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Inductive transcription and protective role of fish heme oxygenase-1 under hypoxic stress

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SUMMARY

Heme oxygenase-1 is the rate-limiting enzyme in the degradation of heme into biliverdin, carbon monoxide and free divalent iron. In this study, we cloned heme oxygenase isoform 1 (*CaHO-1*) from a hypoxia-tolerant teleost fish *Carassius auratus*. The full-length cDNA of *CaHO-1* is 1247 bp and encodes a protein of 272 amino acids. RT-PCR and real-time PCR analysis indicated that *CaHO-1* was predominantly transcribed in posterior kidney, head kidney, gill and intestine, and induction of gene transcription was observed predominantly in posterior kidney under hypoxic stress. Moreover, the hypoxia-induced transcription was confirmed in goldfish larvae and in *in vitro* cultured CAB cells. Fluorescence of the HO-1-GFP fusion protein revealed a cytoplasmic and plasma membrane localization, which was consistent with the putative transmembrane structure. Subsequently, we established a stably transfected CAB/pcDNA3.1-HO-1 cell line and a control CAB/pcDNA3.1 cell line, and found that the number of dead cells was obviously reduced in the pcDNA3.1-HO-1-transfected group following 4 days of hypoxic (1% O_2) treatment in comparison with numerous detached dead cells in the control pcDNA3.1-transfected cells. Furthermore, a significant cell viability difference between the two kinds of transfected cells during hypoxia–reoxygenation was revealed. Therefore, the data suggest that fish HO-1 might play a significant protective role in cells in response to hypoxic stress.

Key words: *Carassius auratus*, heme oxygenase-1, hypoxia, inductive transcription, subcellular localization.

INTRODUCTION

Heme oxygenase, the rate-limiting enzyme in heme catabolism, cleaves heme to form biliverdin IXa, carbon monoxide (CO) and iron (Tenhunen et al., 1968; Yoshida and Kikuchi, 1978). Biliverdin IXa is immediately reduced to bilirubin IXa during the last step of the heme breakdown reaction (Kikuchi et al., 2005). There are three isoforms of heme oxygenase, and they are regulated under separate mechanisms (Shibahara et al., 1993). Heme oxygenase-1 (HO-1) and heme oxygenase-2 (HO-2) are immunologically distinct and the genes are located on different chromosomes (Maines et al., 1986). Heme oxygenase-3 (*HO-3*) may be a retrotransposition of the *HO-2* gene unique to rats (McCoubrey et al., 1997). Among them, the HO-1 isoform is ubiquitously distributed and its expression is strongly induced by a variety of physiological and pathophysiological stimuli in human cells, including heme, heavy metals, inflammatory cytokines, nitric oxide and UV irradiation (Keyse and Tyrrell, 1989; Takahashi et al., 1996; Taketani et al., 1989; Yoshida and Kikuchi, 1978).

Hypoxia causes a series of biochemical and pathophysiological changes, which involve changes of cytokine signaling leading to altered gene transcription and ultimately to membrane damage and cell death (Long et al., 2001; Lushchak and Bagnyukova, 2007; Yachie et al., 1999). However, there are conflicting reports on the response of HO-1 to hypoxic stress in mammals. In cardiac myocytes, retinal pigment epithelial cells and D407 cells, *HO-1* transcription was demonstrated to be increased by hypoxia (Udono-Fujimori et al., 2004; Webster et al., 1993), whereas in primary cultures of human umbilical vein endothelial cells, coronary artery endothelial cells and astrocytes, *HO-1* transcription was revealed to be reduced by hypoxia (Nakayama et al., 2000). These results suggested that HO-1 might play various roles and be involved in many pathways.

Some cyprinid fish have been shown to be highly tolerant of hypoxia, and crucian carp (*Carassius carassius*), goldfish (*Carassius auratus*) and common carp (*Cyprinus carpio*) are the most tolerant species (van den Thillart and van Waarde, 1985). Lushchak and colleagues analyzed the tissue-specific responses of goldfish and common carp to environmentally relevant hypoxia exposure and subsequent aerobic recovery, and observed significant changes of some antioxidant enzyme activities in response to different levels of hypoxia (Lushchak et al., 2001; Lushchak et al., 2005). However, the responding regulatory mechanism was almost unknown in fish, although the functional roles of HO-1 in response to hypoxia have been extensively revealed in mammals. So far, HO-1 has only been described in zebrafish (*Danio rerio*) and Takifugu (*Takifugu rubripes*), and nothing was known about the hypoxic responsiveness of the gene. In this study, we cloned heme oxygenase isoform 1 gene (*CaHO-1*) from the most hypoxia-tolerant goldfish *Carassius auratus* and analyzed the expression pattern at the transcription level *in vitro* and *in vivo*. Moreover, we observed that hypoxic treatment strongly induced *HO-1* over-expression at the transcription level. The data suggest that up-regulation of HO-1 might play a protective role in fish cells under hypoxic conditions.

MATERIALS AND METHODS

Cell culture and hypoxic treatment

Carassius auratus L. blastulae embryonic cells (CAB) were cultured at 27°C in medium 199 supplemented with 10% fetal calf serum, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Normoxic culture was performed in an ordinary incubator under air conditioning, and the oxygen concentration was about 20% O_2. For hypoxic exposure experiments, cell cultures were transferred to a

gas- and temperature-controlled incubator (3110/Thermo; Forma Scientific, Inc., Marietta, OH, USA). Before each hypoxic treatment, oxygen within the incubator was set to the required concentration using nitrogen gas (99.999% purity). Then, open cell culture flasks were placed in the incubator, and oxygen was maintained at the required concentration by constantly flushing with nitrogen gas. The hypoxic exposure experiments were performed at 1% O_2 or 2.5% O_2 for various durations (0, 3, 6, 12, 24, 48 and 72 h).

