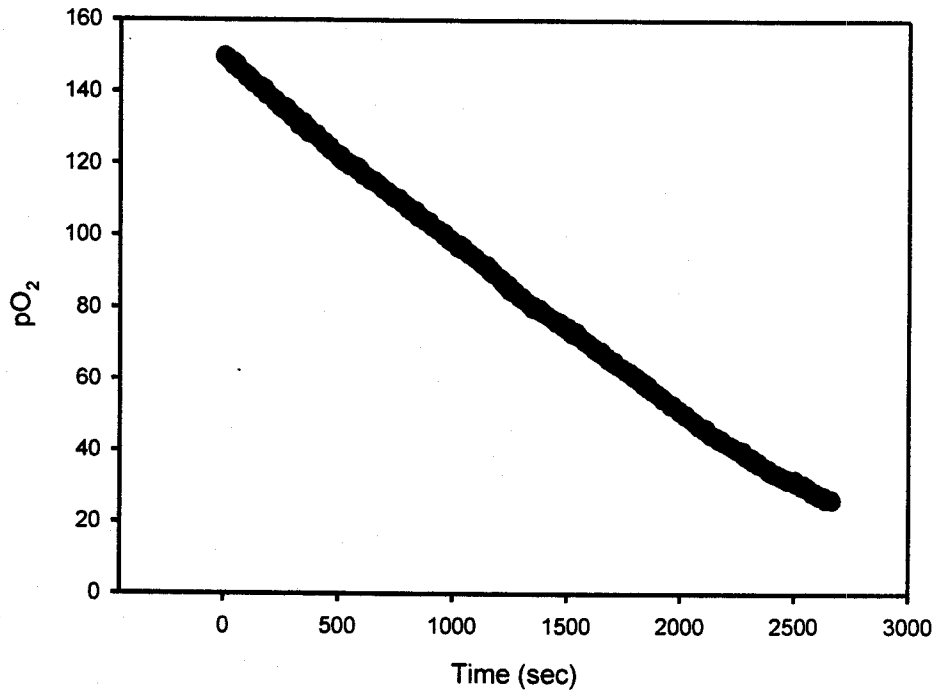
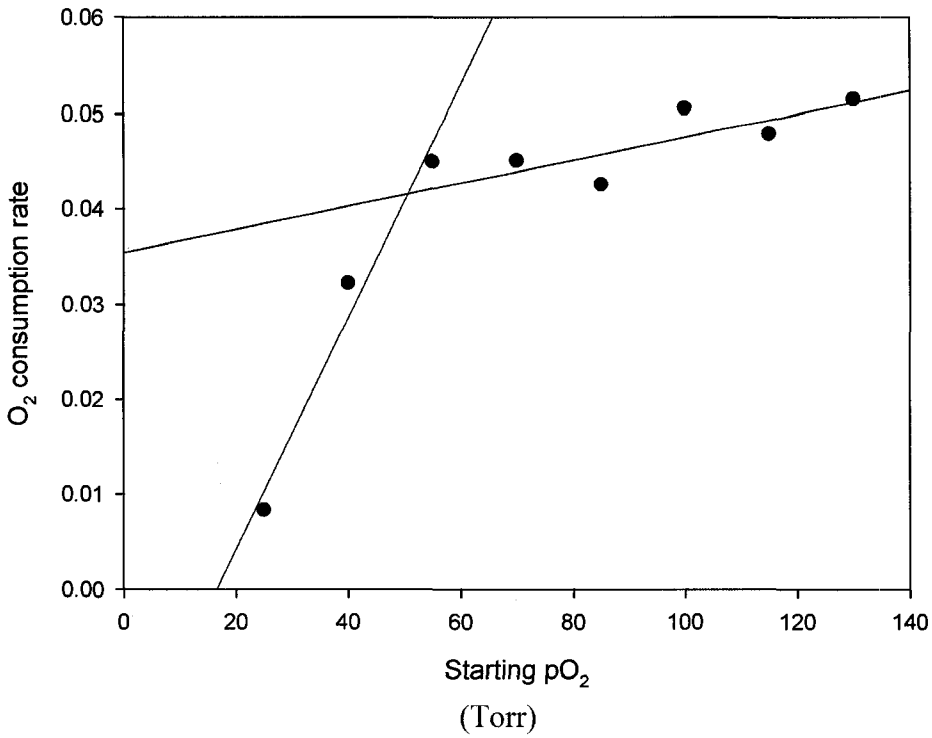


Figure 5: Linear regression, rate of oxygen consumption by 24hpf zebrafish embryos as a function of starting pO_2. Representation of critical pO_2 calculation, (Nov. 27/05, hypoxic group).



4. RT-PCR

Sample collection and treatment

i) Time-course

Embryos were incubated at 28°C for 24 hours and then subjected to hypoxia (35 Torr) or normoxia for various durations: 1, 2, 4, 6, 8 or 24 hours.

ii) Morphants

Embryos were collected immediately following fertilization and micro-injected with antisense morpholino designed against the translational start site of HIF-1α. Control embryos were injected with injection buffer. Injections were done at the 1-2 cell stage (0-1.5 hpf). Following injections, both sets of embryos were incubated at 28°C for 24 hours and then subjected to hypoxia (35 Torr) for 4 hours.

iii) Over-expression

Embryos were collected immediately following fertilization and micro-injected with human HIF-1α RNA with a mutation in the degradation domain to prevent HIF-1α from being degraded during normoxia. Control embryos were injected with injection buffer. Injections were done at the 1-2 cell stage (0-1.5 hpf). Following injections, both sets of embryos were incubated at 28°C for 24 hours and then controls were subjected to hypoxia (35 Torr) for 4 hours, while over-expressing embryos remained in normoxia.

RNA extraction

RNA was extracted using Trizol (Invitrogen) and chloroform, immediately following treatment, and quality and quantity were assessed through spectrophotometry and formaldehyde gel electrophoresis respectively.

cDNA synthesis

The total RNA was used as a template in a cDNA synthesis. Approximately 5 μg of RNA was mixed with 250 ng random primer, 0.5 mM dNTP mix, 1x buffer, 10 mM DTT, 40 units RNase inhibitor and 200 units M-MLV reverse transcriptase. The reaction was incubated at 37°C for one hour and then stopped by heating at 70°C for 15 minutes. The quality of cDNA was assessed by PCR with β-actin primers. β-actin primer sequences were as follows: Forward primer 5'AGAAGATCTGGCATCACACC3' (Tm 55.5°C). Reverse primer 5'TCCATACCCAAGAAGGATGG3' (Tm 54.8°C).

Primers

RT-PCR was performed on 9 selected HIF-1 target genes (**table 1**). Genes were selected based on literature. Primers were designed to amplify a 150-200 bp region of the selected gene, and to have an annealing temperature of approximately 58°C. β-actin was used as a control gene.

Table 1: Primer sequences for RT-PCR

Gene	Primer sequence (F,R)	Tm °C	Product size (bp)	Accession #
β-actin	5′ACTGGTATTGTGATGGACTCTGGTG3′	58	144	NM131031
	5′ACGCTCGGTCAGGATCTTCAT3′	58		
Aldolase	5′ TGATGGTGACCACGACCTTA3′	63	119	NM213215
	5′ TGTTGGGTTTGAGCAGAGTG3′	63		
Transferrin	5′GAACCCGTCAGCACCTACAAGTCA3′	59	182	BC054944
	5′GGCCACCATCAACTGCTAAGAAAT3′	57		
IGFBP-1	5′TACGCAAGACACTGGAGGAACAGG3′	58	205	NM173283
	5′CAGGATGACACACACCAACACTTC3′	60		
IGFBP-2	5′ACCTGTCCGAGTGCGCTTCTTA3′	58	105	NM131458
	5′CAGCAACAAGCCGCAACTCA3′	57		
VEGF	5′ACAGTCACGGAAATAAGACGAGGA3′	56	182	NM131408
	5′TTCGTCCACTTCCAAGCGCA3′	59		
Phosphoglycerate Kinase A	5′GACGTGAAAGGAAAGCGGGT3′	56	148	NM213387
	5′TCATCAGGGCCACAGCTTTG3′	56		
Lactate Dehydrogenase A	5′TGTGAATGTGGCTGGGGTGT3′	56	177	NM131246
	5′CTTTCACACAGGTCAGCTACAGACA3′	56		
Prolyl-4-Hydroxylase A	5′AGGATGAGAAGGGAGCAGCCAA3′	59	121	AY193828
	5′TCCACCGTCAGCAGAGCATT3′	56		
Enolase	5′CGGCAGGATGTCTGTTGTAAGC3′	56	142	BC072713
	5′CTCGTAGATGCCTGTGGATGCT3′	56		
Erythropoietin	5′CGTCCTCGACCATTTCATTAAGGA3′	56	166	DQ278896
	5′ TGACTGGACCTCCTGAGCTTG3′	58		

Standard curves

Standard curves were produced for each primer set to assess the efficiency of the reactions. Zebrafish 24 hpf cDNA was used as a template, and serially diluted over eight concentrations ranging from 1x to 10^{-7} x .

Reactions

RT-PCR reactions of 20 µl were set up using a Qiagen SYBR-Green kit. Master mix included in the kit contains HotStart Taq DNA Polymerase, QuantiTect SYBR Green PCR buffer, SYBR Green 1, ROX (passive reference dye) and 5 mM $MgCl_2$. Reactions consisted of 10 µl master mix, 0.5 µl cDNA and 1 µl of each primer. Reactions were run in a Stratagene MX4000 machine for 40 cycles with an annealing temperature of 58°C.

Analysis

CT (threshold cycle) values were entered into an excel worksheet and values for ΔCT were calculated as $CT_{(gene)}$-$CT_{(actin)}$. ΔΔCT values were calculated for each time-point by comparing the experimental samples to the corresponding control samples. ΔΔCT was calculated as $\Delta CT_{(treated)}$-$\Delta CT_{(control)}$. Efficiencies were calculated by the formula $E=10^{(-1/slope)}$ where the slope is that of the standard curve (Pfaffl, 2001). The efficiency of each primer set was taken into account by applying a mathematical equation suggested by Pfaffl et. al (2001). For experimental-control pairs, average relative mRNA expression was calculated and graphed using SigmaPlot.

Statistics

One-tailed T-tests were performed to determine if relative mRNA expression was significantly different than 1.0. Significance was accepted at $P<0.05$.

5. Western blots

Embryo collection and treatment

Embryos were collected immediately following fertilization, and injected with either morpholino or mutant HIF-1 mRNA (control embryos were injected with injection buffer). Following injection, the embryos were incubated for 8 hours at 28°C and then put into hypoxia (35 Torr) overnight (approximately 16 hours).

Protein extraction

Immediately following hypoxic exposure, oocyte lysis buffer was added to embryos at a concentration of 10 µl of buffer per embryo. Oocyte lysis buffer contains 10 mM phosphate buffer, 150 mM NaCl, 1% Triton and protease inhibitors (200 µM Na orthovandate, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin). Embryos were homogenized by passage through a 22 gauge needle and microfuged for 10 minutes at 4°C. Protein was stored at –20°C.

Protein quantification

Protein was quantified colorimetrically using BC solution (1 ml bicinchoninic acid and 20 µl Copper (II) sulfate). Bovine Serum Albumin was used as standard, at concentrations of 100, 50, 25, 12.5 and 6 mg/ml. 2 µl protein or standard was added to

200 µl BC solution and incubated at 37°C for 1-2 hours and then read by a plate reader (Molecular Devices SpectraMax 190). Values for BSA standards were plotted to create a linear regression to which protein samples were compared for quantification.

Cell lysate preclearing

50 µl Protein agarose A beads slurry (Sigma) was washed two times in 500 µl cold (4°C) oocyte lysis buffer (see *protein extraction* for contents). 500 µl embryo lysate was added to 50 µl washed beads slurry and incubated on ice for 60 minutes. Beads slurry and lysate were centrifuged for 10 minutes at 10000g at 4°C and the supernatant was transferred to a clean 1.5ml tube.

