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Molecular characterization and mRNA expression of two key enzymes of hypoxia-sensing pathways in eastern oysters *Crassostrea virginica* (Gmelin): Hypoxia-inducible factor α (HIF- α) and HIF-prolyl hydroxylase (PHD)

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Abstract

Oxygen homeostasis is crucial for development, survival and normal function of all metazoans. A family of transcription factors called hypoxia-inducible factors (HIF) is critical in mediating the adaptive responses to reduced oxygen availability. The HIF transcription factor consists of a constitutively expressed β subunit and an oxygen-dependent α subunit; the abundance of the latter determines the activity of HIF and is regulated by a family of O₂- and Fe²⁺-dependent enzymes prolyl hydroxylases (PHDs). Currently very little is known about the function of this important pathway and the molecular structure of its key players in hypoxia-tolerant intertidal mollusks including oysters, which are among the animal champions of anoxic and hypoxic tolerance and thus can serve as excellent models to study the role of HIF cascade in adaptations to oxygen deficiency. We have isolated transcripts of two key components of the oxygen sensing pathway - the oxygen-regulated HIF- α subunit and PHD - from an intertidal mollusk, the eastern oyster *Crassostrea virginica*, and determined the transcriptional responses of these two genes to anoxia, hypoxia and cadmium (Cd) stress. HIF- α and PHD homologs from eastern oysters *C. virginica* show significant sequence similarity and share key functional domains with the earlier described isoforms from vertebrates and invertebrates. Phylogenetic analysis shows that genetic diversification of HIF and PHD isoforms occurred within the vertebrate lineage indicating functional diversification and specialization of the oxygen-sensing pathways in this group, which parallels situation observed for many other important genes. HIF- α and PHD homologs are broadly expressed at the mRNA level in different oyster tissues and show transcriptional responses to prolonged hypoxia in the gills consistent with their putative role in oxygen sensing and the

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adaptive response to hypoxia. Similarity in amino acid sequence, domain structure and transcriptional responses between HIF- α and PHD homologs from oysters and other invertebrate and vertebrate species implies the highly conserved functions of these genes throughout the evolutionary history of animals, in accordance with their critical role in oxygen sensing and homeostasis.

Keywords

Oxygen sensing; hypoxia-inducible factor; evolution; expression; intertidal mollusk

Introduction

Oxygen homeostasis is crucial for development, survival and normal function of all metazoans, and complex cellular interaction networks have evolved to permit oxygen sensing and to tightly regulate oxygen homeostasis. Hypoxia-inducible factors (HIF) and prolyl hydroxylases (PHDs) have been identified as critical mediators in the adaptive responses to reduced oxygen availability in animals (Lahiri et al., 2006; Liu and Semenza, 2007; Taylor and Pouyssegur, 2007). HIF is a heterodimeric redox-sensitive transcription factor composed of a constitutively expressed β subunit (HIF-1 β also called the aryl hydrocarbon receptor nuclear translocator, or ARNT) and an α subunit which is regulated by the concentration of intracellular oxygen (Wang and Zhang, 1995; Bruick and McKnight, 2002; Lahiri et al., 2006). The oxygen-sensitive α subunit of the HIF is constitutively transcribed and translated in the cells, but has an extremely short half-life under normoxic conditions. This high normoxic turnover of HIF- α is due to the oxygen-dependent post-translational control by a family of novel prolyl hydroxylases, or PHDs (Aragones et al., 2009). Mammals have three isoforms of HIF- α (HIF-1 α , HIF-2 α and HIF-3 α) and PHD (including PHD1, PHD2 and PHD3 also called EGLN-2, EGLN-1 and EGLN-3, respectively) (Ratcliffe, 2007; Aragones et al., 2009). Different isoforms of HIF- α and PHD are genetically homologous within the group, and play similar although non-redundant roles in cellular oxygen sensing (Appelhoff et al., 2004; Ratcliffe, 2007).

Of the three mammalian HIF- α isoforms, HIF-1 α was historically the first one identified, and its mechanism is perhaps best studied of all the three isoforms (Semenza, 2004; Lahiri et al., 2006; Ratcliffe, 2007). The basic mechanisms of the oxygen-dependent regulation are similar for all HIF- α isoforms. Briefly, PHD uses molecular oxygen to oxidize critical prolyl residues within a central oxygen-dependent degradation domain (ODDD) in HIF- α which prompts polyubiquitination of HIF- α targeting it for proteosomal degradation (Aragones et al., 2009). Declining pO₂ prevents the PHD-mediated oxidation of HIF- α with the subsequent stabilization of the protein, its translocation into the nucleus, and dimerization with HIF-1 β . The HIF dimer binds hypoxia response elements (HRE) at target gene loci and activates a cascade of adaptive responses to hypoxia via regulation of the expression of genes promoting anaerobic glycolysis, oxidative phosphorylation, cell signaling, and (in vertebrates) vasodilatation, angiogenesis and erythropoiesis (Benizri et al., 2008; Taylor and Pouyssegur, 2007). This pathway is typical for HIF-1 α , HIF-2 α and some splice variants of HIF-3 α , while other splice isoforms of HIF-3 α can also encode peptides that antagonize HRE-dependent gene activation (Ratcliffe, 2007). HIF-dependent transcriptional activation of the target genes plays a critical role in a host of developmental, physiological, and pathological processes in animals including hypoxic survival, embryogenesis, tissue repair, immune defense, ischemia and post-ischemic recovery, neurological diseases and cancer (review in: Hochachka et al., 2002; Benizri et al., 2008).

Up to date, most of our knowledge about the structure and function of HIF pathways comes from the model systems such as mammals, insects (*Drosophila melanogaster*) and nematodes (*Caenorhabditis elegans*) (review in: Chuan Shen and Powell-Coffman, 2003; Webster, 2003; Semenza, 2004; Shen et al., 2005; Lahiri et al., 2006). Much less is known about the function of this important pathway and the molecular structure of its key players in hypoxia- and anoxia-tolerant animals such as intertidal invertebrates, and hypoxia-tolerant fish and reptiles (Heise et al., 2006b; Rissanen et al., 2006a; Rissanen et al., 2006b; Li and Brouwer, 2007; Soñanez-Organis et al., 2009). Given the central role of the HIF pathway in oxygen homeostasis and its striking evolutionary conservatism throughout the animal kingdom (Gorr et al., 2006; Hoogewijs et al., 2007), it is likely that a HIF cascade also plays an important role in adaptations to oxygen deficiency in anoxia- and hypoxia-tolerant organisms.