For the hypoxia–reoxygenation studies, cell lines stably expressing the vectors pcDNA3.1-HO-1 and pcDNA3.1 were subjected to 4 days of hypoxia (1% O_2) followed by 1 day of reoxygenation (20% O_2).

Goldfish larvae of ~1.5 cm were divided into two groups (three individuals per group), and treated under either hypoxic (1.5% O_2) or normoxic conditions for 1.5 h. The whole fish was sampled for RNA isolation. Similarly, adult goldfish with a body weight of about 45 g were also divided into two groups (three individuals per group). One group was treated with hypoxia (1% O_2); the second group was in the normoxic condition. At 24 h post-treatment, posterior kidney and gill tissues were isolated for RNA extraction. Three repeated experiments were performed for the larvae and adults.

SMART cDNA synthesis, construction and screening of a subtractive cDNA library

After being treated with hypoxia (1% O_2) and normoxia for 24 h, total RNA of CAB cells was extracted using the SV total RNA isolation system (Promega, Madison, WI, USA). Poly(A)+ RNA was purified with the poly(A) tract mRNA isolation system (Promega) and then used to synthesize SMART cDNA according to the instructions of the BD SMART cDNA library construction kit (Clontech, Mountain View, CA, USA). Using mRNA derived from hypoxic cells as the tester and mRNA from normoxic cells as the driver, a hypoxia-induced subtractive cDNA library was constructed according to the Clontech instructions and our previous reports (Zhang et al., 2003; Zhang et al., 2007). The subtracted cDNAs were ligated into pGEM-T vector (Promega) and transformed into *E. coli* DH5α cells. PCR was used to screen the hypoxia-induced genes from the subtracted cDNA library according to previous reports (Chen et al., 2005).

RNA extraction and reverse transcription

Total RNA from CAB cells, goldfish tissues and goldfish larvae was extracted by the SV total RNA isolation system (Promega). First-strand cDNA was synthesized using random primers and M-MLV reverse transcriptase. Each 25 μl reverse transcriptase reaction contained the following: 2 μg RNA, 1× M-MLV buffer (10 mmol l^{-1} Tris-HCl, 25 mmol l^{-1} KCl pH 8.3, 0.6 mmol l^{-1} $MgCl_2$ and 2 nmol l^{-1} DTT), 10 U of RNasin, 1 μg of hexa-base random primer, 0.5 mmol l^{-1} of each dNTP and 400 U of M-MLV reverse transcriptase (Promega). The reaction was incubated at 37°C for 1.5 h, and subsequently stopped by incubation at 95°C for 5 min.

RACE, semi-quantitative RT-PCR and real-time PCR

RACE (rapid amplification of cDNA ends) was used to clone the full-length *CaHO-1* cDNA. Typically, a pair of primers, HO1-F1 and HO1-R1 (Table 1), was designed according to the sequence of screened EST homologs to zebrafish (*Danio rerio*). Using SMART cDNA as the template, the 5′ sequence was amplified with primers SMART-F and HO1-R1, and the 3′ end was amplified with primers SMART-R and HO1-F1.

Semi-quantitative RT-PCR and real-time PCR were used to analyze the transcription of *CaHO-1* stimulated by hypoxia *in vitro* and *in vivo* by specific primers (Table 1). β-Actin served as a positive control for each cDNA sample. Amplification reactions were performed in a volume of 25 μl containing 1 μl cDNA as template, 0.2 μmol l^{-1} of each primer, 0.5 U of Taq polymerase (MBI, Fermentas, Glen Burnie, MD, USA), 0.1 μmol l^{-1} of each dNTP and 1× Taq polymerase buffer (MBI, Fermentas). PCR conditions were as follows: 94°C for 3 min, 94°C for 30 s and 55°C for 30 s, and 72°C for 30 s for 25–30 cycles, followed by 72°C for 5 min.

Real-time PCR was done in a DNA engine opticon real-time system (MJ Research, Waltham, MA, USA) using a DyNAmo™ SYBR Green qPCR kit (Finnzymes, Espoo, Finland) following the manufacturers' instructions. All reactions were performed in a 20 μl volume (10 μl of 2× master mix, 0.5 μmol l^{-1} each primer and 1 μl cDNA template). A total of 36 cycles were performed, each with similar cycling parameters to the semi-quantitative RT-PCR described above. All samples were analyzed in triplicate and the results were expressed as fold transcription relative to that of the β-actin gene with the $2^{-\Delta\Delta Ct}$ method.

Plasmid construction, subcellular localization and selection of stable transfectants

Two fusion plasmids, HO-1-GFP (amino acids 1–272) and pcDNA3.1-HO-1 (1–272), were generated by fusing PCR fragments of *CaHO-1* with in-frame restriction sites into the pEGFP-N3 and pcDNA3.1 vectors (Clontech) with specific primers (Table 1).