Immunoprecipitation

Zebrafish HIF-1α antibody was added to precleared cell lysate plus beads slurry at a concentration of 1:250. Antibody plus cell lysate was incubated at 4°C for 60 minutes. An additional 50 µl washed protein agarose A beads slurry was added to antibody plus cell lysate and incubated for an additional 60 minutes at 4°C with shaking. Lysate plus beads slurry was centrifuged at 10000g at 4°C for 30 seconds and the supernatant was discarded. Beads were washed 5 times with 500 µl oocyte lysis buffer and then resuspended in 50 ml of 2x protein loading buffer (25% buffer C, 20% glycerol, 4% SDS, 0.005% bromophenol blue, 10% beta-mercaptoethanol). Sample was heated to 100°C for 10 minutes and then centrifuged at 10000g for 5 minutes. The supernatant was then used to run SDS-PAGE and Western blot.

SDS-PAGE

Acrylamide gels were poured one day prior to running and kept at 4°C overnight. 12% resolving gel contained 12% acrylamide/Bis solution (BioRad, Hercules CA), 2.5% Buffer B (1.5M Tris pH 8.8, 0.4% SDS), 0.2% TEMED and 0.005% Ammonium persulfate. 4% stacking gel contained 4% acrylamide/Bis solution, 2.5% Buffer C (0.5M Tris pH 8.8, 0.4% SDS), 0.2% TEMED and 0.005% ammonium persultafe.

Protein was diluted with sterile water to a working concentration of 10 mg/ml. 100 µg (10 µl) was mixed with 10 µl of 2x sample buffer (25% buffer C, 20% glycerol, 4% SDS, 0.005% bromophenol blue, 10% beta-mercaptoethanol) and placed in boiling water for 5 minutes immediately prior to loading 20 µl samples on acrylamide gel.

Gels were run at 4°C in a BioRad system with Tris-glycine buffer. Buffer contained 0.302% Tris base, 1.88% glycine and 0.1% SDS.

Gel transfer

Gel was transferred overnight at 180 mAmps at 4°C in a BioRad wet transfer system, using nitrocellulose membrane from BioRad. Transfer buffer contained 0.58% Tris base,0.29% glycine, 20% methanol and 0.038% SDS.

Gel staining (Coomassie blue)

To verify complete transfer, the gel was stained for 1 hour following transfer with Coomasie blue stain. Stain contains 0.25% Coomassie blue in 50% methanol and 10% acetic acid. The gel was destained overnight. Destain contains 50% methanol and 10% acetic acid.

Antibodies

HIF-1α primary antibodies were applied at a concentration of 1/500 overnight at 4°C or for 1 hour at 37°C with constant shaking. HIF-1α antibodies used were developed against zebrafish, common carp, rainbow trout and human HIF-1α (Santa Cruz; one targeted against the N-terminus of human HIF-1α and one against the C-terminus). Alternate antibodies against human HIF-1α were used in the lab of Dr. Bill Willmore at Carleton University (Novus; targeted against amino acids 432-528 of human HIF-1α) with human cell lysates as positive control.

Secondary antibodies were applied at a concentration of 1/10000 for one hour at room temperature.

Primary and secondary antibodies were diluted in 5% milk in PBST. PBST contains 27.4mM NaCl, 0.54mM KCl, 2mM Na_2HPO_4, 0.4mM KH_2PO_4 and 10% TWEEN.

Gel staining (Silver Nitrate)

Gels were stained with silver stain and potential HIF-1α bands were excised and sent for sequencing. Gels were put into fixing solution (30% ethanol, 5% glacial acetic acid) for one hour and then rinsed with deionized water for 15 minutes. Gels were then put into sensitizing solution (0.02% sodium thiosulphate, pentahydrate) for 1 minute, rinsed and put into silver solution (0.2% silver nitrate) for 20 minutes. Following rinsing, gels were developed for 5-10 minutes (4% potassium carbonate, anhydrous; 0.011% sodium thiosulphate, pentahydrate; 0.025% formaldehyde). Gel was immediately put in stopping solution (4% Tris base, 2% glacial acetic acid). The desired bands were cut out of the gel

and frozen at -20°C until sent for sequencing. Bands were sent to the Ontario Genomics Innovation Centre proteomics services (Ottawa Health Research Institute) for sequencing.

6. General Morphology

Embryos were collected immediately following fertilization, and injected with either morpholino or mutant HIF-1 mRNA (control embryos were injected with injection buffer). Following injection, the embryos were incubated for 8 hours at 28°C and then put into hypoxia (35 Torr) or normoxia overnight (approximately 16 hours). Embryos were photographed at 24, 48, 72 and 96hpf. Stages following hatching (approximately 48hpf) were anesthetized with 4% paraformaldehyde in 1xPBS prior to photographing. 1xPBS contains 27.4mM NaCl, 0.54mM KCl, 2mM Na_2HPO_4 and 0.4mM KH_2PO_4.

Results

1. Determination of Critical PO_2

Critical PO_2 (Pcrit) was determined for control embryos and embryos exposed to 16 h of hypoxia (35 Torr) between 8 and 24 hpf. The embryos pre-exposed to hypoxia exhibited a significant lowering of Pcrit of approximately 10 Torr (**fig. 6**).

The treatment of embryos with HIF-1α antisense oligonucleotide morpholino did not alter the p_{Crit} in the embryos pre-exposed to hypoxia (**fig. 7**).

Figure 6: Critical pO_2 of 24hpf hypoxic embryos after 16 hours treatment with hypoxia (35 Torr), and control embryos as determined by closed system respirometry experiments. Normoxic N=34. Hypoxic N=24.

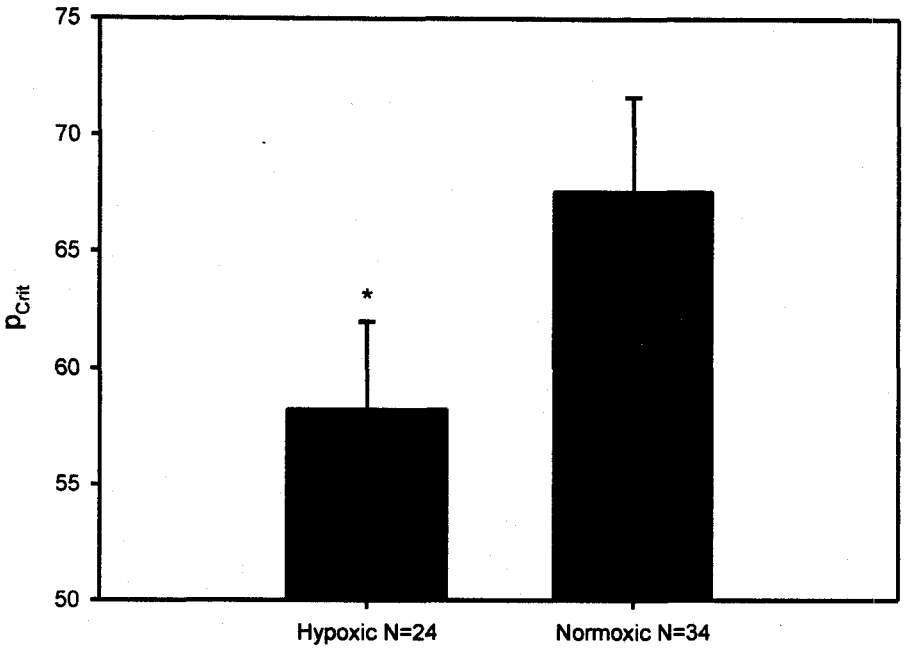
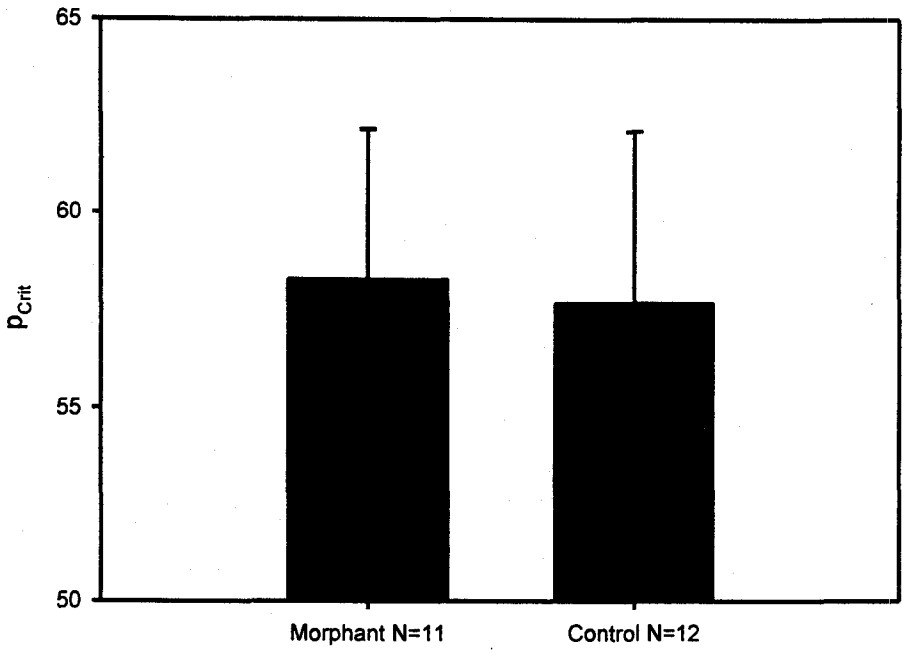


Figure 7: Critical pO_2 of 24hpf morphant embryos and control embryos after 16 hours treatment with hypoxia (35 Torr), as determined by closed system respirometry experiments. Morphant N=11. Control N=12.



2. mRNA expression during hypoxia

Following 24 h incubation at 28°C, embryos were subjected to 1, 2, 4, 6, 8 or 24 h of hypoxia (35 Torr) or normoxia (155 Torr). Real-time RT PCR was performed to determine expression of ten genes in treated embryos relative to controls. While there were trends indicating the induction of all ten genes following hypoxic exposure, none of the inductions were statistically significant (**figs. 8-17**).

Based on the time-course data, 4 h was selected as an appropriate duration of hypoxia to subject morphant and control embryos. Of the ten genes monitored, only one demonstrated a significant induction in control embryos where there was none in morphant embryos. IGFBP-1 expression had a 2-fold increase in control hypoxic embryos compared to morphant hypoxic embryos, and EPO had a decrease, whereas all other genes had no significant difference between control and morphant expression levels (**fig.18**).

Figure 8: Real-time RT-PCR results: mRNA expression of aldolase A in hypoxic zebrafish embryos relative to normoxic zebrafish embryos (+SE). Embryos were grown to 24 hpf and then exposed to varying durations of hypoxia (35 Torr): 1, 2, 4, 6, 8 or 24 hours. Relative mRNA expression was calculated using the formula
$E_{aldolase}$^($H_{aldolase}$-$N_{aldolase}$)/ E_{actin}^(H_{actin}-N_{actin})
where $E_{aldolase}$=1.972, E_{actin}=1.637, H=hypoxic ct value, N=average normoxic ct value.