Intertidal mollusks including oysters are among the animal champions of anoxic and hypoxic tolerance and thus excellent models to study the role of HIF cascade in adaptations to oxygen deficiency. They are periodically exposed to hypoxia and anoxia during the low tide due to the closure of their shells that prevents desiccation but severely limits gas exchange (Newell, 1976; Brinkhoff et al., 1983). Such tidally-induced hypoxia and anoxia can last from several hours to several days depending on the shore height and the state of the tides. This intermittent short-term oxygen deprivation can be superimposed by the long-term severe hypoxia and anoxia (from several weeks to up to several months) triggered by anthropogenic release of nutrients in estuaries and coastal zones (Malakoff, 1998; Tilman et al., 2001; Ferber, 2004; Chan et al., 2008). Intertidal mollusks such as oysters can cope with prolonged environmental hypoxia and anoxia by temporarily switching to anaerobic mode of ATP production, utilizing alternative anaerobic pathways that produce higher ATP yield per unit substrate than “classical” lactate-producing glycolysis and entering a metabolically depressed state to conserve energy resources (Hochachka, 1985, 1988; Grieshaber et al., 1994; Stickle et al., 1989; Storey & Storey, 1990). They can also complement aerobic ATP synthesis with anaerobic pathways in cases when environmental oxygen is not limiting but mitochondrial function is impaired and/or energy demand is excessive leading to tissue hypoxemia (e.g. in response to elevated temperatures, mitochondrial poisons or pollutants) (Pörtner, 2001, 2002; Sokolova and Lannig, 2008; Lannig et al., 2006). Thus, aerobic/anaerobic transitions play a crucial role in metabolic adaptations of marine invertebrates to the life in estuaries and intertidal zones, and the HIF pathway is a likely candidate playing a role in these transitions and regulating the adaptive responses to the environmental oxygen deficiency. However, the molecular mechanisms regulating oxygen sensing (including structure and function of HIF pathway) are not well understood in intertidal mollusks.

The aim of this study was to isolate and characterize transcripts of two key components of the oxygen sensing pathway - the oxygen-regulated HIF- α subunit and PHD - from an intertidal mollusk, the eastern oyster *Crassostrea virginica*, and to determine the transcriptional responses of these two genes to anoxia, hypoxia and Cd stress. In water-breathing invertebrates such as oysters, all these stressors converge on the oxygen deficiency: anoxia and hypoxia - through the limited oxygen availability in the environment, and Cd stress - through tissue-wide hypoxemia resulting from a mismatch between oxygen demand and supply (Pörtner, 2002; Lannig et al., 2006, 2008). Cd was also shown to directly interfere with HIF signaling in mammalian models (Chun et al., 2000; Horiguchi et al., 2000; Belaidi et al., 2008) thus HIF pathway can be a potential candidate involved in physiological and cellular response to Cd in *C. virginica*. We tested this hypothesis that anoxia, hypoxia and Cd levels (50 $\mu\text{g L}^{-1}$ for 30 days) affect HIF pathway in oysters by measuring tissue-specific expression of HIF- α and PHD transcript levels in different tissues of oysters exposed to these stressors. This study of the structurally novel HIF- α and PHD homologs and their transcriptional profiles in a hypoxia- and anoxia-tolerant marine

mollusk, *C. virginica*, provides insights into the evolutionary relationship of HIF- α and PHD homologs of vertebrates and invertebrates, sheds light on the evolutionary pathways of diversification of these two important protein families and provides an important stepping stone for the future comparative functional studies on molecular oxygen sensing pathways in animals and their role in evolution of hypoxia tolerance.

Materials and Methods

Chemicals

Unless otherwise indicated, all chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA), Roche (Indianapolis, IN, USA) or Fisher Scientific (Pittsburg, PA, USA) and were of analytical grade or higher.

Oyster maintenance and tissue collection

Adult oysters (*Crassostrea virginica*; 70–120 mm shell length) were obtained from Taylor Shellfish Farms Inc. (Shelton, WA, USA), transported to the University of North Carolina at Charlotte within 48 h of collection and placed in tanks with artificial seawater (ASW) (Instant Ocean®, Kent Marine, Acworth, GA) at $20\pm 1^\circ\text{C}$ and $30\pm 1\%$. Oysters were acclimated for at least two weeks prior to the experiments. Oysters were exposed to the following experimental conditions: 1) normoxic controls ($\text{PO}_2\sim 153$ Torr, ~ 20 kPa or 7.5 mg O_2 L^{-1}); 2) hypoxia ($\text{PO}_2\sim 36$ Torr, ~ 5 kPa or 1.8 mg O_2 L^{-1}); 3) environmental anoxia induced by shell closure during aerial exposure; 4) water-borne cadmium (50 μg L^{-1} Cd) under normoxic conditions. Details of experimental exposures are given below. During all experimental exposures (except air exposure), oysters were fed *ad libitum* on alternate days with a commercial algal blend (2 mL per oyster) containing *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* with a cell size of $2 - 20$ μm (DT's Live Marine Phytoplankton, Premium Reef Blend Sycamore, IL, USA). All exposures were carried out at $20\pm 1^\circ\text{C}$.

For normoxic controls, oysters were kept in well aerated ASW ($30\pm 1\%$) at $20\pm 1^\circ\text{C}$ for 2–3 weeks. Oxygen concentrations were maintained at $7.3\text{--}7.5$ mg O_2 L^{-1} throughout the exposure.

For hypoxic exposures, oysters were maintained for 2 weeks at $20\pm 1^\circ\text{C}$ in ASW ($30\pm 1\%$) bubbled with a certified gas mixture containing 5% O_2 , 0.04% CO_2 and balance of nitrogen (Roberts Oxygen, Charlotte, NC). Oxygen concentrations were tested every other day and ranged between 20 and 30% of air saturation ($1.5\text{--}2.3$ mg O_2 L^{-1} , on average 1.8 mg O_2 L^{-1} or ~ 36 Torr (~ 5 kPa) PO_2). Water in each tray was changed every other day using ASW equilibrated with the hypoxic gas mixture described above.

To mimic environmental anoxia in the intertidal zone, oysters were exposed to air at $20\text{--}22^\circ\text{C}$ in plastic trays lined with seawater-soaked paper towels, and their shells were closed with a rubber band to prevent gaping. Our earlier studies showed that oxygen levels in oyster hemolymph drop to 0% within 20–40 min of such exposure and remain at this low level throughout 6 days in the air, indicating an onset of deep anoxia (Kurochkin et al., 2009). It is worth noting that while typical periods of air exposure for oysters in the intertidal zone are several hours, they may periodically experience more prolonged air exposures (lasting for several days) during neap tides (Brinkhoff et al., 1983; McLusky, 1989; Doney et al., 2009). Therefore, while a six day exposure to environmental anoxia represents an extreme stress for oysters, it is within the environmentally relevant range of anoxic exposures for this species.

