

Assessment of hypoxia-inducible factor-1 α mRNA expression in mantis shrimp as a biomarker of environmental hypoxia exposure

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Efforts to assess the ecological impacts of the marked increase in coastal hypoxia worldwide have been hampered by a lack of biomarkers of hypoxia exposure in marine benthic organisms. Here, we show that hypoxia-inducible factor-1 α (HIF-1 α) transcript levels in the heart and cerebral ganglion of mantis shrimp (*Oratosquilla oratoria*) collected from hypoxic sites in Tokyo Bay are elevated several-fold over those in shrimp collected from normoxic sites. Upregulation of HIF-1 α mRNA levels in the heart after exposure to sub-lethal hypoxia was confirmed in controlled laboratory experiments. HIF-1 α transcript levels were increased at approximately threefold after 7 and 14 days of hypoxia exposure and declined to control levels within 24 h of restoration to normoxic conditions. The results provide the first evidence for upregulation of HIF-1 α transcript levels in two hypoxia-sensitive organs, heart and cerebral ganglion, in a marine invertebrate exposed to environmental hypoxia. These results suggest that upregulation of HIF-1 α transcript levels is an important component in adaptation of mantis shrimp to chronic hypoxia and is a potentially useful biomarker of environmental hypoxia exposure.

Keywords: hypoxia-inducible factor; biomarker; coastal hypoxia; marine invertebrate; mRNA expression; mantis shrimp

1. INTRODUCTION

Chronic hypoxia exposure (dissolved oxygen, DO: less than or equal to 2 ml l⁻¹ (2.8 mg l⁻¹); see discussion on definition of hypoxia in the electronic supplementary material) has been shown to have deleterious effects on growth, metabolism, reproduction and development as well as population size in aquatic organisms [1–4]. Aquatic benthic organisms are often exposed to environmental hypoxia induced by anthropogenic eutrophication [1,5]. However, the

extent and duration of exposure to low DO conditions cannot be assessed owing to a lack of reliable and sensitive biomarkers of hypoxia exposure in these organisms. Such biomarkers are of critical importance for understanding the responses of mobile benthic organisms to low DO and the long-term effects because their exposure to hypoxia may be intermittent due to migration between hypoxic and non-hypoxic areas [6]. Hypoxia-inducible factor-1 α (HIF-1 α) is an oxygen-sensitive transcription factor which regulates the expression of numerous genes involved in adaptation to hypoxia [7,8], and therefore is a potential molecular biomarker of environmental hypoxia exposure. In fact, increased HIF-1 α mRNA and protein levels have been reported in a marine teleost, Atlantic croaker (*Micropogonias undulatus*), collected from hypoxic field sites [2,3].

Here, we examine the applicability of HIF-1 α transcript levels as a molecular biomarker of chronic hypoxia exposure in a representative marine megabenthic species, mantis shrimp (*Oratosquilla oratoria*). Mantis shrimp is a suitable model species for studying the responses to hypoxic stress since it inhabits the muddy bottom sediment where hypoxia is often evident and is relatively hypoxia-tolerant [4]. In this study, we cloned and characterized mantis shrimp HIF-1 α and then investigated the expression of HIF-1 α mRNA in individuals exposed to low DO both in Tokyo Bay and in the laboratory.

2. MATERIAL AND METHODS

Additional details of the methods are described in the electronic supplementary material.

(a) Collection of field samples

Mantis shrimp were collected with bottom trawls at seven sites in August 2009 from Tokyo Bay (figure 1a). The sites were categorized into four groups: normoxic reference, R (mean temperature: 20.9°C, and DO: 3.5 ml l⁻¹, 67.9% saturation (S)); hypoxic, H (temperature: 18.2°C, DO: 0.6 ml l⁻¹, 11.9% S); transition between hypoxic and normoxic, T (temperature: 18.1°C, DO: 2.3 ml l⁻¹, 42.4% S); and central normoxic (CN) sites where hypoxia occurs intermittently (temperature: 20.0°C, DO: 3.3 ml l⁻¹, 51.4% S; electronic supplementary material, table S1).