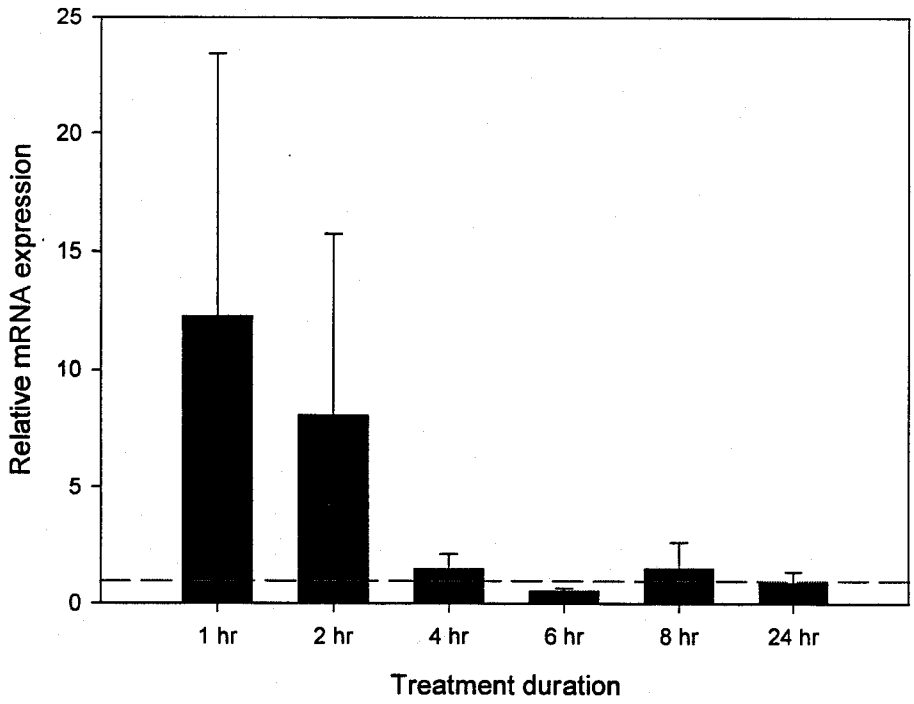


Figure 9: Real-time RT-PCR results: mRNA expression of transferrin in hypoxic zebrafish embryos relative to normoxic zebrafish embryos (+SE). Embryos were grown to 24 hpf and then exposed to varying durations of hypoxia (35 Torr): 1, 2, 4, 6, 8 or 24 hours. Relative mRNA expression was calculated using the formula $E_{transferrin}^{(H_{transferrin}-N_{transferrin})} / E_{actin}^{(H_{actin}-N_{actin})}$
where $E_{transferrin}$=2.186, E_{actin}=1.637, H=hypoxic ct value, N=average normoxic ct value. n=4.

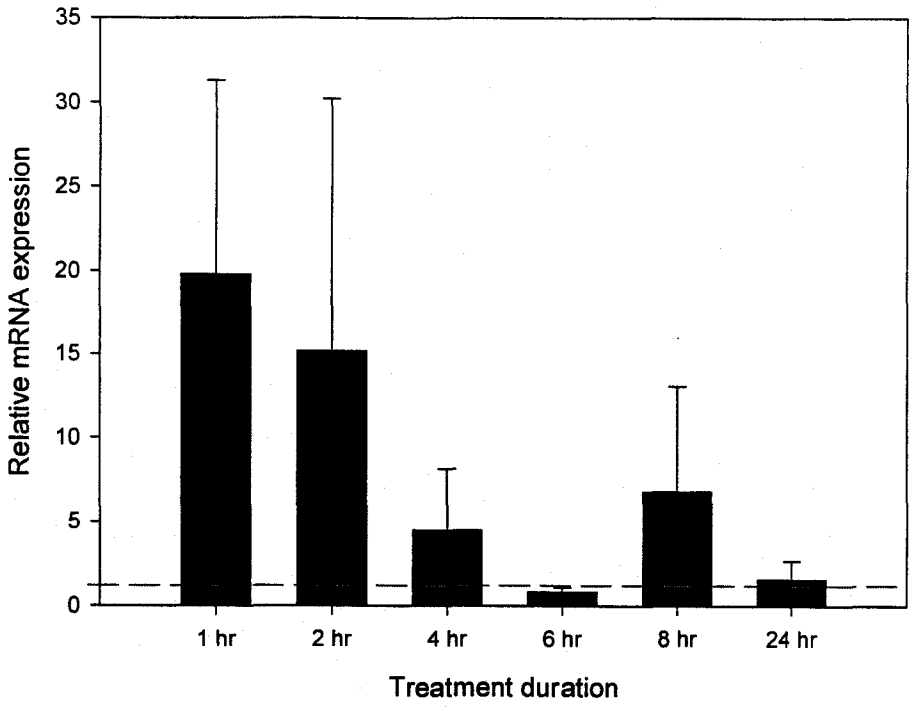


Figure 10: Real-time RT-PCR results: mRNA expression of insulin-like growth factor-2 (IGFBP-2) in hypoxic zebrafish embryos relative to normoxic zebrafish embryos (+SE). Embryos were grown to 24 hpf and then exposed to varying durations of hypoxia (35 Torr): 1, 2, 4, 6, 8 or 24 hours. Relative mRNA expression was calculated using the formula

$2^{(H_{IGFBP-2}-N_{IGFBP-2})}/ E_{actin}^{(H_{actin}-N_{actin})}$

where E_{actin}=1.637, H=hypoxic ct value, N=average normoxic ct value.

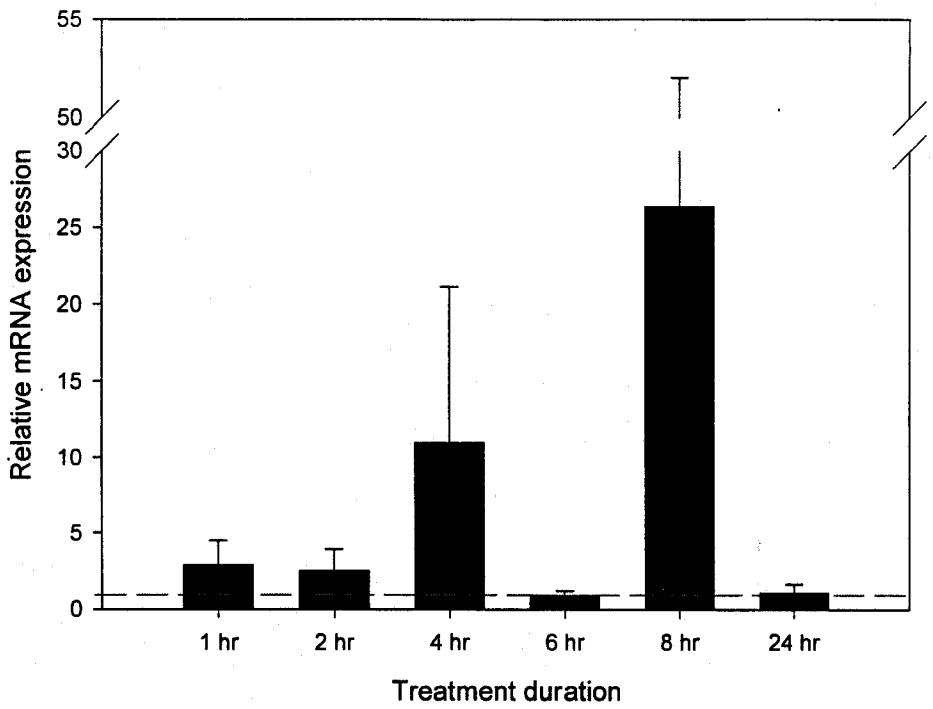


Figure 11: Real-time RT-PCR results: mRNA expression of vascular endothelial growth factor (VEGF) in hypoxic zebrafish embryos relative to normoxic zebrafish embryos (+SE). Embryos were grown to 24 hpf and then exposed to varying durations of hypoxia (35 Torr): 1, 2, 4, 6, 8 or 24 hours. Relative mRNA expression was calculated using the formula
$E_{VEGF}\text{^}(H_{VEGF}\text{-}N_{VEGF})/\ E_{actin}\text{^}(H_{actin}\text{-}N_{actin})$
where E_{VEGF}=2.121, E_{actin}=1.637, H=hypoxic ct value, N=average normoxic ct value. n=4.

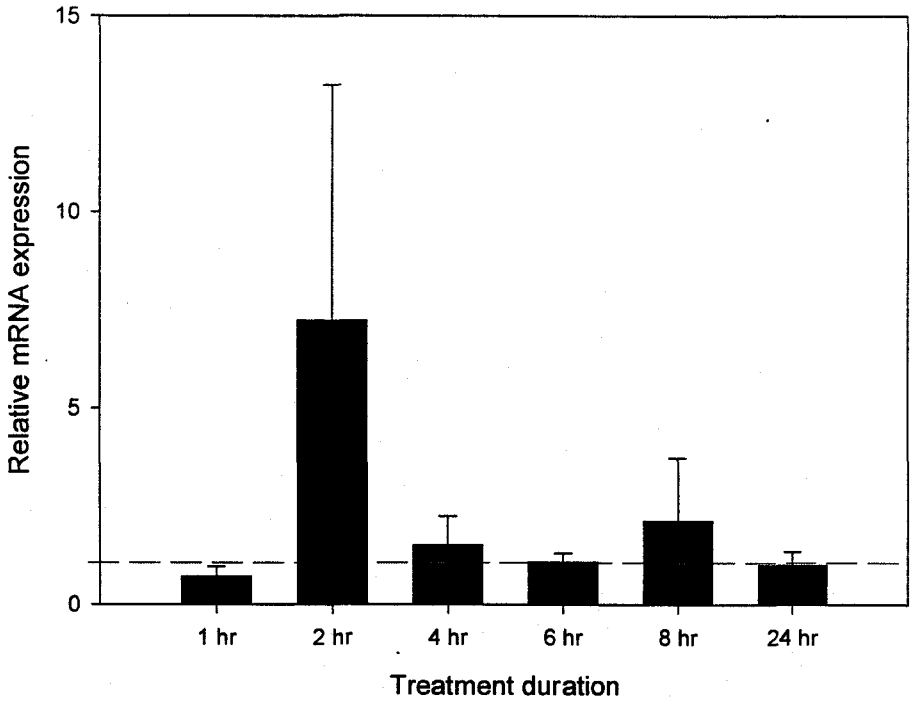


Figure 12: Real-time RT-PCR results: mRNA expression of phosphyglycerate kinase A (PGK) in hypoxic zebrafish embryos relative to normoxic zebrafish embryos (+SE). Embryos were grown to 24 hpf and then exposed to varying durations of hypoxia (35 Torr): 1, 2, 4, 6, 8 or 24 hours. Relative mRNA expression was calculated using the formula
$E_{PGK}\text{^}(H_{PGK}\text{-}N_{PGK})/\ E_{actin}\text{^}(H_{actin}\text{-}N_{actin})$
where E_{PGK}=2.837, E_{actin}=1.637, H=hypoxic ct value, N=average normoxic ct value. n=4.

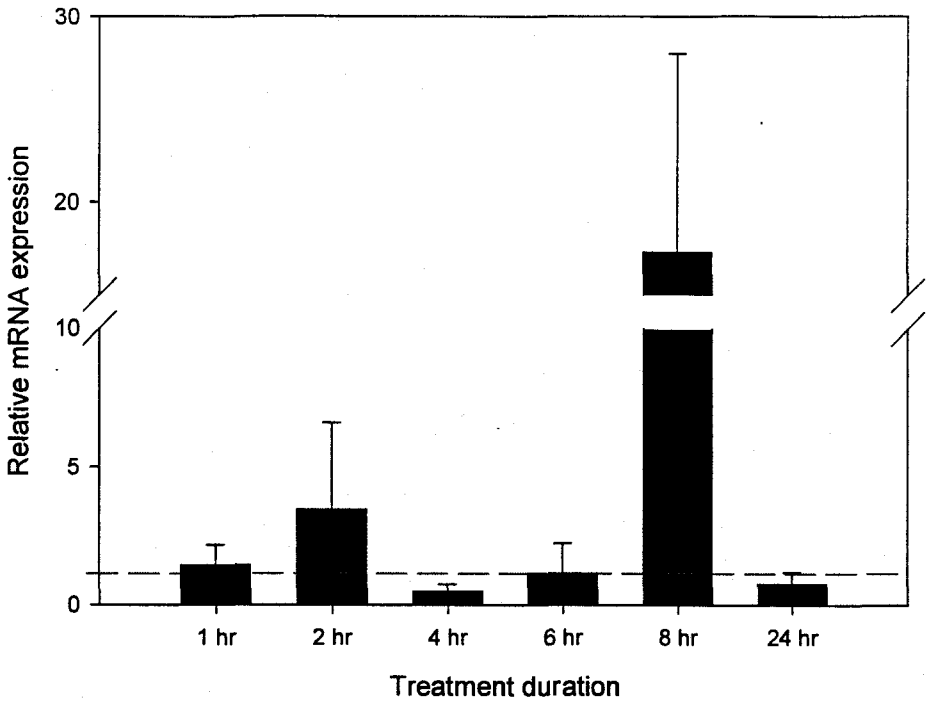


Figure 13: Real-time RT-PCR results: mRNA expression of lactate dehydrogenase A (LDH) in hypoxic zebrafish embryos relative to normoxic zebrafish embryos (+SE). Embryos were grown to 24 hpf and then exposed to varying durations of hypoxia (35 Torr): 1, 2, 4, 6, 8 or 24 hours. Relative mRNA expression was calculated using the formula

$E_{LDH}\text{^}(H_{LDH}\text{-}N_{LDH})/\ E_{actin}\text{^}(H_{actin}\text{-}N_{actin})$

where E_{LDH}=2.318, E_{actin}=1.637, H=hypoxic ct value, N=average normoxic ct value. n=4.