For Cd exposure, oysters were maintained for 30 days at $20 \pm 1^\circ\text{C}$ in ASW ($30 \pm 1\%$) with Cd (as CdCl_2) added to the nominal concentration of $50 \mu\text{g L}^{-1}$. To avoid Cd depletion, ASW was supplemented with Cd twice a week. Our earlier studies using the same exposure regime showed that actual Cd concentration in the seawater are close to the nominal concentration of $50 \mu\text{g L}^{-1}$ under these conditions (Lannig et al., 2006). Cd concentration used in this study is within the range found in polluted estuaries ($15\text{--}80 \mu\text{g L}^{-1}$ Cd; GESAMP, 1987; Crompton, 1997; Frew et al., 1997; Hackney et al., 1998) and was shown to result in physiologically relevant tissue Cd burdens similar to those found in oysters from polluted estuaries (Sokolova et al., 2005; Cherkasov et al., 2006 and references therein).

After all experimental exposures, oysters were dissected, and their tissues collected and immediately shock-frozen in liquid nitrogen until further use.

RNA isolation

Total RNA was extracted from gills, hepatopancreas, mantle and muscle of control, hypoxia-, anoxia- and Cd-exposed oysters by homogenization in ice-cold Tri Reagent (Sigma-Aldrich, Saint Louis, Missouri, USA) or TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA was quantified with NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Tissue to TRI reagent (or Trizol) ratio was kept at or below 1:10 (w:v). This method yielded high purity total RNA with 280/260 absorbance ratio ≥ 1.9 . RNA from gill and hepatopancreas tissues of control oysters was used to isolate full length transcripts of HIF- α and PHD, and from all five studied tissues – for determination of tissue-specific expression of these two genes during normoxia and anoxia. cDNA was obtained from 1–5 μg total RNA using SuperScript[®] III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions.

Isolation and characterization of the full-length HIF- α and PHD transcripts

Gill and hepatopancreas tissues were dissected from ice-cold *C. virginica* and immediately subjected for RNA extraction. Degenerate primers (PHD-1 FW1 and PHD-1 RV, Table 1) were designed against conserved fragments of the PHD protein using sequences from *C. elegans*, *Strongylocentrotus purpuratus*, *Apis mellifera*, *Trichoplax adhaerens* and *D. melanogaster* (NCBI accession numbers [NP_444274](#), [XP_783963](#), [XP_397368](#), [XP_002112390](#) and [NP_649525](#), respectively) and used in PCR under the following conditions: initial denaturation for 5 min at 94°C , 35 cycles of 30 s at 94°C , 30 s at 55°C , 40 s at 72°C , followed by the final extension for 7 min at 72°C . Amplified fragments were gel-purified on 1.5% agarose gel using Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA, USA), and subjected to direct sequencing in forward and reverse directions at BioAnalytical Services Laboratory (University of Maryland Center for Environmental Science, Institute of Marine and Environmental Technology, Baltimore, MD, USA). These sequences were used to design specific primers for 5'- and 3' rapid amplification of cDNA ends (RACE)-PCR of PHD2 with a SMART cDNA amplification kit (BD Biosciences, San Jose, CA). For HIF- α , a partial EST sequence from *C. virginica* (NCBI accession number [CD648099](#)) was used to design specific primers for RACE PCR.

A full-length HIF- α transcript was isolated from freshly extracted gill and hepatopancreas RNA using 5'-end and 3'-end RACE-PCR. For the first PCR of 5'-end RACE, 1 μL of 5' RACE cDNA (containing 50 ng of total RNA) was amplified with CvHiF5R1 (Table 1) and the universal primer (UPM) (BD Biosciences, San Jose, CA, USA) using Advantage Taq enzyme (BD Biosciences). PCR conditions were as follows: initial denaturation for 2.5 min at 94°C , 35 cycles of 30 s at 94°C , 30 s at 55°C , 1 min at 72°C , followed by the final extension for 7 min at 72°C . One microliter of the product of this first PCR reaction was

diluted 10-fold in nuclease-free water and served as the template for the nested PCR. Either CvHiF5R2 or CvHiF5R3 were used as a reverse primer (Table 1), and nested universal primer (NUP) (BD Biosciences) was used as the forward primer under the same PCR conditions as described above. The first 3'-end RACE PCR was carried out using CvHiF3F1 (Table 1) and UPM primers. The PCR product of this first reaction was 10-fold diluted in nuclease-free water and used as the template for the nested PCR with either CvHiF3F2 or CvHiF3F3 as reverse primers (Table 1) and NUP as the forward primer. PCR conditions used for 3'-end RACE-PCR and nested PCRs were identical to those described above for 5'-end RACE-PCR.

For isolation of the PHD transcript from *C. virginica*, the procedures were the same as for HIF- α transcript with the following modifications: gene-specific primer CvPHD1-5R1 (Table 1) was used in the first 5'-end RACE PCR at the annealing temperature of 50°C and primer CvPHD1-5R2 was used for the nested 5'-end RACE PCR at the annealing temperature of 52°C; gene-specific primer CvPHD1-3F1 was used in the first 3'-end RACE PCR at the annealing temperature of 50 °C and primer CvPHD1-3F2 was used for the nested 3'-end RACE PCR at the annealing temperature of 52 °C.

The 3'-end nested RACE PCR yielded one product for HIF- α and two products of different lengths for PHD. The 5'-end nested RACE PCR yielded one product each for HIF- α and PHD. All products of the nested 3'-end and 5'-end RACE PCRs were gel-purified with QIAquick Gel Extraction Kit (QIAGEN, Valencia CA, USA), and cloned in *E. coli* using a pGEMT vector (Promega, Madison, WI, USA). Three positive clones were randomly selected for each gene product and sequenced (University of Maryland Center for Environmental Science, Institute of Marine and Environmental Technology, Baltimore, MD, USA).

Molecular characterization and evolutionary analysis

To elucidate phylogenetic relationships among animal HIF- α and PHD genes, including placement of oyster sequences, representative amino acid sequences were collected from multiple complete and draft assemblies of genomes of vertebrates and invertebrates using GenBank (Benson et al., 2007) and Ensembl (Hubbard et al., 2007) databases (see Supplemental Tables 1 and 2 for the list of species and accession numbers). Because of the saturation of substitutions in the nucleotide sequences, we focused our analyses on amino acid sequences instead. HIF- α and PHD (EGLN) sequences from full genomes of 10 vertebrates (representing major vertebrate groups) and 1 cephalochordate species, as well as representative sequences of 12 invertebrates (including available sequences from mollusks, insects and crustaceans) were used to address relationships between invertebrate and vertebrate homologs. In addition to HIF-1 α sequences, we included representatives of two other homologous genes that are part of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) protein superfamily (Sogawa and Fujii-Kuriyama, 1997), namely, endothelial PAS domain-containing protein 1 (EPAS1, also referred to as HIF-2 α) and HIF-3 α . These isoforms are most closely related to HIF-1 α phylogenetically and structurally (Sogawa and Fujii-Kuriyama, 1997; Tian et al., 1997; Gu et al., 1998), and have the capacity to sense low oxygen tension (Gu et al., 2000). In our preliminary analyses, other members of the bHLH/PAS superfamily were also considered including aryl hydrocarbon receptor nuclear translocator (ARNT), aryl hydrocarbon receptor (AHR) and the single-minded gene (Sim). However, because the duplication events leading to appearance of these families have occurred very early in the animal evolution (Sogawa and Fujii-Kuriyama, 1997; Simionato et al., 2007) resulting in the high degree of divergence of amino acid sequences among these distantly related members of the superfamily, inclusion of these distantly related members of the bHLH/PAS superfamily led to poorly resolved relationships among taxa of interest (data not shown). To avoid this ambiguity, we focused on relationships among the most closely

related animal homologs of oyster HIF- α including HIF-1 α , HIF-2 α and HIF-3 α from vertebrates. If multiple transcripts exist, only the longest one was used in the alignment. Multiple protein sequence alignments were reconstructed using MUSCLE (Edgar, 2004) and manually verified.

