Molecular characterization and *in vivo* expression of hypoxia inducible factor (HIF)-1α in sea bass (*Dicentrarchus labrax*) exposed to acute and chronic hypoxia

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**ABSTRACT** - Aquatic hypoxia is a frequent event and in fish a complex set of physiological and biochemical alterations are employed to cope with this environmental stress. Many of these adjustments depend to a large extent on changes in the expression of genes that encode for physiologically relevant proteins. Genes that are induced by hypoxia appear to share a common mode of transcriptional regulation. This induction depends upon activation of a transcription factor, the hypoxia inducible factor-1 (HIF-1), which is a heterodimer composed of two subunits: α and β.

In this study we report first on the molecular cloning and characterization of HIF-1α in sea bass (*Dicentrarchus labrax*). The full-length sea bass cDNA for HIF-1α was isolated and deposited in the GenBank with accession no. DQ171936. It consists of 3317 base pairs (bp) carrying a single open-reading frame that encompasses 2265 bp of the coding region and 1052 bp of the 3’ UTR.

We then utilized the real-time PCR technology to monitor dynamic changes in levels of HIF-1α transcripts, in response to acute and chronic hypoxic stress. The number of HIF-1α mRNA copies were significantly increased in response to both acute (1.9 mg/L, dissolved oxygen for 4 hours) and chronic (4.3 mg/L, DO for 15 days) hypoxia in sea bass, whereas it remained unchanged in fish exposed to hyperoxic (DO 13.5±1.2 mg/L, 155 % saturation) conditions. This is the first study carried out to investigate the behaviour of HIF-1α gene transcripts during hypoxia in representative of marine hypoxia-sensitive fish species.

**Key words:** Aquaculture, Gene expression, Aquatic hypoxia, Real-time RT-PCR.

**Introduction** - In fish a complex set of physiological and biochemical alterations are employed to cope with hypoxia stress (for a review see Nikinmaa, 2002). Many of these adjustments depend to a large extent on changes in the expression of genes that encode diverse groups of physiologically relevant proteins. Gracey et al., (2001) recently identified alterations in the expression of over 120 genes in hypoxic fish (*Gillichthys mirabilis*). Genes that are induced by hypoxia appear to share a common mode of transcriptional regulation. This induction depends upon activation of a transcription factor, the hypoxia inducible factor-1 (HIF-1). HIF-1 is a heterodimer composed of α and β subunits. HIF-1β is generally found to be constitutively expressed in the nucleus and to be insensitive to changes in O2 availability, whereas stabilization of HIF-1α and its nuclear accumulation are acutely regulated by hypoxia (Uchida et al., 2004). Since the initial characterization of HIF-1α in humans, several additional cDNAs have been isolated in different vertebrates, whereas orthologues from fish have only been identified in a few species. On the other hand, although recent technological developments have made it possible to measure patterns of gene expression, only few published reports are available on tissue expression patterns of HIF-1α in fish exposed to hypoxia. Therefore, in this study we have examined the HIF-1α gene expression pattern in sea bass (*Dicentrarchus labrax*) exposed to conditions of acute and chronic hypoxia. We describe also the molecular cloning and sequencing of HIF-1α for the first time in this marine hypoxia-sensitive species.
Material and methods - Sea bass of mixed sexes were stocked into indoor tanks (3x1x1m) of 2500 L each and allowed to acclimate for 45 days before starting the trial. The tanks were connected to a sea water recirculation system, with strictly controlled water conditions: temperature 21.8±0.9°C, pH 7, total ammonia below 0.2 mg/L, and nitrite <0.02 mg/L. Dissolved oxygen (DO) was maintained at over 99% of the saturation value by adding pure O2 to the system.

Chronic hypoxia and hyperoxia exposure trial - After the acclimation period, 35 fish were transferred into each of three experimental tanks (600 L) connected to the same recirculation system and allowed to acclimate for five days. Then one of the tanks (control) was maintained under normoxic conditions (DO, 8.1±0.3 mg/l, 99-100% saturation), the second one under moderate hypoxic conditions (4.3±0.8 mg/L, 51% of saturation), and the third tank under hyperoxic (DO 13.5±1.2 mg/L, 155 % saturation) conditions. At the beginning of the experiment, the mean body weight of the sea bass was 90.1±24.5 g for the control group, 97.6±13.6 g for the “hypoxia” group, and 95.7±23.8 g for the “hyperoxia” group. Five fish from each of the three groups were sampled at the start of the experiment (day 0), and then after continuous exposure for 24h, 48h, 5days, and 15days to the respective experimental conditions. After 15 days of hypoxia and hyperoxia exposure, the DO levels in the two tanks were adjusted back to the normal, saturated levels and, after a 24 h recovery period, five fish were sampled from each tank.