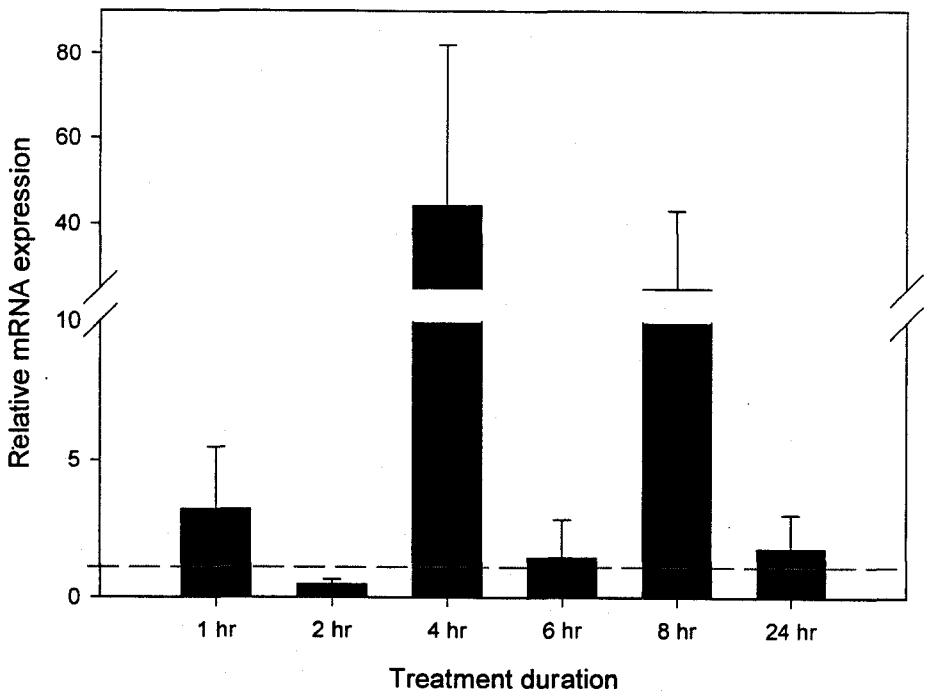


Figure 14: Real-time RT-PCR results: mRNA expression of prolyl-4-hydroxlase A (PHD) in hypoxic zebrafish embryos relative to normoxic zebrafish embryos (+SE). Embryos were grown to 24 hpf and then exposed to varying durations of hypoxia (35 Torr): 1, 2, 4, 6, 8 or 24 hours. Relative mRNA expression was calculated using the formula
E_{PHD}^(H_{PHD}-N_{PHD})/ E_{actin}^(H_{actin}-N_{actin})
where E_{PHD}=2.2, E_{actin}=1.637, H=hypoxic ct value, N=average normoxic ct value. n=4.

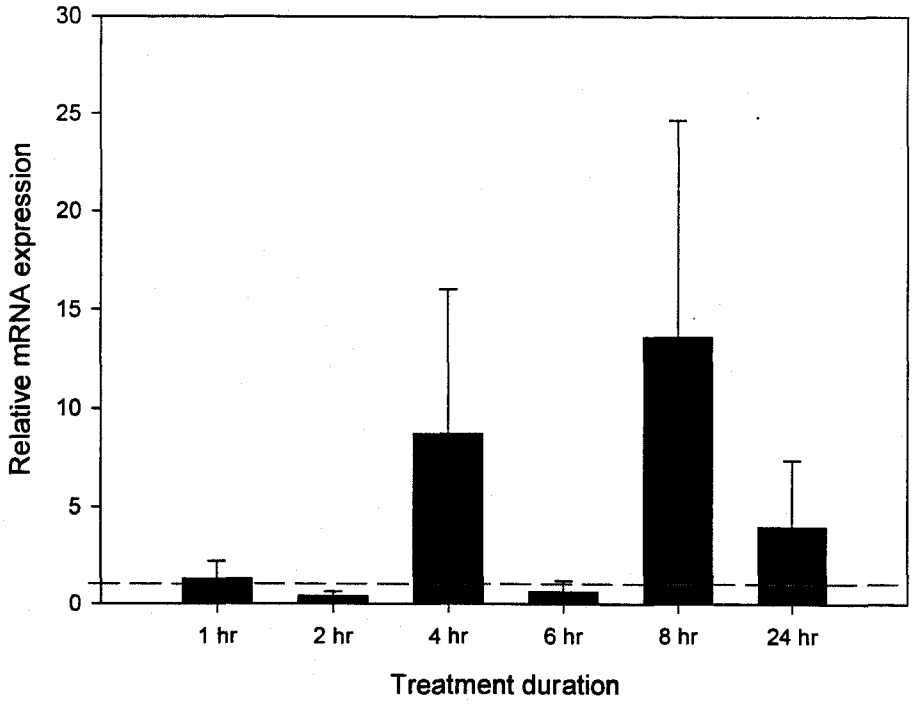


Figure 15: Real-time RT-PCR results: mRNA expression of enolase in hypoxic zebrafish embryos relative to normoxic zebrafish embryos (+SE). Embryos were grown to 24 hpf and then exposed to varying durations of hypoxia (35 Torr): 1, 2, 4, 6, 8 or 24 hours. Relative mRNA expression was calculated using the formula
$E_{enolase}$^($H_{enolase}$-$N_{enolase}$)/ E_{actin}^(H_{actin}-N_{actin})
where $E_{enolase}$=2.492, E_{actin}=1.637, H=hypoxic ct value, N=average normoxic ct value.

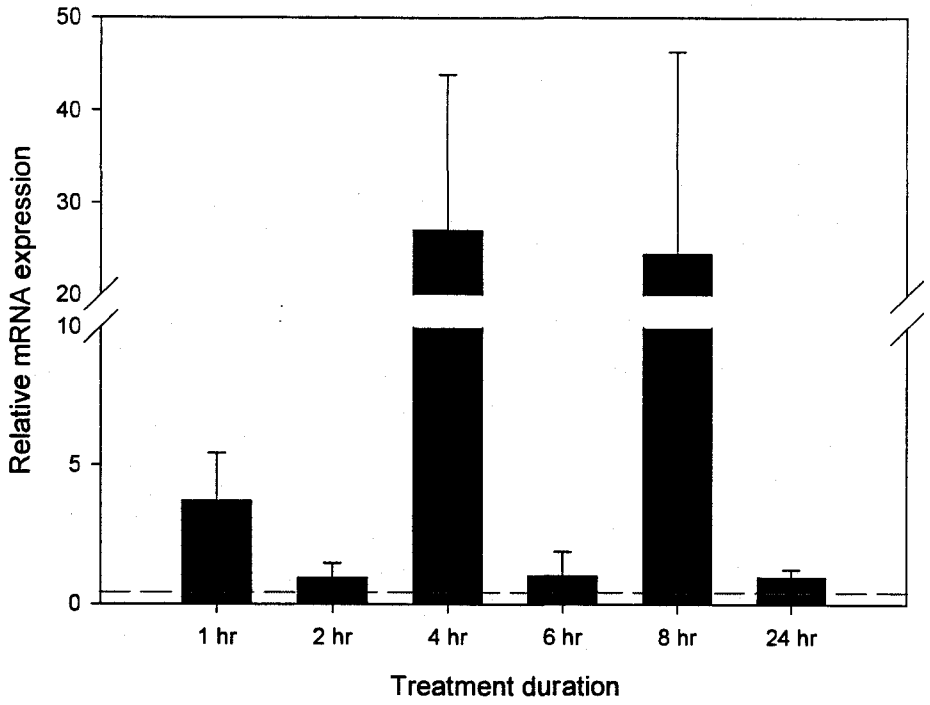


Figure 16: Real-time RT-PCR results: mRNA expression of insulin-like growth factor-1 (IGFBP-1) in hypoxic zebrafish embryos relative to normoxic zebrafish embryos (+SE). Embryos were grown to 24 hpf and then exposed to varying durations of hypoxia (35 Torr): 1, 2, 4, 6, 8 or 24 hours. Relative mRNA expression was calculated using the formula
$2^{(H_{IGFBP\text{-}1}-N_{IGFBP\text{-}1})}/ E_{actin}^{(H_{actin}-N_{actin})}$
where E_{actin}=1.637, H=hypoxic ct value, N=average normoxic ct value.

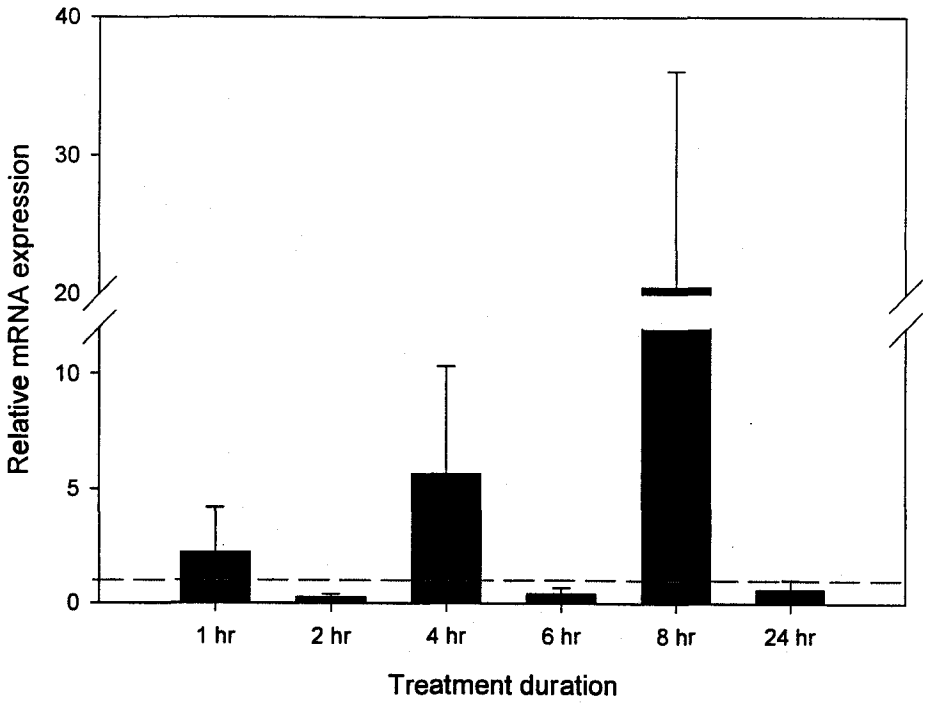


Figure 17: Real-time RT-PCR results: mRNA expression of erythropoietin (EPO) in hypoxic zebrafish embryos relative to normoxic zebrafish embryos (+SE). Embryos were grown to 24 hpf and then exposed to varying durations of hypoxia (35 Torr): 1, 2, 4, 6, 8 or 24 hours. Relative mRNA expression was calculated using the formula $E_{EPO}\text{^}(H_{EPO}\text{-}N_{EPO})/ E_{actin}\text{^}(H_{actin}\text{-}N_{actin})$
where E_{EPO}=1.805, E_{actin}=1.637, H=hypoxic ct value, N=average normoxic ct value.