Phylogenetic trees were reconstructed using the minimum-evolution (ME) method (Rzhetsky and Nei, 1992) as implemented in the MEGA4.0 program (Tamura et al., 2007) and the maximum likelihood (ML) method using PhyML3.0 program (Guindon and Gascuel, 2003). ME tree was built using the Dayhoff amino acid substitution model (Dayhoff et al., 1978; Nei and Kumar, 2000), with 1000 bootstrap pseudo-replications used to evaluate the reliability of internal branches (Felsenstein, 1985). Rate variation among sites was approximated by the gamma distribution with the alpha parameter 1 and 0.8 for HIF- α and PHD homologs, respectively, estimated from the data using PhyML. ML tree was built using the Whelan and Goldman substitution model (Whelan and Goldman, 2001) that also took into account the rate variation among sites and the proportion of invariable sites (i.e., WAG+gamma+inv). To evaluate the reliability of internal branches, the bootstrap test and the approximate likelihood-ratio test (aLRT) based on the non-parametric Shimodaira-Hasegawa-like procedure as implemented in the PhyML (Guindon and Gascuel, 2003; Anisimova and Gascuel, 2006) were used. Sites with gaps were excluded from the analyses, resulting in 275 and 195 shared amino acid sites among 30 HIF-1,2,3 α and 28 PHD aligned sequences, respectively. It is worth noting that while potentially a larger sample of representative HIF- α and PHD homologs from draft animal genomes was available, our aim was to maximize the number of shared amino acid sites within the alignment. Particularly, the PHD alignment was constrained by the length of available partial oyster PHD sequence (putatively translated into 230 amino acids). Further, because of the draft state of some genome assemblies, only those sequences that shared the maximum number of amino acid sites with the oyster were included in the phylogenetic analyses. HIF- α and PHD trees were rooted with (a) HIF- α homologs from *C. elegans* (**F38A6.3**) (Jiang et al., 2001) and *C. briggsae*, and (b) ELG-9 homologs from *C. elegans* (**F22E12.4**), *C. briggsae* (**CBG09602**) and *C. remanei* (**CRE12835**), respectively.

Protein domains were predicted using Pfam protein families database (<http://pfam.sanger.ac.uk/>), with “global and local (merged)” search strategy against Pfam-A families, with E value cut-off of 0.1 (Finn et al., 2008).

HIF- α and PHD mRNA expression—mRNA expression of target genes was determined using quantitative real-time PCR (qRT-PCR) using a LightCycler[®] 2.0 Real Time PCR System (Roche Applied Science, Indianapolis, IN, USA) and QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturers’ instructions. The qRT-PCR reaction mixture consisted of 5 μ L of 2 $\times$ QuantiTect SYBR Green master mix, 0.3 μ M of each forward and reverse primers, 1 μ L of 10 $\times$ diluted cDNA template and water to adjust to 10 μ L. Ten microliters of reaction mixture were loaded into LightCycler 20 μ L capillaries (Roche Applied Science, Indianapolis, IN, USA) and subjected to the following cycling: 15 min at 95 $^{\circ}$ C to denature DNA and activate Taq polymerase; 50 cycles of 15 s at 94 $^{\circ}$ C, 20 s at 55 $^{\circ}$ C and 15 s at 72 $^{\circ}$ C. SYBR Green fluorescence (acquisition wavelength 530 nm) was measured at the end of each cycle for 2 s at the read temperature of 78 $^{\circ}$ C (to melt all primer dimers but not the amplified gene product). Serial dilutions of a cDNA standard were amplified in each run to determine amplification efficiency (Pfaffl, 2001), and an internal standard of cDNA was included to test for run-to-run amplification variability. Expression of target genes was normalized relative to the expression of β -actin or 18S RNA and against the internal standard as described elsewhere (Pfaffl, 2001; Sanni et al., 2008). A single sample of cDNA isolated from the gills of a control oyster was used as an internal reference in all qRT-PCR assays. Specific primers were designed to amplify cDNA using *C. virginica*

sequences obtained in this study (for HIF- α and PHD) or published in NCBI (for β -actin and 18S rRNA, accession numbers **X75894.1** and **L78851.1**, respectively). Expected product lengths in qRT-PCR were 150, 200, 134 bp and 124 bp for HIF- α , PHD, β -actin and 18S rRNA, respectively. Amplification efficiencies were 2.05 ± 0.13 (N=4), 2.36 ± 0.14 (N=5), 1.98 ± 0.04 (N=4) and 1.90 ± 0.08 (N=4) for HIF- α , PHD, β -actin and 18S rRNA, respectively. Pilot studies demonstrated that relative expression of target gene mRNAs (HIF- α or PHD) show qualitatively similar patterns when standardized to β -actin or to 18S ribosomal (rRNA) mRNA (data not shown). However, 18S rRNA mRNA expression was significantly affected by tissue and experimental condition (ANOVA: $F_{3, 83}=5.01$, $p=0.003$ and $F_{3, 83}=5.55$, $p=0.002$, respectively), whereas β -actin mRNA expression did not vary between tissues and experimental conditions (ANOVA: $F_{3, 83}=1.71$, $p=0.172$ and $F_{3, 83}=1.52$, $p=0.216$, respectively). Therefore, in all further analyses the target gene mRNA expression was standardized relative to β -actin mRNA.

Statistical analysis

Generalized Linear Model (GLM) ANOVA followed by Fisher's least square difference (LSD) post-hoc test was used for determination of the effects of acclimation temperature, Cd and hypoxia on relative HIF- α and PHD mRNA expression in oysters. The following comparisons were performed using LSD test: 1) within each tissue type, all experimental treatment groups were compared to each other and to the respective controls; 2) within each experimental treatment group, mRNA levels for the target genes were compared between different tissues. Statistical analyses were performed using SAS 8.2 software (SAS Institute, Cary NC, USA). Differences were considered significant if the probability for Type I error (P) was less than 0.05.