Acute-hypoxia exposure - Five fish from the same, previously described stock were transferred into each of two 100L tanks connected to the recirculation system and after five days of acclimation were exposed to severe hypoxia (1.9±0.2 mg/L). Fish from the first tank were sampled 4 hours after the target level of DO was achieved, as soon as they lost the equilibrium, whereas fish from the second tank were at the same moment, immediately reoxygenated normally for 24 hours and sampled at the end of the recovery period.

For the molecular biology analysis, brain, heart, liver, kidney, spleen, and muscle were isolated and stored at -80°C. Total RNA (tRNA) was extracted from the tissues and reverse transcribed into cDNA. To perform PCR, an aliquot of the resulting cDNA was amplified with GoTaq Polymerase and each of the designed primer sets. The PCR products from HIF-1α primer amplifications were then cloned using the pGEM®-T cloning vector system, and sequenced in both directions (T7 and SP6). The full-length cDNA for HIF-1α was subsequently isolated by 3' and 5' RACE method. The number of HIF-1α gene transcript copies was quantified via real-time RT-PCR by comparing them with a standard graph (Fig. 1) constructed using the known copy number of mRNAs of this gene. For this, a forward and a reverse primer were used to create templates for the in vitro transcription of cRNAs. The cRNAs produced by in vitro transcription were then used as quantitative standards in the analysis of experimental samples. Real-time Asays-by-Design™ PCR primers and gene-specific Taqman® fluorogenic probes were designed by Applied Biosystems. The data were statistically compared using one-way analysis of variance (ANOVA). The level of statistical significance was set at P<0.05.

Results and conclusions - The full-length sea bass cDNA for HIF-1α was isolated and deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) with accession no. DQ171936. It consists of 3317 base pairs (bp) carrying a single open-reading frame that encompasses 2265 bp of the coding region and 1052 bp of the 3' UTR, including a common (AATAAA) polyadenylation signal upstream of the poly(A) tail. Conceptual translation of the cDNA predicts a protein of 755 amino acids (aa), similar to the HIF-1α of other species as indicated by sequence analysis on the NCBI database. Real-time RT-PCR using the standard curve established for HIF-1α cRNA, (Fig. 1) revealed high levels of HIF-1α transcripts in liver, lower in brain, muscle, heart, and negligible levels in other tissues such as spleen and kidney (Fig. 2). The absolute amounts of mRNA for HIF-1α in the liver in response to chronic hypoxia...

Figure 1. Standard curve for HIF-1α.

\[ y = -3.3738x + 44.873 \]

R² = 0.9963
Eff = 86.4%
hypoxic stress as estimated by real-time PCR are presented in Fig. 3. These data demonstrate that HIF-1α is induced from hypoxia. At a hypoxic stress level of 4.3 mg/L DO and up to 24-hour exposure, HIF-1α mRNA levels fluctuated insignificantly as compared with normal levels, whereas they were significantly (P<0.05) increased 48 hours after exposure. Longer exposure to hypoxia (from 5 to 15 days) contributed to a further increase in the HIF-1α mRNA copy number. Recovery for 24 hours significantly decreased the HIF-1α mRNA levels, which returned to control values within 24 hrs of exposure to normoxic conditions.

Severe hypoxic stress (DO 1.9 mg/L) for 4 hours dramatically increased the number of HIF-1α transcripts (P<0.05) in the liver, whereas recovery for 24 hrs decreased the mRNA copy number of HIF-1α to pre-treatment levels (Fig. 4). HIF-1α mRNA copy numbers were similar for normoxic and hyperoxic conditions (data not shown). This transcriptional factor response seems to be less affected by hyperoxic conditions.

In conclusion, we isolated and characterized HIF-1α in a hypoxia-sensitive marine fish species. We also demonstrated that acute- and chronic-term hypoxia induces changes in the expression of this gene and that these expression patterns are rapidly reversed upon re-exposure to normoxia. We recognize that HIF-1α mRNA levels in our study do not measure physiological effects produced by the protein. Due to this, our hypothesis that HIF-1α is an important trigger of the hypoxia response in the sea bass will have to be confirmed by future investigations.