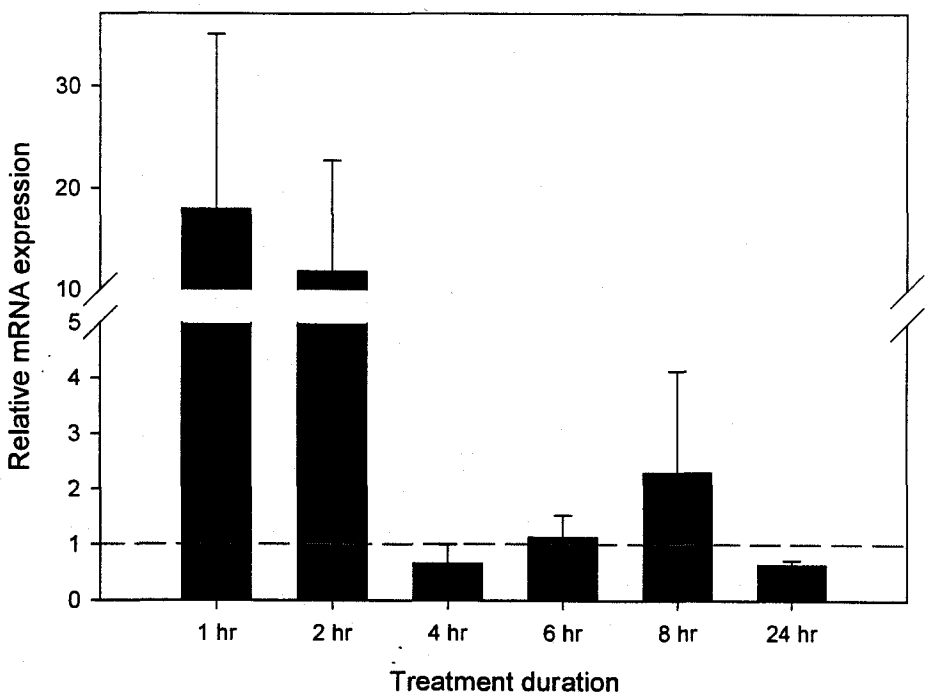
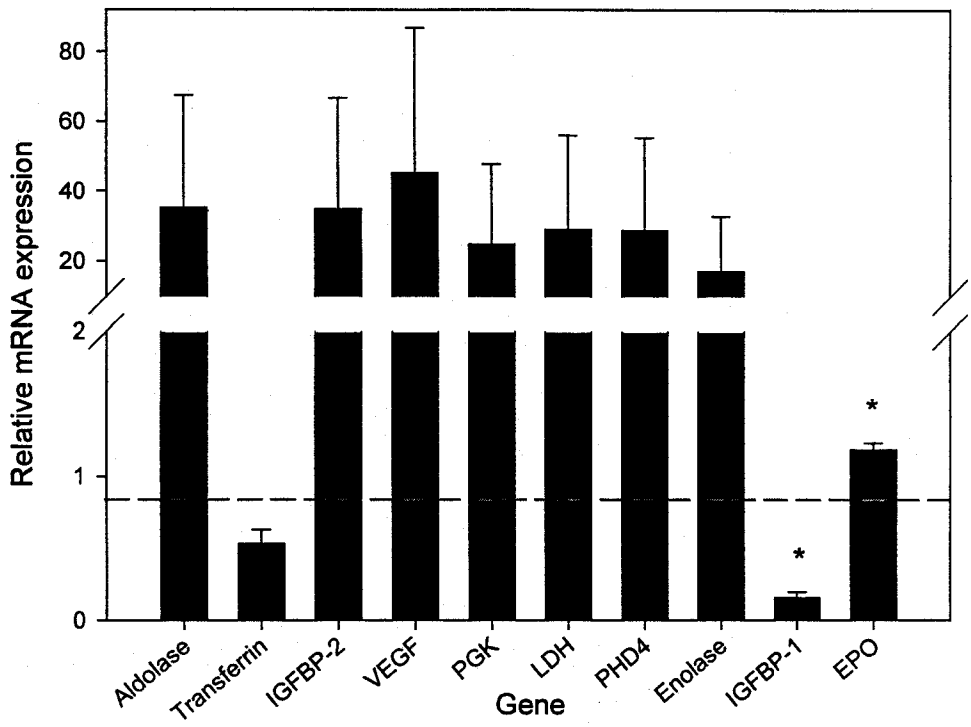


Figure 18: Real-time RT-PCR results: mRNA expression of ten hypoxia-regulated genes in HIF-1α-morphant zebrafish embryos relative to hypoxic zebrafish embryos (+SE), N=6. Embryos were grown to 24 hpf and then exposed 4hours of hypoxia (35 Torr). Relative mRNA expression was calculated using the formula
$2^{(M_{gene}-C_{gene})}/2^{(M_{actin}-C_{actin})}$
where M= morphant ct value, C=average control ct value.



3. Western Blots

Several HIF-1α antibodies were used in an attempt to determine the protein levels of HIF-1α in normoxic, hypoxic and morphant zebrafish embryos. Antibodies included two targeted against zebrafish HIF-1α, two against human HIF-1α, two against carp HIF-1α and one against rainbow trout HIF-1α. One of the antibodies against zebrafish HIF-1α produced two bands on a western blot that were in the correct size range to be HIF-1α (**fig. 19**). To determine whether or not either of these bands was in fact HIF-1α, the bands were excised from an acrylamide gel, silver stained, and sent for sequencing. Neither of the bands turned out to be HIF-1α, and therefore none of the antibodies tried was able to successfully detect HIF-1α in zebrafish embryos.

Three antibodies were used to determine the protein levels of human HIF-1α in normoxic and hypoxic cell lysates as well as zebrafish embryos over-expressing a mutant form of human HIF-1α. Two antibodies, both obtained from Santa Cruz, were ineffective in detecting HIF-1α in hypoxic samples. An antibody used in the lab of Dr. Bill Wilmore, obtained from Novus, was able to detect HIF-1α in hypoxic human cell lysates, but not in zebrafish embryos treated to over express human HIF-1α (**fig. 20**). These results suggest that the zebrafish embryos were not expressing the human HIF-1α protein.

Figure 19: Western blot showing bands at 85 and 100 kDa in normoxic (N), hypoxic (H) and morphant (M) samples. Bands were excised and sent for sequencing to determine whether or not they were the desired HIF-1α band.

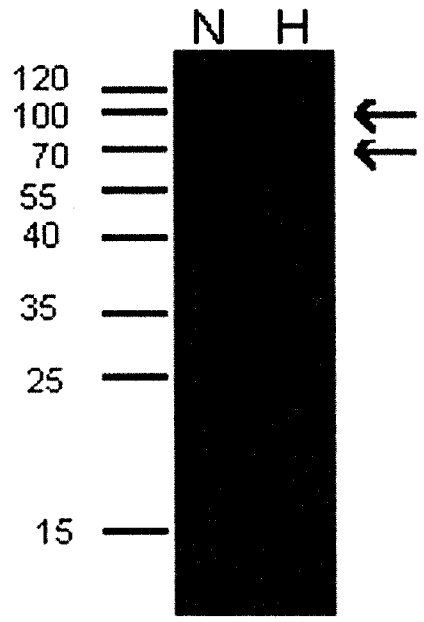
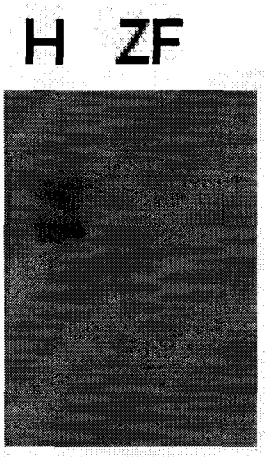


Figure 20: Western blot showing bands at 85 and 100 kDa in hypoxic human cell lysates (H) and no bands in zebrafish cell lysates (ZF)



4. General Morphology

Embryos were observed at various time-points (24, 48 and 72 hpf) after exposure to normoxia (155 Torr) or hypoxia (35 Torr). General morphology was observed in each group at each time-point. Other than a severe developmental delay in hypoxic embryos, embryos appeared normal after hypoxic exposure (not shown).

Embryos were injected with either HIF-1α morpholino or control and observed at various time-points (24, 48 and 72 hpf) after exposure to hypoxia (35 Torr). General morphology was observed in each group at each time-point. There did not appear to be any significant difference in morphant morphology compared to control embryos (not shown).

Discussion

1. Preconditioning:

Through respirometry experiments, it was demonstrated that zebrafish embryos can be preconditioned to hypoxia by exposing them to non-lethal levels of hypoxia (35 Torr) for 16 h immediately prior to the respirometry run. Embryos left overnight in hypoxia had a significantly lower p_{crit} than embryos left in normoxia overnight. This coincides with the findings of Rees et al (2001) who reported that adult zebrafish could be exposed to non-lethal levels of hypoxia, and later be exposed to more severe levels of hypoxia and be more likely to survive.

Morphant embryos, deficient of HIF-1α, were demonstrated to have a p_{crit} similar to the control, preconditioned group. Thus, it would appear that preconditioning was still occurring in the absence of HIF-1α. These findings suggest that, assuming the morphant embryos were indeed HIF-α deficient, HIF-1α does not play a significant role in the preconditioning process or perhaps more precisely that HIF-1α is not an essential requirement for hypoxic preconditioning. Rees et. al (2001) deduced that preconditioning in adult zebrafish depended on several factors, including environmental and genetic factors.

It was impossible to maintain exact conditions of the respirometry experiments over the two years during which experiments were performed, which could have had an impact on the outcomes. Water temperature, external temperature and air pressure, and conditions directly surrounding the experimental apparatus, such as light, noise and movement, may all have played a role in the behavior of the embryos. Another factor to be taken into consideration is the multiple respirometers used for experiments. These experiments

required a lot of troubleshooting. Two home-made set-ups with differing internal volumes (3 and 5 ml), and one commercial set-up were used for preliminary data, although a single, commercial system was used for the morphant experiments. The first experimental set-up had a volume of 5 ml, which proved to be too large for the purposes of these experiments. The change in pO_2 took up to 45 min between each data collection point causing the experiments to take up to 10 h to complete. Ultimately, the volume of the chamber was reduced to 3 ml to speed up the process.

Changes in water temperature caused significant problems in the initial experiments. Because the fiber optic O_2 electrodes are highly sensitive to fluctuations in temperature, it was crucial for water temperature to be constant during an experiment. High demand on the heated water system caused the temperature of thermoregulation water flowing through the respirometry system to drop unexpectedly and drastically, thereby forcing termination of numerous experiments. The temperature of the warm water in the system was changed to 28°C, which was the desired temperature for the experiments, thereby eliminating the need to mix warm and cold water to obtain the correct temperature. The commercial respirometer included a tank of heated water which was kept at 28°C, and the chambers were submerged in this tank to maintain the temperature of the experiment at the ideal temperature for the embryos, thus eliminating problems relating to water temperature. Another way of managing the problem of fluctuating water temperature was to change the method with which experiments were preformed. Originally, each starting pO_2 was measured individually, and the embryos were then deprived of fresh water and oxygen consumption was measured. Following each measurement, embryos were allowed to acclimate to a new starting pO_2, and then once again cut off from fresh water flow and allowed to consume oxygen while the consumption rate was measured. Each

time the water flow was opened and fresh water was allowed to flow into the embryo chamber, there was the possibility of a slight change in the temperature of water from the measurement before. With the new method, the embryos were allowed to acclimate to the chamber one time before the run, and then the water flow was stopped and embryos were allowed to consume all of the oxygen before flow was restored. The resulting measurement of oxygen consumption was then broken into segments of 15 Torr and the p_{Crit} was calculated in the same manner as with the previous method. With this new method, the only water temperature fluctuations to affect the experiment would be that of the thermoregulation water, which was being controlled by the heated tank in which the chambers were submerged.