Results

Molecular characterization of eastern oyster HIF- α and phylogenetic relationships among animal HIF- α homologs

Amino acid alignment of the putative translation of HIF- α gene from eastern oyster with other HIF protein sequences from representative invertebrate and chordate genomes and comparison with the PFAM database reveals a helix-loop-helix (HLH) DNA-binding domain and two PAS fold domains shared among all sequences, as well as hypoxia-inducible factor-1 domain shared among all but *C. elegans* sequences (Fig. 1). Notably, HLH domain was also detected in all three human HIF sequences, although with less significant PFAM matches (with cut-off E values < 0.2). Both N- and C-terminus oxygen-dependent degradation domains (ODDDs) that mediate rapid degradation of HIF- α under normoxic conditions were detected in the sequence from *C. virginica*. They were located downstream of the second PAS domain, similarly to those in HIF- α sequence from grass shrimp (Li and Brouwer, 2007) and in a reported sequence from a closely related Pacific oyster species (100% amino acid sequence identity between these three sequences at 6-aa long N-ODDD, and 85% sequence identity between C-ODDD motifs of shrimps and oysters). These domains were also shared with human sequences, with both N- and C-ODDD domains present in orthologous positions in HIF-1 α and EPAS1 (HIF-2 α) sequences. Interestingly, N-ODDD was missing in the human HIF-3 α while C-ODDD shared only ~70% amino acid identity with corresponding C-ODDD domain between human HIF-3 α and HIF-1 α . There were no detectable ODD domains identified in sequences from *Caenorhabditis*.

Minimum evolution (ME) tree of representative animal HIF amino acid sequences, reconstructed using Dayhoff model, is shown on Fig. 2. Here HIF- α protein sequence from the eastern oyster is clustered with the sequence from the Pacific oyster with a 100%

bootstrap support. This molluscan clade is clustered with other invertebrate sequences, although the relationships among major groups of arthropods (i.e., insects and crustaceans) are not strongly supported. Notably, sequence of a HIF- α homolog from lancelet (cephalochordate) was clustered closer to vertebrate clade than to invertebrate sequences, although this internal branch was relatively weakly supported by 68% bootstrap value (Fig. 2). Vertebrate sequences formed a separate cluster, supported by a 70% bootstrap value. Among vertebrates, HIF sequences were clustered into three clades corresponding to HIF-1 α , HIF-2 α and HIF-3 α supported by high bootstrap values of 100%, 100% and 91%, respectively. Vertebrate HIF-1 α and HIF-2 α clades were clustered together, although the respective internal branch received relatively weak bootstrap support of 66%. Within each vertebrate clade, fish sequences are clustered together with a strong bootstrap support (100%), basally to sequences from tetrapods (mammals, birds and reptiles). Likewise, sequences from mammals (human and chimp) and birds (chicken and zebrafish) are appropriately clustered together with 100% bootstrap support (Fig. 2). Essentially the same clustering pattern was revealed by a ML tree reconstructed using WAG+gamma+inv substitution model (Supplementary Figure 1), with two oyster sequences clustered together (supported by 100% bootstrap value). Notably, on the ML tree, oyster sequences clustered basally to the lancelet and vertebrate HIF sequences, although this clustering was very weakly supported by 51% bootstrap value, and likely reflects lack of taxa closely related to mollusks available for this analysis. Within vertebrate clade (supported by 80% bootstrap), HIF-3 α clustered separately from HIF-1 α and HIF-2 α on the ML and the ME trees (cf. Fig. 2 and Supplementary Figure 1). Similar ML tree topology was also obtained using the LG model that has been shown to outperform both WAG and JTT substitution models (Le and Gascuel 2008) (results not shown).

We also reconstructed the ME tree using a subset of 26 HIF sequences that maximizes the number of shared amino acid sites to 518 amino acids (i.e., shorter sequences of lancelet and other species were excluded). In agreement with above results, oyster sequences were clustered together with other invertebrate sequences, while vertebrate clade was strongly supported by 86% bootstrap value, with HIF-1 α and HIF-2 α vertebrate homologs clustered together with 74% bootstrap support (data not shown).

To maximize the number of taxa, we also included available partial HIF ETS sequences from other molluscan and annelid species (Supplementary Figure 2), which resulted in a tree topology similar to that presented on Fig. 1, although the analysis involving ETS sequences could only be conducted on 177 shared amino acid sites. This tree shows a stronger support of oyster clade being clustered with other invertebrate species, with 75% bootstrap support of the molluscan clade.

Molecular characterization of eastern oyster PHD and phylogenetic relationships among animal PHD/EGL-9 homologs

Comparison of the amino acid alignment of the putative translation of partial PHD gene from the eastern oyster with other PHD/EGL-9 homologous protein sequences from representative invertebrate and vertebrate genomes with the PFAM database reveals presence of shared 2-oxoglutarate, Fe²⁺-dependent (2OG-Fe(II)) oxygenase superfamily domain (Fig. 3). Within this domain, oyster sequence shared about 70% amino acid sequence identity with respective domains in three human homologs, and about 61% sequence identity with respective domains in nematode homologs. Within this domain, a stretch of 11 amino acids (FWSDRRNPHVEV) including several predicted catalytic residues (Aravind and Koonin, 2001) was perfectly conserved in all surveyed sequences (Fig. 3).

ME tree of representative animal EGL-9 homologous amino acid sequences, reconstructed using Dayhoff model, is shown on Fig. 4. Here PHD protein sequence from oyster is

clustered outside of both the insect clade and the chordate clade (the latter including the lancelet), although with very weak bootstrap support < 50%. This lack of resolution of phylogenetic relationships can be attributed to poor sampling of taxa closely related to oysters (and mollusks overall). In fact, to the best of our knowledge, sequence of PHD homolog from *C. virginica* obtained in this study is the first PHD sequence reported from mollusks. Interestingly, within vertebrate clade, PHD1, PHD2 and PHD3 sequences form three strongly-supported clusters indicating that duplications events and subsequent diversification of EGL-9 homologs occurred during the evolution of vertebrates. Notably, clustering of oyster sequence outside of either vertebrate cluster suggests that this gene is equally distant to either one of the three vertebrate loci. Similar clustering pattern was observed on a ML tree reconstructed with WAG+gamma+inv model, although the weakly supported positions of oyster and lancelet sequences as basal to the vertebrate clade were switched (Supplemental Figure 3).

It is worth noting that analysis involving ETS sequences (similar to that conducted for HIF- α and shown on Supplementary Figure 2) could not be done on PHD/EGLN because only short EGLN-like EST sequences were available for molluscs and annelids, resulting in less than 100 shared amino acid sites with other PHD/EGLN sequences and hindering the reconstruction of a reliable phylogenetic tree. Therefore, our discussion of HIF- α and PHD evolution is based on the results obtained with the longest shared sequences. To further improve the resolution of deep divergences among invertebrates, new full-length sequences representatives of other major proteostome taxa need to be identified and included in the analysis, particularly those of lophotrochozoans (e.g., Annelida and Platyhelminthes).