For the subcellular localization assay, CAB cells were transfected with HO-1-GFP or empty pEGFP-N3 using lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). At 48 h after transfection, the cells were rinsed with PBS (pH 7.4), fixed with 4% paraformaldehyde for 15 min and observed using a confocal laser scanning microscope (Leica, Wetzlar, Germany).

CAB cells were transfected with pcDNA3.1-HO-1 or empty vector pcDNA3.1 using the above-mentioned method. Following transfection for 48 h, G418 (Amresco, Solon, OH, USA) was added to the medium at a final concentration of 400 μg ml^{-1}. After 4 weeks of selective culture, the transfected cells were confirmed by semi-quantitative RT-PCR detection for transcription of the *HO-1* gene. The stably pcNDA3.1- and pcDNA3.1-HO-1-transfected CAB cells were termed CAB/pcDNA3.1 and CAB/pcDNA3.1-HO-1.

Cell viability assay

The viability of CAB/pcDNA3.1 and CAB/pcDNA3.1-HO-1 cells cultured in the 96-well culture plates was assessed using a 3-(4,5-

Table 1. Primers used for the experiments

Primer name	Sequence (5′–3′)	Usage
HO1-F1	CCCACACCTAACGCAAACTGG	Gene cloning
HO1-R1	GCTGGATGAGGCAACCAGAGC	RT-PCR
HO1E-F2	CCGCTCGAGCGATGGAATCCACGAAAA	Plasmid construction
HO1E-R2	TCCCCGCGGGAAAAGCATAAACTCCCATT	Plasmid construction
HO1D-F3	CCGCTCGAGATGGAATCCACGAA	Plasmid construction
HO1D-R3	GGGGTACCAAAAGCATAAACTCCC	Plasmid construction
HO1-F4	GGAAGAGAGTAACAGTGCC	Real-time PCR
HO1-R4	AGCACTTCCTGTCTCTGCTG	RT-PCR
SMART-F	AACGCAGAGTACGCGGG	RACE-PCR
SMART-R	CAGAGTACT16	RACE
β-Actin F	CACTGTGCCCATCTACGAG	Real-time PCR
β-Actin R	CCATCTCCTGCTCGAAGTC	RT-PCR
T7	TAATACGACTCACTATAGGG	RT-PCR

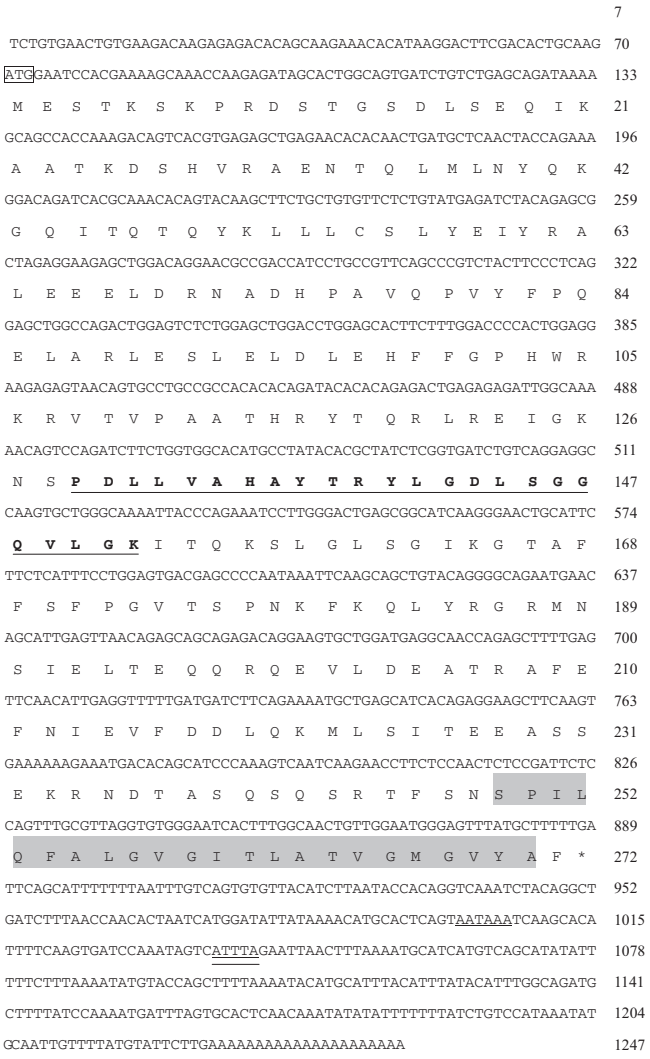

Fig. 1. Nucleotide sequence and deduced amino acid sequence of *CaHO-1*. The nucleotides (upper row) and deduced amino acids (lower row) are numbered on the right. A putative transmembrane segment sequence is shaded, and a poly(A) signal (in the 3′ UTR) is underlined. The start codon (ATG) is boxed and the stop codon (TGA) is indicated by an asterisk. An unstable motif (ATTTA) is doubly underlined. Heme oxygenase signature is in bold and underlined.

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay-based cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) at various time points (0, 1, 2, 3, 4 and 5 days) according to the manufacturer's instructions (Fukuda et al., 2007; Mei et al., 2008). CCK-8 solution (10 µl) was added to each well and incubation was carried out at 27°C for 4 h; the OD was read at 450 nm using a microplate reader (Tecan Sunrise, Zurich, Switzerland). The morphological changes were observed under a phase contrast microscope (Leica).