(b) Laboratory hypoxia experiments

Two-week hypoxia exposure experiments were conducted under controlled laboratory conditions at 21.4°C ~ 22.2°C to reveal the time-course of HIF-1 α mRNA expression. Two DO levels were selected: 4.2 ml l⁻¹, 82.5 per cent S for normoxic conditions and 1.2 ml l⁻¹, 23.6 per cent S for the hypoxic treatment which was achieved by bubbling nitrogen gas into the aquarium (electronic supplementary material, table S2). Mantis shrimp were sampled at 0, 1, 3, 7 and 14 days after the reduction of DO levels. After two weeks of hypoxia exposure, the DO level in the hypoxic treatment tank was restored to normoxic conditions and shrimp were sampled at 3, 6, 12 and 24 h later.

(c) Cloning, sequencing and quantitative real-time PCR

The molecular protocols used to clone HIF-1 α were similar to those described previously [9]. Briefly, total RNA was extracted from pooled cerebral ganglia and treated with DNase to prevent genomic DNA contamination. The PCR products were ligated, cloned into a vector, transformed into competent cells and sequenced. The full-length sequence of mantis shrimp HIF-1 α cDNA was obtained by 5'- and 3'-RACE. Sequence identity was verified using the BLAST program and NCBI database. The expression of HIF-1 α mRNA among different tissues was determined by RT-PCR analysis using gene-specific primers (electronic supplementary material, table S3). HIF-1 α mRNA levels in mantis shrimp heart and cerebral ganglion were quantified by quantitative real-time PCR (qRT-PCR) using actin as an internal control and are expressed as relative values per 2.5 ng total RNA using the efficiency corrected comparative Ct method.

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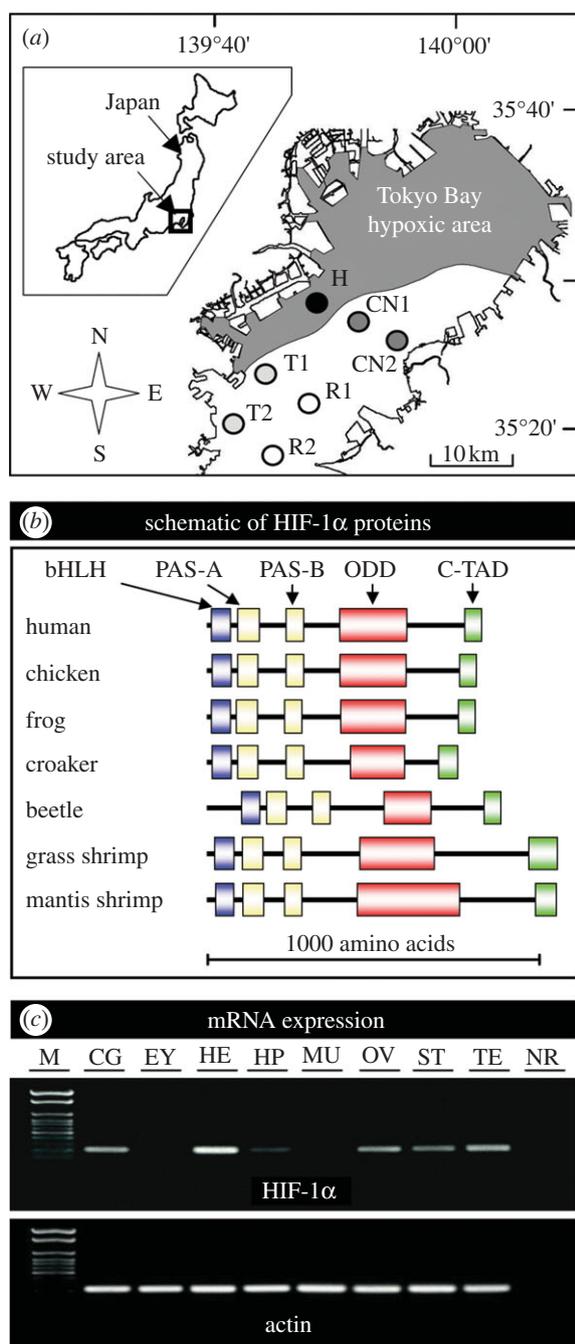