Tissue specific HIF- α and PHD mRNA expression

HIF- α and PHD were expressed at the mRNA level in all studied oyster tissues (Fig. 5). Both experimental conditions and tissue had a significant effect on expression of HIF- α and PHD in oysters (ANOVA: $F_{4, 74}=4.52$, $P=0.003$ and $F_{4, 73}=3.57$, $P=0.010$ for tissue effects on HIF- α and PHD, respectively; $F_{3, 74}=2.94$, $P=0.038$, and $F_{3, 73}=16.41$, $P<0.0001$ for the overall effect of experimental conditions on HIF- α and PHD, respectively). HIF- α mRNA was approximately equally expressed in the four studied tissues (gill, hepatopancreas, mantle and muscle) under normoxic conditions ($PO_2 \sim 153$ Torr or ~ 20 kPa), as well as after prolonged (6 days) anoxia ($PO_2 < 1$ Torr or < 0.1 kPa) and after 30 days of Cd exposure ($50 \mu\text{g L}^{-1}$ Cd) ($P>0.05$ for comparisons between different tissue types within the respective experimental conditions). In contrast, during hypoxia ($PO_2 \sim 36$ or ~ 5 kPa), HIF- α was significantly elevated in the gills compared to other tissues (Fig. 5A).

Expression of PHD mRNA tended to be higher in hepatopancreas compared to other tissues under all experimental conditions (9–24 times higher than in gills, 2–12 times higher than in mantle and 4–7 times higher than in muscle) except hypoxia where PHD mRNA expression was equally high in gills and hepatopancreas and significantly above the levels found in mantle and muscle (2- and 7-fold higher, respectively) (Fig. 5B). Overall, PHD mRNA expression was elevated in hypoxia compared to normoxia and anoxia in the gill tissue (Fig. 5A). PHD mRNA expression was significantly lower in gills and hepatopancreas of Cd-exposed oysters compared to their hypoxic counterparts; differences from the normoxic controls were not significant due to the high individual variation of PHD mRNA levels in normoxic gills (Fig. 5B).

Discussion

Evolutionary relationships of HIF- α and PHD genes from *C. virginica*

Due to insufficient taxon sampling, the evolutionary relationships between the homologs of HIF- α from different groups of animals remain poorly resolved (e.g., Sogawa and Fujii-Kuriyama, 1997; Li and Brouwer, 2009). Some recent studies that focused exclusively on vertebrate genes achieved good tree resolution (Rytkönen et al., 2007; Cao et al., 2008; Rytkönen et al., 2008); however, they do not provide an insight into the relationship between the three isoforms HIF-1 α , HIF-2 α and HIF-3 α and their invertebrate homologs. Similarly, very little is known about relationships between PHD homologs from invertebrates and vertebrates (e.g., Taylor, 2001). Availability of numerous completely sequenced or draft assemblies of vertebrate and invertebrate genomes provided us with an opportunity to overcome these limitations, and to conduct more detailed phylogenetic analyses of the relationships of these genes in animals.

Analysis of the domain structure of HIF- α homologs from oysters, shrimp, human and a nematode indicate that the main functional domains of this protein (including HLH DNA-binding domain, PAS folds and HIF-1 domain) are conserved between invertebrates and vertebrates. Furthermore, HIF- α homologs from oysters and shrimps shared N- and C-terminal oxygen-dependent degradation domains (ODDD) with the vertebrates; in contrast, N-ODDD was not detected in nematodes. In vertebrates, ODDDs play a key role in PHD-dependent post-translational regulation of HIF- α (Semenza, 2004), and their absence in HIF- α homolog from nematodes may suggest a difference in the oxygen-dependent regulation of this gene. While molecular mechanisms responsible for the oxygen-sensing function of this protein are not fully understood in *C. elegans*, experimental data indicates that HIF is required for their hypoxic survival suggesting that fundamental features responsible for function of this protein in hypoxic environment are likely to be present in nematodes despite the absence of N-ODD domain (Jiang et al., 2001; Shen et al., 2005).

PHD (EGLN) oyster sequences share a 2OG-Fe(II) oxygenase superfamily domain that is common to other members of EGLN family with prolyl hydroxylase functions (Aravind and Koonin, 2001). Our analysis also identified several catalytically important residues outside the 2OG-Fe(II) oxygenase superfamily domain that are highly conserved between PHD homologs from oysters, humans and a nematode (Fig. 3). This structural similarity suggests possible functional similarity between PHD homologs from oysters and those from the better studied mammalian and nematode models. In mammals, all three PHD isoforms are involved in oxygen homeostasis with each enzyme playing a specific non-redundant role in the regulation of HIF-1 α and HIF-2 α ; PHD1 and PHD3 also appear involved in other cellular processes unrelated to their oxygen-sensing function (Berra et al., 2003; Appelhoff et al., 2004). PHD homologs from the model invertebrates (*C. elegans* and *Drosophila*) are also involved in the oxygen-dependent regulation of HIF- α proteins (Gorr et al., 2006). This suggests that oxygen sensing is an ancient function of PHD homologs common to all three PHD isoforms from vertebrates and invertebrate PHD/EGL-9, and thus likely to be also present in the molluscan PDH homologs.

Our study clearly shows that invertebrate HIF- α homologs are ancestral to the vertebrate branch containing HIF-1 α , -2 α and -3 α genes (Fig. 2 and Suppl. Fig. 1). There is no indication in our phylogenetic trees that HIF-1 α homologs from tetrapods are closer to the ancestral forms of this gene than HIF-1 α from fish, contrary to the conclusions derived from a previous study that focused exclusively on HIF-1 α sequences from vertebrates (Rytkönen et al., 2007). Similar to HIF- α homologs, PHD sequence from oyster clustered basally outside of vertebrate PHD (EGLN) clade, strongly supported by 100% bootstrap values of the respective internal branches in both ME (Fig. 4) and ML trees (Suppl. Fig. 3). Within

vertebrates, HIF-1 α and HIF-3 α are clustered together and separately from HIF-2 α (EPAS1), although the respective internal branch was only weakly supported by 66% and 58% bootstrap support values in ME and ML phylogenetic trees, respectively. Notably, none of invertebrate HIF- α sequences clustered inside the vertebrate lineage indicating lack of direct orthology between these genes and corresponding vertebrate HIF- α homologs. Thus, the commonly used annotations of the respective invertebrate genes as HIF-1 α or PHD2 are not strictly speaking correct, since they are no closer to HIF-1 α or PHD2 of vertebrates than to any other homolog from the respective branch (e.g., HIF-2 α or -3 α , or PHD1 and PHD3). Thus, it is more appropriate to refer to the invertebrate sequences as HIF- α and PHD homologs.