RESULTS

Molecular characterization of *Ca*HO-1

The full-length *CaHO-1* cDNA was generated by 3′ and 5′ RACE. As shown in Fig. 1, it comprises 1247 bp, and has an open reading frame (ORF) encoding a 272 amino acid (aa) peptide with a putative transmembrane segment (from 249 to 271 aa). In the 3′ UTR, there is an AU sequence (ATTTA), a motif possibly involved in mRNA instability (Shaw and Kamen, 1986). Pairwise comparison showed that *CaHO-1* was highly similar to *HO-1* genes deposited in public databases, especially to zebrafish (*Danio rerio*) *HO-1*, which is also a cyprinid fish (Table 2). Multiple alignments revealed that, like mammalian HO-1 proteins, the putative *Ca*HO-1 protein has a heme oxygenase domain (from aa 14 to 218) and a heme oxygenase signature motif (from aa 129 to 152; Fig. 2) (Maines and Gibbs, 2005). There is a relatively high level of sequence conservation in the putative oxygenase signature motif. Overall, *Ca*HO-1 is 45–86% identical to homologous proteins from birds, mammals and fish, with 53–88% identity at the level of the heme oxygenase domain and 83.3–95.8% identity at the level of the heme oxygenase signature motif (Table 2).

Inductive transcription of *CaHO-1 in vitro* under hypoxia

To evaluate whether hypoxia can induce *CaHO-1* transcription *in vitro*, we detected *CaHO-1* transcript levels in CAB cells after exposure to 2.5% O_2 and 1% O_2. Real-time PCR analysis showed that both hypoxic conditions induced a significant increase in *CaHO-1* transcription. Upon 2.5% O_2 treatment, the transcript level of *CaHO-1* was initially steady, then rose sharply, reaching a peak at 24 h, and thereafter decreasing back to the basal level at 48 h and 72 h (Fig. 3A). When the treatment was changed to the 1% O_2 condition, the transcription of *CaHO-1* increased quickly at 6 h, and continued to increase up to 72 h (Fig. 3B). The relative gene transcription of *CaHO-1* at 72 h after exposure to 1% O_2 was about 30-fold the gene transcription peak caused by 2.5% O_2 treatment.

Inductive transcription of *CaHO-1 in vivo* under hypoxia

To reveal the transcription pattern of *CaHO-1 in vivo*, *CaHO-1* transcripts were first detected in various tissues from a healthy goldfish. As shown in Fig. 4A, *CaHO-1* was predominantly transcribed in posterior kidney, head kidney, gill and intestine, and a lower level of transcription was also detected in heart, spleen, brain and liver. Interestingly, after the healthy fish were treated with hypoxia (1% O_2) for 24 h, inductive transcription was observed in the examined tissues including posterior kidney and gill, but predominant induction appeared in posterior kidney with 7.8-fold higher levels than the control (Fig. 4B). Moreover, we also examined *CaHO-1* transcription change in ~1.5 cm larvae under hypoxia. When the larvae were exposed to hypoxia (1.5% O_2) for 1.5 h, obvious *CaHO-1* transcription induction was also observed in the hypoxia-treated larvae in comparison with the larvae under the normoxic condition (Fig. 4C).

Subcellular localization of *Ca*HO-1

To probe the intracellular localization of *Ca*HO-1 protein in CAB cells, we constructed an expression plasmid containing the full-length ORF of *Ca*HO-1 and HO-1-GFP, which can express a fusion green fluorescent protein with full-length *Ca*HO-1. After CAB cells were transfected with HO-1-GFP, strong green fluorescence was observed by confocal microscopy to be aggregated in the cytoplasm (Fig. 5A) and plasma membrane (Fig. 5B), whereas the control vector-expressed EGFP was ubiquitously distributed in the CAB cells (Fig. 5C). The data indicated that HO-1-GFP fusion protein was localized in the cytoplasm and plasma membrane of CAB cells, which was consistent with the putative transmembrane structure predicted by the bioinformatics approach.

Effect of *Ca*HO-1 over-expression on viability of CAB cells under hypoxia–reoxygenation

To assess whether *Ca*HO-1 was involved in the hypoxia-mediated effect on cell growth and survival, we established a stably transfected

CAB/pcDNA3.1-HO-1 cell line and a control CAB/pcDNA3.1 cell line. First, the CAB/pcDNA3.1-HO-1-transfected cell line was confirmed by detecting the *CaHO-1* transcripts through semi-quantitative RT-PCR using the *CaHO-1* gene-specific primer HO1-R4 and the plasmid primer T7, whereas no *CaHO-1* transcripts were detected in the control CAB/pcDNA3.1-transfected cell line (Fig. 6A). Then, after 4 days of hypoxia (1% O_2) treatment, *Ca*HO-1 over-expression was observed in the CAB/pcDNA3.1-HO-1-transfected cells in comparison with that in the control CAB/pcDNA3.1-transfected cells.