Figure 1. (a) Mantis shrimp sampling sites in Tokyo Bay. Hypoxic area (DO : less than or equal to 2 ml l^{-1}) is shaded. CN, central normoxic sites, where hypoxia occurs intermittently; H, hypoxic; T, transition between hypoxic and normoxic; and R, normoxic reference. (b) Schematic of HIF-1 α proteins (see electronic supplementary material for GenBank accession no.) showing conserved domains: bHLH, basic helix–loop–helix; PAS, Per–Arnt–Sim-A and -B; ODD, oxygen-dependent degradation domain; and C-TAD, c-terminus transactivation domain. (c) Expression of HIF-1 α mRNA in mantis shrimp tissues collected from site H. Equality of template amplification in the RT-PCR reaction was confirmed with actin. M, marker; CG, cerebral ganglion; EY, eye; HE, heart; HP, hepatopancreas; MU, muscle; OV, ovary; ST, stomach; TE, testis; NR, negative reaction.

(d) Statistical analysis

Significant differences in relative HIF-1 α mRNA levels between sampling sites for field studies and treatment groups for laboratory experiments were analysed by Dunnett's test.

3. RESULTS

(a) Molecular characterization and tissue-specific expression

The full-length mantis shrimp HIF-1 α cDNA consists of 3742 bp nucleotides with an open reading frame encoding 1050 amino acid residues (GenBank accession no. HM032914) with presumed domains that are conserved in HIF-1 α proteins from other species (figure 1b, see the electronic supplementary material for additional descriptions of characterization and phylogenetic relationship of mantis shrimp HIF-1 α protein). Interestingly, the amino acid sequence of mantis shrimp HIF-1 α , like that of other arthropods, is larger than that of vertebrate species, mainly because of the larger size of the oxygen-dependent degradation domain (figure 1b, see additional details in the electronic supplementary material).

HIF-1 α transcript levels in mantis shrimp collected from a hypoxic site in Tokyo Bay showed highest expression in the heart followed by the cerebral ganglion and gonads, whereas the transcript was weakly expressed in the hepatopancreas and stomach (figure 1c).

(b) Hypoxia-inducible factor-1 α mRNA expression: field and laboratory studies

HIF-1 α mRNA levels in the heart of shrimp collected from T, CN and H sites were 3.2, 5.5 and 5.3-fold higher than those in shrimp from reference site R, where differences in the mRNA levels were significant except between sites R and T (figures 1a and 2a). On the other hand, significant upregulation of HIF-1 α mRNA levels in the cerebral ganglion was evident only for shrimp collected at site H, where levels were 2.9-fold higher than those at site R (figure 2b).

There were no significant changes in HIF-1 α mRNA levels in shrimp heart until the third day of hypoxia exposure, but they were significantly elevated 2.8-fold after 7 and 14 days of hypoxia exposure. HIF-1 α transcript levels remained high after 3 h of normoxic recovery but had declined to control levels after 24 h in normoxic conditions (figure 2c).

4. DISCUSSION

The results of the present study show that HIF-1 α transcript levels in the heart and cerebral ganglion are upregulated several-fold in mantis shrimp collected from hypoxic sites in Tokyo Bay when compared with those collected from normoxic sites. A similar increase in HIF-1 α mRNA expression in mantis shrimp heart was observed after prolonged hypoxia exposure in controlled laboratory studies, suggesting that the upregulation in field-caught shrimp is induced by chronic exposure to environmental hypoxia. To our knowledge, this is the first report showing increased expression of HIF-1 α mRNA in an invertebrate species under hypoxic conditions in the marine environment.