All of these compounding factors had varying effects on the outcome of the experiments; however these factors would have been present during all experiments. Conditions would not necessarily have been identical from day to day or from experiment to experiment, so although I attempted to always run one treated and one control group side by side, there were always days when one or both of the experiments did not work, and therefore not every control group has a corresponding treatment group and vice versa.

2. Gene expression:

A time course was originally done and analyzed by real time RT-PCR to determine what duration exposure to hypoxia expression of each of the observed genes was most significantly induced. Time points selected were 1, 2, 4, 6, 8 and 24 h of hypoxic or normoxic exposure. The most consistent trend in gene expression patterns following hypoxic exposure occurred in the 4 hour samples. 4 h was therefore selected as the duration of hypoxia treatment for morphant- and control-injected embryos being used for

experiments assessing mRNA levels. The data from these experiments showed that the majority of the observed relative gene expression levels were not significantly different in morphants and controls. IGFBP-1 was the only gene that had lower expression in morphants than controls. Interestingly, EPO was the only gene whose expression was higher in morphant embryos than in controls. EPO expression was expected to decrease in HIF-1α deficient embryos due to the belief that it is induced by hypoxia, presumably through the action of HIF-1α (Krantz, 1991). It could be that zebrafish EPO has an alternate mode of action, or is influenced by additional factors not observed in mammalian systems.

From these data, it was concluded that IGFBP-1 and EPO are the only genes among the ten tested, that are appreciably regulated by HIF-1α during hypoxia in zebrafish embryos. This goes against the literature, which reports a vast number of genes, including several of the genes assessed in this thesis, to be induced in hypoxia, directly through the action of HIF-1α. Indeed, previous studies have demonstrated that several genes, including Ald, PGK and LDH were not induced by hypoxia in samples absent of HIF-1α (Ryan et. al, 1998), and that Ald, PGK, LDH and Enolase were specifically upregulated by HIF-1 (Greijer et. al, 2005). Recently, it was shown that while IGFBP-1 is induced by HIF-1, other IGFBPs, including IGFBP-2, are HIF-1-independent (Kajimura et. al, 2004; Kajimura et. al, 2006). These findings are consistent with the data presented here.

There are a few possible explanations for why the majority of gene expression data in this thesis are not consistent with previous findings. Because prior studies were preformed on mammalian systems (primarily mice), it is possible that zebrafish, and perhaps other fish species, react differently to hypoxia at the genetic level than do

mammals, or that the zebrafish isoforms of these genes are regulated by other factors. Another possible explanation for the discrepancies is the chosen method of gene analysis. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) has become the most commonly used method of characterizing patterns of gene expression. The accuracy of a real time RT-PCR reaction is significantly dependent on the variability between reagents and users, and consequently the technique is not a reliable means of clinical diagnosis Bustin, 2002). It is a very sensitive technique capable of quantifying mRNA levels in tissue samples that contain partially degraded mRNA. However, mispriming and primer dimerization can significantly reduce the sensitivity of the reaction (Bustin, 2002).

Several means of normalization can be used to control error in real time RT-PCR. The most popular method is the use of a reference gene, most frequently a housekeeping gene such as β-actin, glyceraldehydes-3-phosphate (GADPH) or 18S ribosomal RNA (Huggett et. al, 2005). For many years, these genes have been used as references for qualitative or semi-quantitative assays such as Northern blots. These housekeeping genes are known to be expressed at high levels in all cells and were able to serve as a control for the turning off of a gene of interest. For quantitative change measured by real time RT-PCR, the choice of control gene can be problematic as there is evidence that some, if not most of these housekeeping genes are regulated. For example, β-actin is differentially expressed in different samples of human leukemia, (Blomberg et. al, 1987). β-actin, as well as all other housekeeping genes, are only appropriate as reference genes if they are not regulated by the experimental conditions (Bas et. al, 2004; Huggett et. al, 2005), in this case hypoxia. No evidence has been found to show that β-actin is in fact regulated by hypoxia. The importance of validating the chosen reference gene in any experimental

model is widely recognized (Dheda et. al, 2004; Huggett et. al, 2005), as there is evidence that the use of an inappropriate reference gene can severely alter the experimental outcome (Tricarico et. al, 2002; Bas et. al, 2004; Dheda et. al, 2005). The problem with validating reference genes is that a reliable reference gene is required. However, in order to establish a gene's suitability as a reference gene, that is, that it is not regulated, another reference gene is needed. The question arises as to how many reference genes and validations are needed in order to confidently validate the first gene.

The fact that no extensive validation was done, other than searching the literature for possible hypoxic regulation of the reference gene, leads to the possibility that perhaps β-actin was not the best choice of reference. Primers were tested for efficiency by doing standard curves on serially diluted cDNA. Efficiencies were calculated by the formula $E=10^{(-1/slope)}$ (Pfaffl, 2001). The efficiency of each primer set was taken into account by applying a mathematical equation suggested by Pfaffl et. al (2001). The ratio between the relative expression of the target gene in relation to the relative expression of the reference gene, in this case β-actin, can be calculated as:

$$\text{Ratio} = \frac{(E_{target})^{\text{dCT}target\ \text{(control-sample)}}}{(E_{ref})^{\text{dCT}ref\ \text{(control-sample)}}}$$

where E is the efficiency of the primer (Pfaffl, 2001). This is a more accurate method of calculation than the "delta-delta method", which assumes perfect and identical efficiencies of 100%, or E=2, for target and reference genes (Pfaffl, 2001).

Large standard error reduces the chance of observing significance in gene expression data. To minimize error, conditions were maintained as near to identical as possible. The same reagents were used throughout all experiments, as well as the same program on the same real-time machine. What differed between runs were the cDNA, primer aliquots

(fresh aliquots were made prior to each run to avoid contamination) and the number of times the kit reagents had been removed from the freezer preceding the run. Reactions contained a total of four reagents, and cDNA was added to the master mix rather than to individual tubes, ensuring that each reaction had an identical amount of template. Contamination was kept to a minimum, with a set of RNase-free pipette tips reserved solely for RT-PCR, and with the tip ejectors removed from all pipettes. No-template controls were performed for every primer set for each run, and all showed no contamination.

Despite all measures taken, there was a large amount of variation between samples in both morphant and control groups.

Following the time-course gene expression experiment, it was noted that the most consistent trend appeared to be in the samples that underwent 4 hours of hypoxia. It was therefore decided that 4 hours of hypoxic exposure was the most appropriate treatment for embryos to undergo prior to gene expression analysis. It is possible that 4 hours was not a long enough treatment duration, and that the embryos were not exposed to hypoxia for long enough to acquire a significant induction of the genes in either control or morphant samples. This would explain why there was no difference between morphant and control groups.

There is also a slight chance that the injection of the morpholino was not effective at achieving HIF-1α knockdown. Morpholino was co-injected with GFP mRNA, and embryos were checked for GFP expression, indicating that the majority of injections were successful. It has been shown that antisense-morpholino technology is an effective method of gene knockdown in zebrafish embryos, and that injections can be done in the yolk of the embryo rather than the cell, while still maintaining the effectiveness

(Nasevicius & Ekker, 2000). It therefore follows that if the HIF-1α knockdown wasn't successful, then the morpholino did not fully penetrate or the morpholino solution injected was degraded.

Another area for error to occur is in the hypoxic exposure of the embryos. It is marginally possible that embryos kept in the incubator rather than subjected to hypoxia may still have been exposed to a level of hypoxia. Embryos are stored in Petri dishes with approximately 50 ml of water, which should be sufficient to deliver the required amount of oxygen to the embryos. However, if too many embryos are kept in a single dish, or if embryos clump into a single area in the dish, oxygen will be limited to all embryos. This could lead to a hypoxic response similar to that of the embryos being treated with hypoxia, although would not lead to a response as severe as the treated embryos. It could be that the embryos in normoxic conditions were actually in a certain degree of hypoxia, and responded as such, with elevated expression levels of the HIF-1 target genes.

3. Westerns:

Western blots were meant to confirm the effectiveness of the morpholino injections in knocking down HIF-1α expression in hypoxic embryos, and to confirm effectiveness of over-expression of mutant human HIF-1α in normoxic embryos. A human HIF-1 antibody purchased from Santa Cruz was used against zebrafish embryos, both morphants and over-expressing HIF-1α. Control human cell lysates, hypoxic and normoxic, were used as positive and negative controls. This antibody did not yield bands in either zebrafish or human samples, normoxic or hypoxic. Another human HIF-1α antibody,

purchased from Novus biologicals, was used at Carleton University in Dr. Bill Willmore's lab. The positive control yielded two bands at approximately 120 kDa, while the zebrafish samples did not yield any bands. The two bands represent different post-translational modifications of the protein. The immunogen targeted by the antibody (human HIF-1α amino acids 432-528) was specific to the human HIF-1α sequence, in a domain not conserved in zebrafish. It was therefore not surprising that the antibody did not detect HIF-1α in the hypoxic zebrafish sample. It would have been expected, however, that the zebrafish embryos expressing the mutant form of human HIF-1α would have been detected by the Novus antibody. Since this was not the case, it can be concluded that the embryos were not, in fact, expressing the human HIF-1α. Experiments with the over-expression of HIF-1α in normoxic embryos were therefore terminated.

Blots were also done using a trout HIF-1α antibody and two common carp HIF-1α antibodies, neither of which detected HIF-1α in the zebrafish samples. Two zebrafish-HIF-1α-specific antibodies were used under several conditions. SDS-PAGE was done with several different percent acrylamide gels, and with various different gel recipes including ready-made solutions (Protogel). Different sized gels were run at varying speeds and at different temperatures. Gels were run at room temperature or at 4°C. Transfer conditions were also manipulated in several ways, including voltage, duration, temperature, transfer apparatus and transfer buffer. Primary antibodies were applied at either room temperature or 37°C for one hour, over at 4°C overnight. Primary antibody concentrations ranged from 1/200 to 1/1000, and were applied in 2.5% or 5% Carnation skim milk powder.

Secondary antibody was applied at room temperature for 1-2 hours, with concentrations ranging from 1/5000 to 1/20000. Different aliquots and different stocks of secondary antibodies were tried.

Protein was extracted using three different buffers, and immunogen concentration was increased by immunoprecipitation prior to blotting. Both PVDF and nitrocellulose membranes were used, and exposure was done via a chemidoc system as well as via Kodak film.

Once conditions were optimized, the two zebrafish-HIF-1α antibodies were yielding several bands, two of which were the correct size to be HIF-1α. The samples were run by SDS-PAGE and the gel was silver stained. The possible HIF-1α bands, one at 85 kDa and one at 110 kDa, were excised and sent for sequencing. These bands turned out to not be HIF-1α, and sequencing results did not specify what they were. Ultimately, none of the six HIF-1α antibodies were successful in detecting HIF-1α in zebrafish samples.

4. Morphology

Phenotypic comparison between morphant and control embryos at various time points (24, 48 & 72 hpf) showed no striking differences resulting from the absence of HIF-1α. It has been previously shown that HIF-1α plays an important role in the proper development of mouse embryos (Tyer et al, 1998), with embryos deficient in HIF-1α demonstrating phenotypic abnormalities in many areas of the vascular system, including defective blood vessels and myocardium, and improperly formed neural tube (Tyer et al, 1998). Defects in the mouse embryo caused by HIF-1α deficiency may therefore not be visible on the surface. It follows that if the zebrafish embryo exhibits the

same defects when devoid of HIF-1α, it may only be visible in the vascular system, and not under normal microscopic conditions. It is also possible that these defects don't exist in the zebrafish embryo, and are only found in mammalian species.