Simultaneously, the cells that underwent hypoxia-induced death were observed under the phase contrast microscope. In comparison with numerous detached dead cells in the control pcDNA3.1-transfected group, the number of dead cells was obviously reduced in the pcDNA3.1-HO-1-transfected cell population after 4 days of hypoxia (1% O_2) treatment. Moreover, we analyzed in detail the effects of *Ca*HO-1 on cell growth and survival during hypoxia treatment and reoxygenation by using a cell viability assay (see Materials and methods). As shown in Fig. 6D, when the transfected cells were exposed to 1% hypoxia for 4 days and then the normal oxygen treatment was resumed for 1 day (reoxygenation), the cell growth curves were similar and obvious proliferation inhibition was observed during the initial 2 days; however, a significant cell viability difference was present from the third day of treatment between the two kinds of transfected cells. As the hypoxic treatment was extended, the proliferation inhibition in the pcDNA3.1-HO-1-transfected cells was obviously weaker than that in the pcDNA3.1-transfected cells. On the fourth day, the mean cell viability in the pcDNA3.1-HO-1-transfected cells was 0.68±0.10, whereas it was 0.48±0.04 in the pcDNA3.1-transfected cells. Significantly, after normal oxygen was resumed for 1 day, the mean cell viability in the pcDNA3.1-HO-1-transfected cells was 0.93±0.10, whereas it was only 0.67±0.01 in the pcDNA3.1-HO-1-transfected cells. This significant difference provides evidence that the over-expressed *Ca*HO-1 in CAB cells might play a protective role in hypoxia-induced cell death.

Table 2. Comparison of *Ca*HO-1 with the other six HO-1 members

Species	Overall identity	Heme oxygenase domain	Heme oxygenase signature
Human (*Homo sapiens*)	48	56	91.7
Mouse (*Mus musculus*)	47	53	91.7
Rat (*Rattus norvegicus*)	46	53	91.7
Chick (*Gallus gallus*)	45	56	91.7
Zebrafish (*Danio rerio*)	86	88	95.8
Fugu (*Takifugu rubripes*)	60	69	83.3

Values are percentage identity.

DISCUSSION

Inductive transcription of *HO-1* under hypoxic stress

Hypoxia is an important stress. In humans and other air-breathing vertebrates, hypoxia leads to rapid adaptive changes in metabolic organization. Since water has a low oxygen capacity (1/30th compared with air) and poor oxygen diffusibility (Nikinmaa, 2002), many fish species have evolved behavioral, anatomical, physiological, biochemical and molecular adaptations that enable them to cope with periods of hypoxia (Nilsson and Ostlund-Nilsson, 2004). Goldfish are one of a few vertebrates that tolerate anoxia. Hypoxia has been revealed to cause significant morphological and gene transcription changes in fish (Gracey et al.,

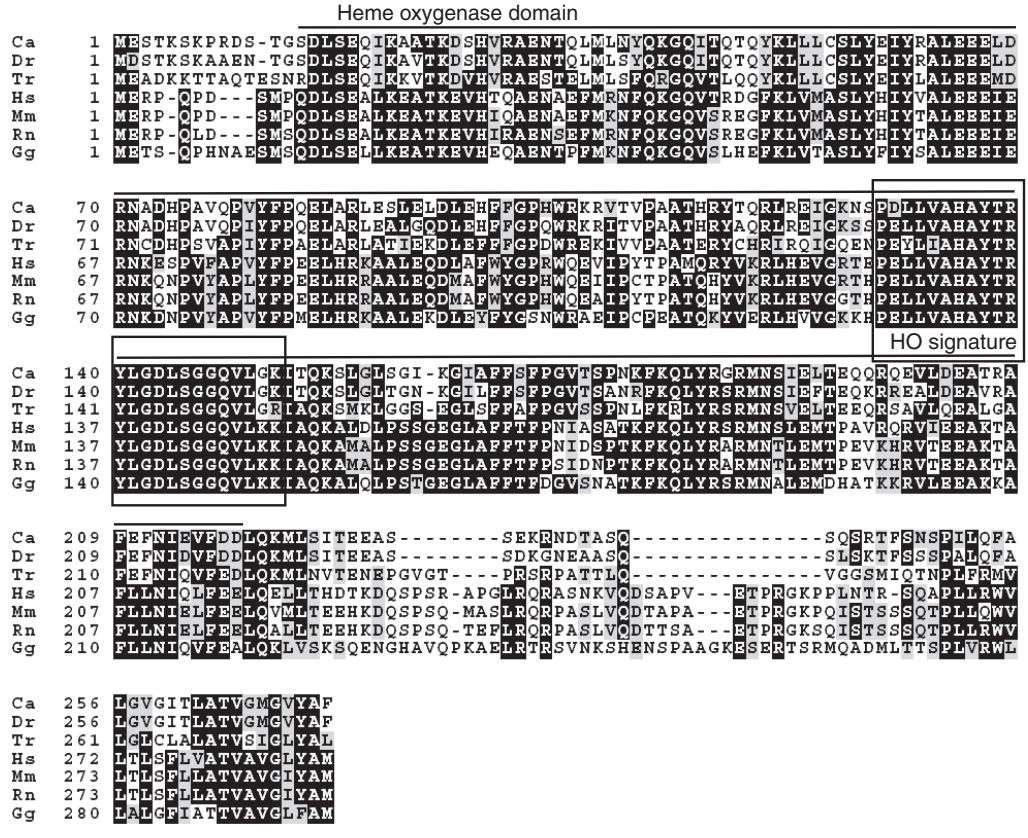

Fig. 2. Multiple alignment of *Ca*HO-1 amino acid sequence with that of other HO-1 proteins from fish, human, mouse, rat and chick. Ca, *Carassius auratus* L., accession number FD483976; Dr, *Danio rerio*, BC061954; Tr, *Takifugu rubripes*, AF022814; Hs, *Homo sapiens*, AY460337; Mm, *Mus musculus*, BC010757; Rn, *Rattus norvegicus*, BC091164; Gg, *Gallus gallus*, NM_205344. Missing amino acids are denoted by dashes (–). Shading is used to highlight regions with different levels of sequence identity: identical amino acids in at least four sequences are in black, and similar amino acids in at least four sequences are in gray. The heme oxygenase domain is indicated by lines above the aligned sequences. The heme oxygenase signature (HO signature) is boxed.