Our study shows that duplication events leading to subsequent diversification into three copies in vertebrate genomes for both HIF- α and PHD (EGLN) homologs have likely occurred during the evolution of vertebrates after the split between cephalochordate and chordates about 750 MYA (Hedges and Kumar, 2003). Sequence diversification of HIF- α and PHD (EGLN) homologs within vertebrates indicates that oxygen sensing pathways diversified in the vertebrates after their split from cephalochordates. This raises a possibility that despite overall amino acid sequence similarity, functional features and regulation of invertebrate representatives of HIF- α and PHD loci may be different from that of their vertebrate counterparts and combine some features from all three vertebrate homologs. Interestingly, previous studies in invertebrate (insects and nematodes) and vertebrate (fish and frogs) ectotherms show that HIF- α homologs also play a role in adaptation to heat, cold and in embryonic and larval development indicating that these proteins may have important cellular functions beyond oxygen sensing (Vuori et al., 2004; Morin et al., 2005; Heise et al., 2006a; Heise et al., 2006b; Rissanen et al., 2006b; Heise et al., 2007). Further studies are needed to elucidate the relative importance and relationships between different cellular functions of HIF- α -related proteins, and invertebrate HIF- α homologs could provide a useful model for the ancestral protein combining these multiple functions.

Tissue-specific mRNA expression of HIF- α and PDH homologs in oysters

In the absence of the specific molluscan antibodies against HIF- α and PHD, we have used mRNA levels to determine whether transcriptional regulation of HIF pathway contributes to the cellular response to hypoxia and anoxia in oysters. While in mammalian cell lines the primary mechanism that regulates HIF- α abundance occurs at the post-translational level, via the oxygen- and PHD-dependent proteosomal degradation of HIF- α protein, recent studies in fish and crustaceans, as well as some *in vivo* mammalian models indicate that transcription of HIF-1 α gene can also be regulated by hypoxia and other stressors and thus may play a role in the adaptive response to oxygen deficiency complementing a more rapid post-transcriptional regulation of HIF-1 α protein (Ke and Costa, 2006; Rissanen et al., 2006b; Sollid et al., 2006; Nikinmaa et al., 2008; Soñanez-Organis et al., 2009).

Transcriptional regulation also plays an important role in regulation of HIF-3 α in mammals indicating that at least some HIF- α isoforms are regulated at both transcriptional and post-translational levels (Heidbreder et al., 2003). Although measuring transcript levels is likely a conservative estimate of the pathway's response that occurs an addition to (and usually later than) the post-transcriptional regulation of HIF- α abundance by PHDs, understanding of the changes in HIF- α and PHD mRNA may shed light on the role of the transcriptional mechanisms in response of HIF pathway to oxygen limitation in oysters.

Our study showed that mRNA of HIF- α and PHD homologs of oysters are constitutively expressed in all studied oyster tissues. HIF- α transcript levels tend to be higher in the gills, whereas PHD transcripts are more abundant in hepatopancreas tissues of oysters irrespective of the experimental treatments. Earlier studies in vertebrates (including mammals and fish) have also demonstrated tissue-specific expression of HIF-1 α at the mRNA and protein levels

(Stroka et al., 2001; Rissanen et al., 2006b; Soñanez-Organis et al., 2009). It has been proposed that high basal expression of HIF-1 α in normoxia and hypoxic regulation of its mRNA levels found in hypoxia-tolerant fish and mammals (such as mole rat) but not in their hypoxia-sensitive counterparts may be an adaptive feature enhancing hypoxia tolerance of animals (Rissanen et al., 2006b). This is consistent with the high abundance of HIF- α transcripts in hypoxia-tolerant eastern oysters in all studied tissues, comparable with the transcript abundance of common housekeeping genes such as β -actin. In the future, it would be important to evaluate whether this pattern also holds true for HIF- α protein once antibodies specific to HIF- α homolog from oysters become available.

In eastern oysters, transcript levels of HIF- α homolog were not significantly affected by prolonged anoxia, hypoxia or Cd exposure in most tissues except the gills where the mRNA levels of HIF- α homolog increased after 2 weeks of exposure to moderate hypoxia (PO₂~36 Torr or ~5 kPa). This absence of a strong transcriptional response may be due to the long acclimation period under the experimental conditions ranging from 6 days of anoxia to several weeks in hypoxia and Cd exposure. In vertebrates, HIF-1 α response to hypoxia is often transient and observed only during the first few hours after the onset of hypoxic conditions (Stroka et al., 2001; Rissanen et al., 2006b). In ectotherms such as fish this transcriptional response can also be modified by other factors such as acclimation temperature. Thus, in crucian carp *Carassius carassius*, temperature and hypoxia interactively affected HIF-1 α expression so that HIF-1 α mRNA levels increased transiently in the gills and/or the kidney of the carp after 6–48 h of hypoxia (0.7 mg O₂ L⁻¹) at two extreme acclimation temperatures (8°C and 26°C) but not at 18°C (Rissanen et al., 2006b). A transient increase in HIF-1 α transcript levels were also found after 4 h of copper exposure (1.65 μ M) in the rainbow trout (*Oncorhynchus mykiss*) which disappeared after 24 h of exposure (Heerden et al., 2004). In contrast, moderate hypoxia (1.5 and 2.5 mg O₂ L⁻¹) had no effect on the mRNA levels of HIF- α homolog in the white shrimp *Litopenaeus vannamei* during the 1st hour of exposure, whereas after 24 h at 1.5 mg O₂ L⁻¹ mRNA levels of HIF- α homolog were significantly suppressed in hepatopancreas and muscle (Soñanez-Organis et al., 2009). Anoxia induced either by nitrogen atmosphere or by freezing for 24 h led to a strong upregulation of transcript levels of HIF- α homologs in a gall fly larva (*Eurosta solidaginis*) (Morin et al., 2005). Overall, the transcriptional response of HIF- α homologs to oxygen deficiency appears to be variable depending on the level of hypoxia, duration of the exposure and species-specific differences which in turn may be related to the degree of their hypoxia tolerance.

Expression of mRNA of PHD homologs was largely unaffected by prolonged anoxia or Cd exposure in the studied tissues of eastern oysters. However, after 2 weeks of exposure to moderate hypoxia (PO₂~36 Torr or ~5 kPa) mRNA levels of oyster PHD increased in the gill tissues similar to the situation found for the HIF- α homolog. Such relatively high sensitivity of the oxygen-sensing pathway to hypoxia in the gills (compared to other tissues) may reflect the fact that gills are the main organ involved in gas exchange in oysters and therefore, play a key role in sensing hypoxia and the compensatory response to it. Elevated expression and activity of PHDs was previously found during hypoxia exposure in mammalian cells and in a nematode *C. elegans* and may serve as a feedback mechanism providing protection against chronic hypoxia (Ginouvé et al., 2008) and allowing for a rapid HIF- α breakdown upon reoxygenation (D'Angelo et al., 2003). This upregulation of PHDs is at least partially mediated by HIF transcriptional regulation (Aprelikova et al., 2004), consistent with the observed parallel increase in mRNA levels of HIF- α and PHD homologs in hypoxic oyster gills.