Our results confirm that HIF-1 α mRNA is ubiquitously expressed in the tissues of aquatic organisms, but the relative expression in different tissues varies. HIF-1 α mRNA expression in sea bass (*Dicentrarchus labrax*) was high in the brain, heart, liver and muscle, whereas that in the kidney and spleen was low [10]. White shrimp (*Litopenaeus vannamei*) HIF-1 α mRNA levels were low in

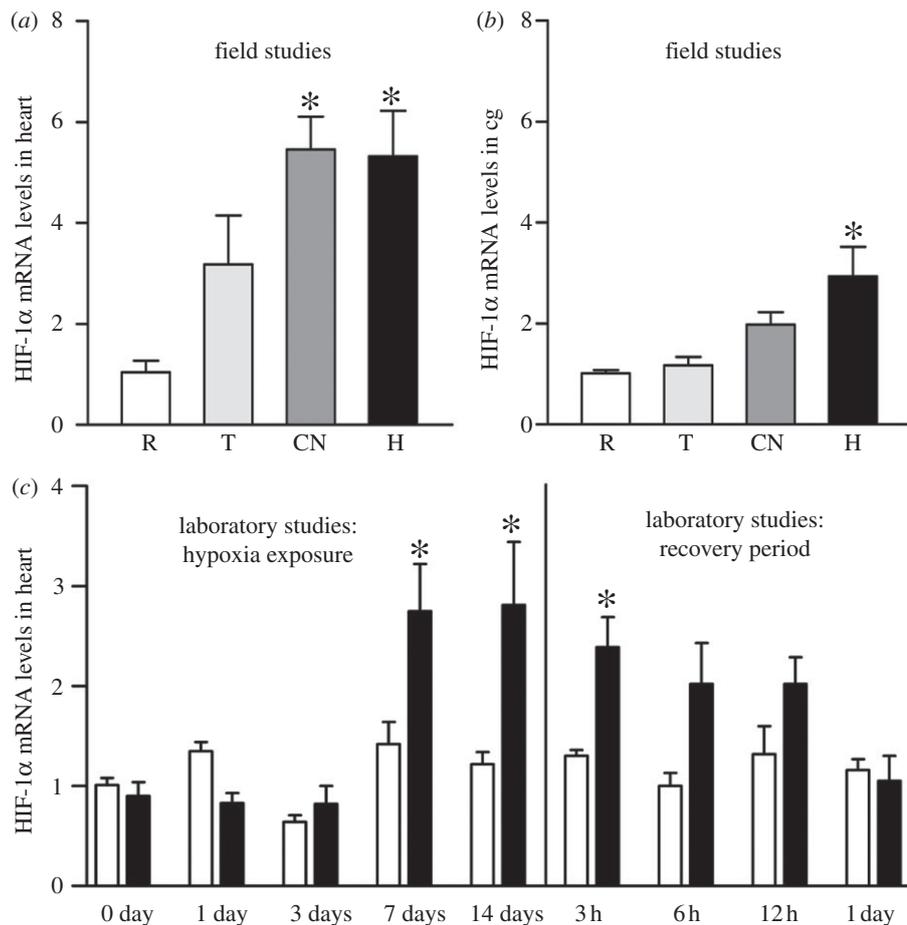


Figure 2. Relative HIF-1 α mRNA levels in (a) heart and (b) cerebral ganglion (cg) in mantis shrimp collected from the sampling sites in Tokyo Bay (see site description in figure 1a). (c) Effects of 14-day laboratory exposure to normoxia (DO: greater than 4.2 ml l⁻¹, white bars), hypoxia (DO: 1.2 ml l⁻¹, black bars) and recovery period on relative HIF-1 α mRNA levels in heart. After 14-day hypoxia exposure, DO of hypoxic treatment was restored to normoxic level (24 h recovery period). Bars mean \pm s.e.m. Field data were combined within each site group due to limited sample sizes. Differences in relative mRNA levels in mantis shrimp from site R and other sites ($n = 4-8$), or between normoxic conditions and other treatments ($n = 6$) were tested by Dunnett's test, * $p < 0.05$.