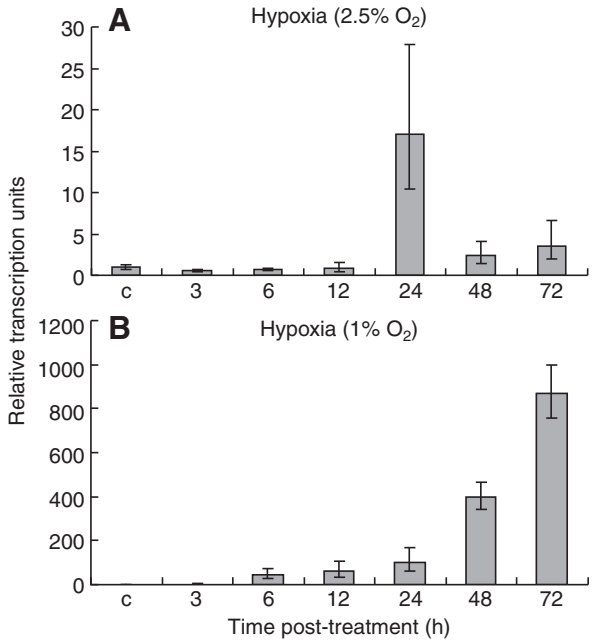

Fig. 3. Inductive transcription of *CaHO-1 in vitro*. Two groups of CAB cells were treated with 2.5% O_2 (A) and 1% O_2 (B) for 3, 6, 12, 24, 48 and 72 h. Cells were harvested immediately and total RNA was isolated as outlined in Materials and methods. The levels of *CaHO-1* mRNA were determined by real-time PCR. Error bars represent standard deviations obtained by measuring each sample three times from three independent experiments.

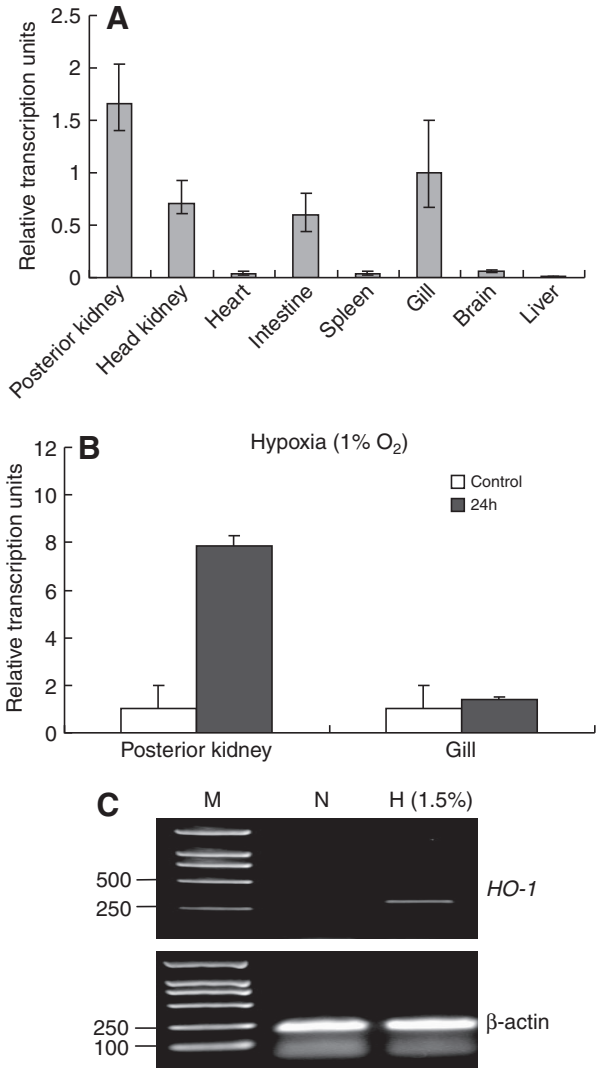

Fig. 4. Gene transcription of *CaHO-1 in vivo*. (A) Total RNA from different tissues (posterior kidney, head kidney, heart, intestine, spleen, gill, brain and liver) of goldfish under normoxia (20% O_2) was analyzed by real-time PCR. Error bars represent standard deviations obtained by measuring each tissue from three fish. (B) Inductive transcription of *CaHO-1* in posterior kidney and gill. Total RNA from posterior kidney and gill of goldfish subjected to normoxia (as control) and hypoxia for 24 h was analyzed by real-time PCR. Error bars represent standard deviations obtained by measuring each tissue from three fish. The data shown have been normalized to β-actin gene transcription. (C) Inductive transcription of *CaHO-1* in goldfish larvae. Total *CaHO-1* transcripts of one normoxic (N) and one hypoxic (H) goldfish larvae of ~1.5 cm length, from a total of three in each group, following exposure to hypoxia (1.5% O_2) for 1.5 h, were analyzed by RT-PCR. Lane M, DL2000 DNA marker lane, kb (Takara). The data shown have been normalized to β-actin gene transcription.