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Appendix A

Gene and primer sequences for real-time RT PCR

β-actin

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Aldolase

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aaaaaaaaaaaaa

Transferrin

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IGFBP-2

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Vascular Endothelial Growth Factor

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Phosphyglycerate Kinase A

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Lactate Dehydrogenase

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Prolyl-4-hydroxylase A

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Enolase

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Erythropoietin

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Appendix B

HIF-1 sequence alignments

Zebrafish HIF-1α nucleotide sequence (GenBank Accession AY326951)

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Zebrafish HIF-2α nucleotide sequence (GenBank Accession DQ375242)

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tctacatctt cataaattat atacatgtac tatttttta gttgcaagta agttgatgaa
cttaagtatt tttggaggag tttaaatgtt gcgttgtcgt ttttggccgg ctgtctctct
gcttgacgga taattttgct gaaactgttt tcaacatgtt ctactctagt cggtttctct
atcaacatca gacaaaaaaa aaaaaaaa

Zebrafish HIF-1α amino acid sequence (AY326951)

MDTGVVTEKKRVSSERRKEKSRDAARSRRGKESEVFYELAHQLP
LPHNVTSHLDKASIMRLTISYLRMRKLLNSDEKEEKEENELESQLNGFYLKALEGFLM
VLSEDGDMVYLSENVSKSMGLTQFDLTGHSIFEFSHPCDHEELREMLVHRTGSKKTKE
QNTERSFFLRMKCTLTSRGRTVNIKSATWKVLHCAGHVRVHEGSEASEDSGFKEPPVT
YLVLICEPIPHPSNIEVPLDSKTFLSRHTLDMKFSYCDERITELMGYEPDDLLNRSVY
EYYHALDSDHLTKTHHNLFAKGQATTGQYRMLAKKGGFVWVETQATVIYNPKNSQPQC
IVCVNYVLSGIVEGDVVLSLQQTVTEPKAVEKESEETEEKTSELDILKLFKPESLNCS
LESSTLYNKLKEEPEALTVLAPAAGDAIISLDFNNSDSDIQLLKEVPLYNDVMLPSSS
EKLPLSLSPLTPSDSIPALTKLETGGEDFPFSSASDRVPDPTNTPSTSGLGSSGPNSP
MDYGFPVEPDISSEFKLDLVEKLFAIDTEAKTPFSTQPMEDLDLEMLAPYIPMDDDFQ
LRIPSPLDPLPSATHSVSAMSSLFQPLPSSPASPASSTSSTVKQEASSRAPSPLHLLQ
EVCSAPVSPFSGSRDASPVRSSTPQSSSQLNNREMSPKMLAFQNIQRKRKLNEVTSLS
EAVGLGALLHSVDSAIDPGKRAKVLEVKGSSVLGGNKTILILPSDVASRLLSSSLEGS
GGLPQLTRYDCEVNAPVQDRHHLLQGEELLRALDQVN

Rainbow trout HIF-1α amino acid sequence (AF304864)

MDTGVVPEKKSRVSSDRRKEKSRDAARCRRGKESEVFYELAQEL
PLPHSVTSNLDKASIMRLAISYLHMRNLLSTDNEEEQEEREMDSQLNGSYLKAIEGFL
MVLSEDGDMIYLSENVNKCLGLAQIDLTGLSVFEYTHPCDHEELREMLVHRTGTSKKS
KEPNTERSFFLRMKCTLTNRGRTVNVKSATWKVLHCSDHVRVHESPAEQIPGGHKEPS
VPYLVLVCDPIPHPSNIEAPLDTKTFLSRHTLDMKFTYCDERITELMGYDPEDLLNRS
VYEYYHALDSDHLMKTHHNLFAKGQVSTGQYRMLAKRGGFVWVETQATVIYNNKNSQP
QCVVCVNYVLSGIEEEKMMLSLEQTEDMRPVKKELEEEESSEPEVSPVLLKEEKSPEL
DVIKLFTRAVETQPLSSLYDRLKEEPEALTLLAPAAGDTIISLDFSSPDSDILQKEVP
LYKDVMLPSTSDKLALPLSLLPPSDQHLVPNTSVDTTEVSTGPDSSSTPGSHSFTEPD
SPLDFCFPMESDINAEFKLDMVETLFAINPEPKTPFTLQAMEDLDLEMLAPYIPMDDD
FQLRTLSPEEPLSCGPAQPLECSSLCSSVRLTQEVHSYPGSPFNAPGSLTASPALAAS
PALAAPEPADSPCPASLLTKTVPQMDREISLRSLASQNAQRKRKMSLSQAVGIGGLLQ
DHPGPGKKLKVSELSHADAPFNRTILLLPTDLASRLLGISSEGSGSPFTLPQLTRYDC
EVNAPVGGRQLLLQGEELLSALDQVN

Common Carp HIF-1α amino acid sequence (ABC24677)

MDSGVVTEKKRVSSERRKEKSRDAARSRRGKESEVFYELAHQLPLPHNVTSHLDKASIMRLTISYLRMRK
LLSSDEEEKENELDCQLNSFYLKALEGFLMVLSEDGDMVYLSENVSKSMGLTQFDLTGHSIFEFSHPCDH
EELREMLVHKTGSKKTKEQNTERNFFLRMKCTLTSRGRTVNIKSATWKVLHCAGHVRVQESSEDSGDSGF
KEPPLTYLVVICEPIPHPSNIEVPLDSKTFLSRHTLDMKFSYCDERITELMGYEPDDLLNRSVYEYYHAL
DSDHLTKTHNNLFAKGQATTGQYRMLAKKGGFVWLETQATVIYNPKNSQPQCIVCVNYVLSGIVEGDVVL
SLQQTMTEPKAEEKENQEMEDEASEVHILKLFKPESLKCPVKSSELYEKLKEEPEALNVLAPSAGDTIIS
LDFNNSDSDMQLLKEVPLYNDVMLPSSSEKLPISLSPLTPLDYAPVLTKLETGAEDFPFCSASDRGPDST
NSSSTSGLGSSVPNSPMEYCFQVDSDISSEFKLDLVEKLFAIDTEAKTPFSSQAMEDLDLEMLAPYIPMD
DDFQLRIPSSLDLLPPGPHSVSAMSSLFQPLSSPVSPASSSSSSVKQEHSSQAPSPLHLLKEVCNAPVSP
FSGSRDVSPARSPTPKSSNQLNNRELSPKMLAVQNAQRKRKLEEVTSLSEAVGLNALLQSVDSAIAPGKR
ALVLELKGSGVLGGNKTILILPSDVASRLLCSSLESSHGLPQLTRYDCEVNAPVQDRHHLLQGEELLRAL
DQVNWS

Human HIF-1α amino acid sequence (NM001530)

MEGAGGANDKKKISSERRKEKSRDAARSRRSKESEVFYELAHQL
PLPHNVSSHLDKASVMRLTISYLRVRKLLDAGDLDIEDDMKAQMNCFYLKALDGFVMV
LTDDGDMIYISDNVNKYMGLTQFELTGHSVFDFTHPCDHEEMREMLTHRNGLVKKGKE
QNTQRSFFLRMKCTLTSRGRTMNIKSATWKVLHCTGHIHVYDTNSNQPQCGYKKPPMT
CLVLICEPIPHPSNIEIPLDSKTFLSRHSLDMKFSYCDERITELMGYEPEELLGRSIY
EYYHALDSDHLTKTHHDMFTKGQVTTGQYRMLAKRGGYVWVETQATVIYNTKNSQPQC
IVCVNYVVSGIIQHDLIFSLQQTECVLKPVESSDMKMTQLFTKVESEDTSSLFDKLKK
EPDALTLLAPAAGDTIISLDFGSNDTETDDQQLEEVPLYNDVMLPSPNEKLQNINLAM
SPLPTAETPKPLRSSADPALNQEVALKLEPNPESLELSFTMPQIQDQTPSPSDGSTRQ
SSPEPNSPSEYCFYVDSDMVNEFKLELVEKLFAEDTEAKNPFSTQDTDLDLEMLAPYI
PMDDDFQLRSFDQLSPLESSSASPESASPQSTVTVFQQTQIQEPTANATTTTATTDEL
KTVTKDRMEDIKILIASPSPTHIHKETTSATSSPYRDTQSRTASPNRAGKGVIEQTEK
SHPRSPNVLSVALSQRTTVPEEELNPKILALQNAQRKRKMEHDGSLFQAVGIGTLLQQ
PDDHAATTSLSWKRVKGCKSSEQNGMEQKTIILIPSDLACRLLGQSMDESGLPQLTSY
DCEVNAPIQGSRNLLQGEELLRALDQVN

Zebrafish/Rainbow trout HIF-1α amino acid sequence alignment

63.4% identity

```
Zebrafish      1 MDTGVVTEKK-RVSSERRKEKSRDAARSRRGKESEVFYELAHQLPLPHNVTSHLDKASIM
Rainbow        1 MDTGVVPEKKSRVSSDRRKEKSRDAARCRRGKESEVFYELAQELPLPHSVTSNLDKASIM
                 ****** *** **** ********** **********  ***** *** *******

Zebrafish     60 RLTISYLRMRKLLNSDEKEEKEENELESQLNGFYLKALEGFLMVLSEDGDMVYLSENVSK
Rainbow       61 RLAISYLHMRNLLSTDNEEEQEEREMDSQLNGSYLKAIEGFLMVLSEDGDMIYLSENVNK
                 ** **** ** **  *  ** ** *  ***** **** ************ ****** *

Zebrafish    120 SMGLTQFDLTGHSIFEFSHPCDHEELREMLVHRTG-SKKTKEQNTERSFFLRMKCTLTSR
Rainbow      121 CLGLAQIDLTGLSVFEYTHPCDHEELREMLVHRTGTSKKSKEPNTERSFFLRMKCTLTNR
                   ** * **** * **  ***************** *** ** *************** *

Zebrafish    179 GRTVNIKSATWKVLHCAGHVRVHEGSEASEDSGFKEPPVTYLVLICEPIPHPSNIEVPLD
Rainbow      181 GRTVNVKSATWKVLHCSDHVRVHESPAEQIPGGHKEPSVPYLVLVCDPIPHPSNIEAPLD
                 ***** **********  ******        * *** * **** * ********* ***

Zebrafish    239 SKTFLSRHTLDMKFSYCDERITELMGYEPDDLLNRSVYEYYHALDSDHLTKTHHNLFAKG
Rainbow      241 TKTFLSRHTLDMKFTYCDERITELMGYDPEDLLNRSVYEYYHALDSDHLMKTHHNLFAKG
                  ************* ************ * ****************** **********

Zebrafish    299 QATTGQYRMLAKKGGFVWVETQATVIYNPKNSQPQCIVCVNYVLSGIVEGDVVLSLQQTV
Rainbow      301 QVSTGQYRMLAKRGGFVWVETQATVIYNNKNSQPQCVVCVNYVLSGIEEEKMMLSLEQTE
                 *  ********* *************** ******* ********** *    *** **

Zebrafish    359 T-EPKAVEKESEET-----------EEKTSELDILKLFKPESLNCSLESSTLYNKLKEEP
Rainbow      361 DMRPVKKELEEEESSEPEVSPVLLKEEKSPELDVIKLFTRAVETQPL--SSLYDRLKEEP
                    *   * * **            ***  ***  ***        *  * **  *****

Zebrafish    407 EALTVLAPAAGDAIISLDFNNSDSDIQLLKEVPLYNDVMLPSSSEKLPLSLSPLTPSDS-
Rainbow      419 EALTLLAPAAGDTIISLDFSSPDSDI-LQKEVPLYKDVMLPSTSDKLALPLSLLPPSDQH
                 **** ******* ******   **** * ****** ****** * ** * ** * ***

Zebrafish    466 IPALTKLETGGEDFPFSSASDRVPDPTNTPSTSGLGSSGPNSPMDYGFPVEPDISSEFKL
Rainbow      478 LVPNTSVDT--------TEVSTGPDSSSTPGSHSF--TEPDSPLDFCFPMESDINAEFKL
                     *   *             **   **         * ** *  ** * **  ****

Zebrafish    526 DLVEKLFAIDTEAKTPFSTQPMEDLDLEMLAPYIPMDDDFQLRIPSPLDPLPSATHSVSA
Rainbow      528 DMVETLFAINPEPKTPFTLQAMEDLDLEMLAPYIPMDDDFQLRTLSPEEPLSCGPAQPLE
                 * ** ****  * ****  * **********************  **  **

Zebrafish    586 MSSLFQPLPSSPASPASSTSSTVKQEASSRAPSPLHLLQEVCSAPVSPFSGSRDASPVRS
Rainbow      588 CSSLCSSVRLT--QEVHSYPGSPFNAPGSLTASPALAASPALAAP-EPADSPCPASLLTK
                  ***             *          *   **          **  *      **

Zebrafish    646 STPQSSSQLNNREMSPKMLAFQNIQRKRKLNEVTSLSEAVGLGALLHSVDSAIDPGKRAK
Rainbow      645 TVPQM-----DREISLRSLASQNAQRKRKM----SLSQAVGIGGLLQDHPG---PGKKLK
                   **       ** *   ** ** *****     *** *** * **        ***  *

Zebrafish    706 VLEVKGSSVLGGNKTILILPSDVASRLLSSSLEGSGG---LPQLTRYDCEVNAPVQDRHH
Rainbow      693 VSELSHADA-PFNRTILLLPTDLASRLLGISSEGSGSPFTLPQLTRYDCEVNAPVGGRQL
                 * *          * *** ** * *****  * ****    ***************  *

Zebrafish    763 LLQGEELLRALDQVN
Rainbow      752 LLQGEELLSALDQVN
                 ******** ******
```