muscle and the hepatopancreas compared with those in gills [11]. We found that HIF-1 α mRNA expression in mantis shrimp was relatively high in the heart, cerebral ganglion and gonads compared with that in other tissues. The differences in the HIF-1 α transcript levels after hypoxia exposure between the various tissues may be a reflection of the requirement for a greater physiological response in certain tissues during adaptation to hypoxic conditions. For example, the cardiovascular system is critical for effective uptake of oxygen in hypoxic environments [12]. Therefore, the marked upregulation of HIF-1 α transcript levels in the heart of mantis shrimp may reflect an HIF-induced enhancement of cardiovascular system functions such as angiogenesis and vasodilation to achieve efficient oxygen transport for survival under chronic hypoxia [7,8]. In contrast, the relatively low HIF-1 α expression in the hepatopancreas of mantis shrimp collected from hypoxic sites in Tokyo Bay may indicate that physiological adaptation of this tissue to low oxygen conditions is not as dependent on HIF-1 α -induced changes in gene expression. Similarly, HIF-1 α mRNA levels in the hepatopancreas of grass shrimp (*Palaemonetes pugio*) exposed to hypoxia for 3–14 days were not significantly elevated over those in shrimp exposed to normoxic

conditions [13]. These results suggest that measurement of HIF-1 α mRNA levels in the hepatopancreas of marine benthic crustacea is not a sensitive biomarker of environmental hypoxia exposure.

The time-course of upregulation of HIF-1 α mRNA expression in the heart of mantis shrimp under low DO conditions is similar to that observed in the ovary in a relatively hypoxia-tolerant teleost, Atlantic croaker [9], with both showing maximum increases after 7-day hypoxia exposure. The hepatic HIF-1 α transcript levels in sea bass, a hypoxia-sensitive teleost, also increase after 2–15 days of moderate hypoxia exposure (51% S) [10]. However, a significant downregulation of HIF-1 α mRNA was evident in the gills and muscle of white shrimp after 24 h hypoxia exposure, which may reflect negative feedback regulation of mRNA levels [11]. Interestingly, mantis shrimp HIF-1 α mRNA levels, which showed a threefold increase after two weeks of hypoxia exposure, had returned to control levels within one day of restoration of normoxic conditions. Similarly, the increased HIF-1 α transcript levels in croaker and sea bass after hypoxia exposure had returned to the control levels after one day of recovery under normoxic conditions [9,10]. These

rapid reversals of the increased HIF-1 α mRNA in mantis shrimp, croaker and sea bass to baseline levels after the return to normoxic conditions are not surprising, because maintenance of HIF-1 α -regulated responses such as the switch to anaerobic metabolism are energetically costly [8].

The observation that HIF-1 α mRNA levels were upregulated in the heart and cerebral ganglion of mantis shrimp exposed to environmental hypoxia in Tokyo Bay was consistent with our predictions. However, an unexpected finding was that HIF-1 α mRNA levels were also upregulated in hearts of mantis shrimp collected from the central normoxic areas (sites CN) during early August. Hypoxia occurred intermittently in these central areas during the summer (electronic supplementary material, figure S2). These field observations and findings from our laboratory studies suggest that mantis shrimp collected from the CN sites might have migrated within the past 24 h from northern hypoxic areas. An alternative possibility is that the bottom DO concentration in the central areas might have recovered from hypoxic conditions just a few hours prior to the sampling time, which was insufficient time for the elevated tissue HIF-1 α mRNA concentrations to return to control basal levels.

Our results are consistent with the earlier findings of upregulation of HIF-1 α transcript levels in croaker from hypoxic areas in a northern Gulf of Mexico estuary and coastal zone compared with mRNA levels in individuals collected from normoxic areas [2,3]. Taken together, the results suggest that measurement of HIF-1 α transcript levels is a useful molecular biomarker of environmental hypoxia exposure in both marine teleost and crustaceans which will be valuable in assessments of the ecological impacts of chronic hypoxia in the estuarine and coastal environments.

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