2001; Sollid et al., 2003), but the transcription pattern of fish *HO-1* in response to hypoxic stress remains unknown *in vitro* and *in vivo*.

Here, we identified a fish *HO-1* gene, *CaHO-1*, from a goldfish cell line in response to hypoxic stress. Most significantly, we have characterized the inductive transcription pattern under hypoxic stress for the first time. *In vivo*, *CaHO-1* is predominantly transcribed and responsive to hypoxia in the posterior kidney of goldfish. This result is quite similar to earlier observations of a hypoxia-responsive gene *CITED3* in grass carp (Ng et al., 2003), where the highest transcription level of *gcCITED3* mRNA was detected in kidney and low transcription levels were detected in brain, heart and liver under normoxia, while a marked and persistent increase was found in kidney and gill with a lower level after exposure to hypoxia. Moreover, the hypoxia-induced transcription was also confirmed in goldfish larvae, which is more sensitive than that in adults. Additionally, we observed various levels of tolerance of larvae to different hypoxic conditions.

Involvement of HO-1 in oxygen-sensing mechanisms

Many oxygen-dependent cellular phenomena have been characterized, but the molecular mechanisms for oxygen sensing are poorly understood. One possibility is that oxygen sensing is a membrane-bound NADPH oxidase-like system, which contains a heme protein, cytochrome *b*558 (Nikinmaa, 2002). Coincidently, HO-1-catalyzed heme procedure requires the concerted activity of NADPH–cytochrome P450 reductase to provide reducing equivalents to support the reduced state of iron (Fe^{2+}) and activation of molecular oxygen (Song et al., 2006). Significantly, the current study revealed that *Ca*HO-1 is not only a cytoplasmically distributed protein but also a membrane-localized protein, which may help it move to the membrane to be involved in this membrane-bound NADPH oxidase-like system. Therefore, *Ca*HO-1 may be a potential oxygen sensor involved in the oxygen-sensing interaction.

Role of HO-1 under hypoxic stress

HO-1 is an inducible gene whose transcription is increased in response to a variety of cellular stresses and stimuli including ischemia, hypoxia, oxidative stress and inflammatory cytokines (Ferrándiz and Devesa, 2008). Previous data suggested that the HO-1-mediated protective role might depend on the cellular

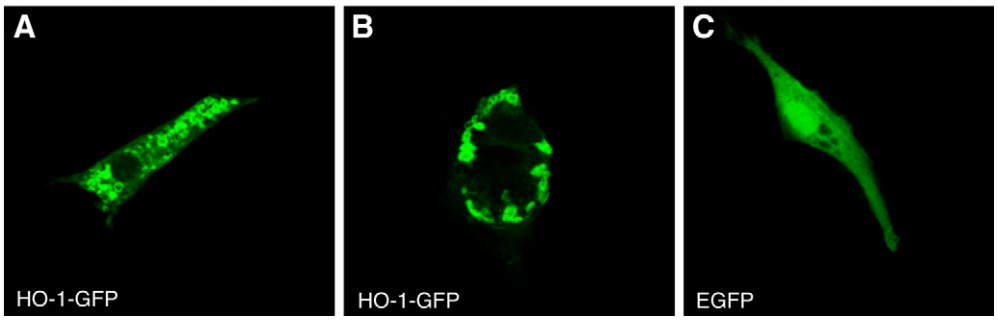

Fig. 5. Subcellular localization of *Ca*HO-1 by confocal microscopy. (A,B) CAB cells transfected with HO-1-GFP showing cytoplasmic (A) and plasma membrane (B) localization. (C) CAB cells transfected with empty vector pEGFP-N_3.

milieu in terms of whether an increase of HO is beneficial or detrimental to the cell (Maines and Gibbs, 2005). In goldfish, Lushchak and colleagues analyzed and evaluated the tissue response of the antioxidant system during anoxia and reoxygenation (Lushchak et al., 2001). They observed significant changes of some antioxidant enzyme activities in some tissues under the anoxia conditions, and suggested that regulation of the antioxidant system during anoxia might constitute a biochemical mechanism that minimizes oxidative stress following reoxygenation. In this study, the hypoxia-induced injury and reoxygenation-induced recovery were also demonstrated in the goldfish larvae.

In order to quantitatively determine the potentially protective effect of *Ca*HO-1 in fish, hypoxic treatment and reoxygenation were