Zebrafish/common carp HIF-1α amino acid sequence alignment

88.5% identity

```
Zebrafish      1 MDTGVVTEKKRVSSERRKEKSRDAARSRRGKESEVFYELAHQLPLPHNVTSHLDKASIMR
Carp           1 MDSGVVTEKKRVSSERRKEKSRDAARSRRGKESEVFYELAHQLPLPHNVTSHLDKASIMR
                 ** ************************************************************

Zebrafish     61 LTISYLRMRKLLNSDEKEEKEENELESQLNGFYLKALEGFLMVLSEDGDMVYLSENVSKS
Carp          61 LTISYLRMRKLLSSDEEEK--ENELDCQLNSFYLKALEGFLMVLSEDGDMVYLSENVSKS
                 ************ *** *   ****  *** ****************************

Zebrafish    121 MGLTQFDLTGHSIFEFSHPCDHEELREMLVHRTGSKKTKEQNTERSFFLRMKCTLTSRGR
Carp         119 MGLTQFDLTGHSIFEFSHPCDHEELREMLVHKTGSKKTKEQNTERNFFLRMKCTLTSRGR
                 ******************************* ************* **************

Zebrafish    181 TVNIKSATWKVLHCAGHVRVHEGSEASEDSGFKEPPVTYLVLICEPIPHPSNIEVPLDSK
Carp         179 TVNIKSATWKVLHCAGHVRVQESSEDSGDSGFKEPPLTYLVVICEPIPHPSNIEVPLDSK
                 ******************* * ** * ******** **** ******************

Zebrafish    241 TFLSRHTLDMKFSYCDERITELMGYEPDDLLNRSVYEYYHALDSDHLTKTHHNLFAKGQA
Carp         239 TFLSRHTLDMKFSYCDERITELMGYEPDDLLNRSVYEYYHALDSDHLTKTHNNLFAKGQA
                 ************************************************* ********

Zebrafish    301 TTGQYRMLAKKGGFVWVETQATVIYNPKNSQPQCIVCVNYVLSGIVEGDVVLSLQQTVTE
Carp         299 TTGQYRMLAKKGGFVWLETQATVIYNPKNSQPQCIVCVNYVLSGIVEGDVVLSLQQTMTE
                 **************** ****************************************** **

Zebrafish    361 PKAVEKESEETEEKTSELDILKLFKPESLNCSLESSTLYNKLKEEPEALTVLAPAAGDAI
Carp         359 PKAEEKENQEMEDEASEVHILKLFKPESLKCPVKSSELYEKLKEEPEALNVLAPSAGDTI
                 *** ***  * *   **  ********** *   ** ** ********* **** *** *

Zebrafish    421 ISLDFNNSDSDIQLLKEVPLYNDVMLPSSSEKLPLSLSPLTPSDSIPALTKLETGGEDFP
Carp         419 ISLDFNNSDSDMQLLKEVPLYNDVMLPSSSEKLPISLSPLTPLDYAPVLTKLETGAEDFP
                 *********** ********************** ******* *  * ******* ****

Zebrafish    481 FSSASDRVPDPTNTPSTSGLGSSGPNSPMDYGFPVEPDISSEFKLDLVEKLFAIDTEAKT
Carp         479 FCSASDRGPDSTNSSSTSGLGSSVPNSPMEYCFQVDSDISSEFKLDLVEKLFAIDTEAKT
                 * ***** ** **  ******** ***** * * *  **********************

Zebrafish    541 PFSTQPMEDLDLEMLAPYIPMDDDFQLRIPSPLDPLPSATHSVSAMSSLFQPLPSSPASP
Carp         539 PFSSQAMEDLDLEMLAPYIPMDDDFQLRIPSSLDLLPPGPHSVSAMSSLFQPL-SSPVSP
                 *** * ************************* ** **   ************* *** **

Zebrafish    601 ASSTSSTVKQEASSRAPSPLHLLQEVCSAPVSPFSGSRDASPVRSSTPQSSSQLNNREMS
Carp         598 ASSSSSSVKQEHSSQAPSPLHLLKEVCNAPVSPFSGSRDVSPARSPTPKSSNQLNNRELS
                 *** ** **** ** ******** *** *********** ** ** ** ** ****** *

Zebrafish    661 PKMLAFQNIQRKRKLNEVTSLSEAVGLGALLHSVDSAIDPGKRAKVLEVKGSSVLGGNKT
Carp         658 PKMLAVQNAQRKRKLEEVTSLSEAVGLNALLQSVDSAIAPGKRALVLELKGSGVLGGNKT
                 ***** ** ****** *********** *** ****** ***** *** *** *******

Zebrafish    721 ILILPSDVASRLLSSSLEGSGGLPQLTRYDCEVNAPVQDRHHLLQGEELLRALDQVN
Carp         718 ILILPSDVASRLLCSSLESSHGLPQLTRYDCEVNAPVQDRHHLLQGEELLRALDQVN
                 ************* **** * ************************************
```

Zebrafish/human HIF-1α amino acid sequence alignment

58.4% identity

```
Zebrafish      4 GVVTEKKRVSSERRKEKSRDAARSRRGKESEVFYELAHQLPLPHNVTSHLDKASIMRLTI
Human          5 GGANDKKKISSERRKEKSRDAARSRRSKESEVFYELAHQLPLPHNVSSHLDKASVMRLTI
                 *    **  ***************** ****************** ******* *****

Zebrafish     64 SYLRMRKLLNSDEKEEKEENELESQLNGFYLKALEGFLMVLSEDGDMVYLSENVSKSMGL
Human         65 SYLRVRKLLDAGDLDI--EDDMKAQMNCFYLKALDGFVMVLTDDGDMIYISDNVNKYMGL
                 **** ****         *     * * ****** ** ***  **** * * ** * ***

Zebrafish    124 TQFDLTGHSIFEFSHPCDHEELREMLVHRTGS-KKTKEQNTERSFFLRMKCTLTSRGRTV
Human        123 TQFELTGHSVFDFTHPCDHEEMREMLTHRNGLVKKGKEQNTQRSFFLRMKCTLTSRGRTM
                 *** ***** * * ******* **** ** *  ** ***** ****************

Zebrafish    183 NIKSATWKVLHCAGHVRVHEGSEASEDSGFKEPPVTYLVLICEPIPHPSNIEVPLDSKTF
Human        183 NIKSATWKVLHCTGHIHVYDTNSNQPQCGYKKPPMTCLVLICEPIPHPSNIEIPLDSKTF
                 ************ **  *          * * ** * *************** *******

Zebrafish    243 LSRHTLDMKFSYCDERITELMGYEPDDLLNRSVYEYYHALDSDHLTKTHHNLFAKGQATT
Human        243 LSRHSLDMKFSYCDERITELMGYEPEELLGRSIYEYYHALDSDHLTKTHHDMFTKGQVTT
                 **** ******************  ** ** *****************  * *** **

Zebrafish    303 GQYRMLAKKGGFVWVETQATVIYNPKNSQPQCIVCVNYVLSGIVEGDVVLSLQQTVTEPK
Human        303 GQYRMLAKRGGYVWVETQATVIYNTKNSQPQCIVCVNYVVSGIIQHDLIFSLQQTECVLK
                 ******** ** ************ ************** ***   *   *****    *

Zebrafish    363 AVEKESEETEEKTSELDILKLFKPESLNCSLESSTLYNKLKEEPEALTVLAPAAGDAIIS
Human        363 PVE---------SSDMKMTQLFTKVE---SEDTSSLFDKLKKEPDALTLLAPAAGDTIIS
                  **          *      **       *   * *  *** ** *** ******* ***

Zebrafish    423 LDF--NNSDSDIQLLKEVPLYNDVMLPSSSEKLP---LSLSPL----TP----SDSIPAL
Human        411 LDFGSNDTETDDQQLEEVPLYNDVMLPSPNEKLQNINLAMSPLPTAETPKPLRSSADPAL
                 ***  *    * * * ************  ***    *  ***    **    *   ***

Zebrafish    470 T-----KLETGGEDFPFSSASDRVPDPTNTPSTSGLGSSGP--NSPMDYGFPVEPDISSE
Human        471 NQEVALKLEPNPESLELSFTMPQIQDQTPSPSDGSTRQSSPEPNSPSEYCFYVDSDMVNE
                       ***   *    *       * *  **      * *  ***  * * *  *   *

Zebrafish    523 FKLDLVEKLFAIDTEAKTPFSTQPMEDLDLEMLAPYIPMDDDFQLRIPSPLDPLPSATHS
Human        531 FKLELVEKLFAEDTEAKNPFSTQDT-DLDLEMLAPYIPMDDDFQLRSFDQLSPLESSSAS
                 *** ******* ***** *****   ******************    * ** *   *

Zebrafish    583 VSAMS-----SLFQPL----PSSPASPASSTSSTVKQEASSR---------APSPLHLLQ
Human        590 PESASPQSTVTVFQQTQIQEPTANATTTTATTDELKTVTKDRMEDIKILIASPSPTHIHK
                     *       **      *   *     *    *     *          *** *

Zebrafish    625 EVCSAPVSPF--SGSRDASPVRS----------STPQSSSQLN----------NREMSPK
Human        650 ETTSATSSPYRDTQSRTASPNRAGKGVIEQTEKSHPRSPNVLSVALSQRTTVPEEELNPK
                 *  **  **     ** *** *           * * *   *            *  **

Zebrafish    663 MLAFQNIQRKRKLNEVTSLSEAVGLGALLHSVD--SAIDPGKRAKVLEVKGSSVLG-GNK
Human        710 ILALQNAQRKRKMEHDGSLFQAVGIGTLLQQPDDHAATTSLSWKRVKGCKSSEQNGMEQK
                  ** ** *****     **  *** * **   *   *        *   * *   *   *

Zebrafish    720 TILILPSDVASRLLSSSLEGSGGLPQLTRYDCEVNAPVQDRHHLLQGEELLRALDQVN
Human        770 TIILIPSDLACRLLGQSMDESG-LPQLTSYDCEVNAPIQGSRNLLQGEELLRALDQVN
                 **   *** * ***  *   ** ***** ******** *    ***************
```