Conclusions—The novel HIF- α and PHD homologs from eastern oysters *C. virginica* show significant sequence similarity and share key functional domains with the earlier

described HIF and PHD isoforms from vertebrates and invertebrates implying the highly conserved function of these genes throughout the evolutionary history of animals. These genes are broadly expressed at the mRNA level in different oyster tissues and show transcriptional response to prolonged hypoxia in the gills consistent with their putative role in oxygen sensing and the adaptive response to hypoxia. Diversification of HIF and PHD isoforms which occurred in vertebrates suggests functional diversification and specialization of the oxygen-sensing pathways in this group, which parallels situation observed for many other important genes (e.g. Sokolova and Sokolov, 2005 for mitochondrial uncoupling proteins, and Ivanina et al., 2010 for nitric oxide synthase, to give but a few examples). Such increased gene diversity could have played an important role in evolution of the new gene functions and thus in adaptive radiation and evolutionary success of the vertebrates.

Supplementary Material

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Fig. 1. Multiple sequence alignment of partial amino acid sequence of HIF- α from eastern oyster with HIF sequences from Pacific oyster, shrimps, nematodes (*C. elegans* and *C. briggsae*) and human

Helix-loop-helix (HLH) DNA binding domain and PAS fold domains are shown with striped and white boxes, respectively, as per PFAM prediction and Li and Brouwer (2007). Identical residues are shown with dots (.), and gaps are indicated as dashes (-). The mRNA sequence for HIF- α transcript from *C. virginica* is deposited in GenBank (NCBI accession number [HM441076](#)).

Fig. 2. Minimum evolution tree of representative metazoan HIF- α sequences based on the Dayhoff distance

Support of internal branches was evaluated using the bootstrap method, and only values higher than 50% are shown. HIF- α sequences from nematodes were used as outgroup. Each sequence is identified by the name of the species and its Ensembl or GenBank accession number. Detailed list of sequences used is given in Suppl. Table 1.

Fig. 3. Multiple sequence alignment of partial amino acid sequence of PHD from eastern oyster with EGLN-1,-2 and -3 sequences from human and EGL-9 homolog from *Caenorhabditis elegans* Shared 2-oxoglutarate- and Fe²⁺-dependent oxygenase domain (2OG-FeII_Oxy) is shown with white and shaded boxes, as per PFAM prediction and Aravind and Koonin (2001), respectively. Asterisks (*) and underscores () mark predicted catalytic residues and residues that share physicochemical properties among the entire 2OG-Fe(II) dioxygenase superfamily, respectively, per Aravind and Koonin (2001). The mRNA sequence for PHD homolog from *C. virginica* is deposited in GenBank (NCBI accession number [HM441077](#)).

Fig. 4. Minimum evolution tree of representative metazoan PHD sequences based on the Dayhoff distance

Support of internal branches was evaluated using the bootstrap method, and only values higher than 50% are shown. EGLN-9 sequences from nematodes were used as outgroup. Each sequence is identified by the name of the species and its Ensembl or GenBank accession number. Detailed list of sequences used is given in Suppl. Table 2.

Fig. 5. Tissue-specific mRNA expression of HIF- α (A) and PHD (B) in *C. virginica* under different experimental conditions

mRNA expression was determined using qRT-PCR in tissues of control oysters (normoxia), oysters maintained for 2 weeks in 5% oxygen (hypoxia), exposed for 6 days in air with no gapping (anoxia) or for 30 days to 50 $\mu\text{g L}^{-1}$ Cd in normoxia (Cd). HP – hepatopancreas. A post-hoc LSD test was used to carry out the following comparisons: 1) within each tissue type, all experimental treatment groups were compared to each other and to the respective controls; 2) within each treatment group, mRNA levels for the target genes were compared between different tissues. For the first type of comparisons, asterisks in panel B indicate values significantly different from the respective hypoxic values in the same tissue type ($P < 0.05$); all other experimental conditions were not significantly different from each other or from the respective controls ($P > 0.05$). For the second type of comparisons, different letters denote values that are significantly different between the tissues within each experimental condition ($P < 0.05$). No letters mean that there were no significant differences in gene expression between different tissues within the given experimental condition (e.g. in normoxic, hypoxic and Cd-exposed groups) ($P > 0.05$). $N = 4-10$.

Table 1

Primer sequences and PCR product characteristics used for isolation of gene fragments and for quantitative real time PCR (qRT-PCR) of HIF- α and PHD homologs from *C. virginica*

Gene		Primers	T _{ann} , °C
HIF- α	HIF-1F326 ^a	CTGCTCCGATCTGATCAACA	55
	HIF-1R ^a	CATACGTGTCCCCCTGTCTT	
	CvHIF5R1 ^b	TGGGAAGTAGAACTCGTCGACTTTG	55
	CvHIF5R2 ^b	ATTGTGTCTGCTGAGGAAGGTCATC	55
	CvHIF5R3 ^b	GCCTCATGATGGATGCCTTGTCCAG	55
	CvHIF3F1 ^b	AAGTTTTCTGGGAAGTTGGTCAAGG	55
	CvHIF3F2 ^b	TCCACTTCCCAGCAGTCTTACAGTC	55
	CvHIF3F3 ^b	ATGAGGGCCCCCTTACATCCCCA	55
PHD	PHD-1 FW1 ^c	ATGGTTGCNTGYTAYCCNGG	55
	PHD-1 RV ^c	GGGTTCTACDRTCNSWCCARAA	
	PHD2-F ^a	CGTCATGTCGATAATCCAAAC	55
	PHD2-R ^a	ATGTGGATTGCGTCTATCTGACCA	
	CvPHD1-3F1 ^b	CGTCATGTCGATAATCCAAAC	50
	CvPHD1-3F2 ^b	TGGTCAGATAGACGCAATCCACA	52
	CvPHD1-5R1 ^b	ATGTGGATTGCGTCTATCTGACCA	50
	CvPHD1-5R2 ^b	GTTGAGGTAATAAATACAGGTGAT	52
β -actin	Act-Cv-F437 ^a	CACAGCCGCTTCCTCATCCTCC	55
	Act-Cv-R571 ^a	CCGGCGGATTCCATACCAAGG	55
18S rRNA	18SrRNA-FW	GGTAACGGGGAATCAGGGTTCGAT	55
	18SrRNA-RV	TGTTATTTTCGTCACCTCCCCGT	55

All primer sequences are given in 5'-to 3'-end orientation.

^a primers used for qRT-PCR,

^b primers used for RACE PCR,

^c degenerate primers.

For primers used in qRT-PCR, expected length of amplified product (bp) and amplification efficiency (E) are given. T_{ann} — annealing temperature.