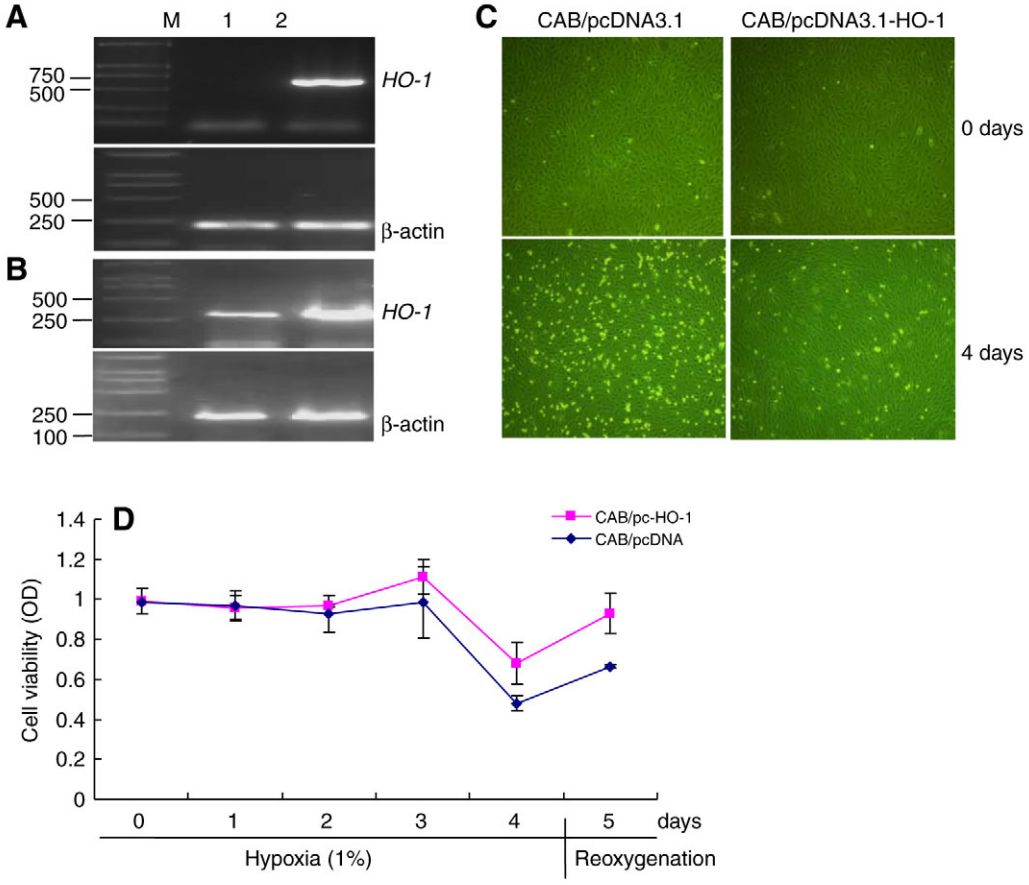

Fig. 6. Suppression of hypoxia-induced cell death by over-expression of *Ca*HO-1. (A) Confirmation of the stably transfected CAB cells by semi-quantitative RT-PCR using T7 primer and *CaHO-1*-specific primer HO1-R4. A band (0.67 kb) was only detected in CAB cells stably transfected with plasmid pcDNA3.1 carrying the full-length ORF of *HO-1* gene; β-actin was employed as an internal control. Lane M, DL2000 DNA marker; lane 1, CAB/pcDNA3.1 cell; lane 2, CAB/pcDNA3.1-HO-1 cell. (B) Over-expression of *HO-1* in stably transfected CAB cells under hypoxic treatment. Total RNA was isolated from CAB/pcDNA3.1 and CAB/pcDNA3.1-HO-1 cells after 4 days of hypoxia (1% O_2). The transcription of *CaHO-1* was then evaluated by semi-quantitative RT-PCR (lane 2). CAB/pcDNA3.1 cells were employed as a control (lane 1). (C) Morphological observation of hypoxia-induced cell death under phase contrast microscope. (D) Viability of CAB/pcDNA3.1 and CAB/pcDNA3.1-HO-1 cells under the hypoxia–reoxygenation treatment. Cell viability was examined by a modified MTT assay using a CCK-8 kit each day (see Materials and methods). The experiments were repeated at least three times and the symbols represent the mean values of triplicate wells, with standard deviations.

also performed in the stably transfected CAB cell lines using a CCK-8 assay. Interestingly, we observed that hypoxia could inhibit CAB cell growth and proliferation, which might serve to divert important energy resources away from growth towards those metabolic processes more essential for hypoxia survival, and induced death after continuing hypoxic treatment. Conversely, reoxygenation can rescue the damage to some extent by promoting proliferation. More interestingly, CAB cells with HO-1 over-expression could suppress the hypoxia-induced cell viability decrease in response to hypoxia and retain a higher proliferation after reoxygenation than the cells with empty-vector expression. Therefore, *Ca*HO-1 may be a protein involved in the protective response against hypoxic stress in CAB cells.

The diversity of fishes and their habitats might promote the solving of the problem of hypoxia tolerance in various interesting ways, and an extensive range of molecular adaptations to hypoxia may have evolved in fish that might be not paralleled in other vertebrate groups. With regard to HO-1, it is likely that the current state of knowledge only scratches the surface of its function in fish. Further studies need to be done to determine the molecular mechanisms of the strong induction by hypoxia, especially in its physiological role(s) in relation to hypoxia adaptation and tolerance in fish.

CONCLUSION

A fish heme oxygenase isoform 1 gene (*HO-1*) was cloned and characterized from a hypoxia-tolerant teleost fish *Carassius auratus*. Significantly, hypoxia-induced expression was observed in adults and larvae, and in the *in vitro* cultured CAB cells. It was localized to the cytoplasm and plasma membrane. Interestingly, in comparison with the numerous detached dead cells in the control pcDNA3.1-transfected group, the number of dead cells was obviously reduced in the pcDNA3.1-HO-1-transfected group after 4 days of hypoxic (1% O_2) treatment. A significant cell viability difference was also revealed between the two kinds of transfected cells during hypoxic treatment and reoxygenation. Therefore, the data suggest that fish HO-1 might play a significant protective role for the cells in response to hypoxic stress.

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