Characterization of three IGFBP mRNAs in Atlantic croaker and their regulation during hypoxic stress: potential mechanisms of their upregulation by hypoxia

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Rahman MS, Thomas P. Characterization of three IGFBP mRNAs in Atlantic croaker and their regulation during hypoxic stress: potential mechanisms of their upregulation by hypoxia. Am J Physiol Endocrinol Metab 301: E637–E648, 2011. First published July 5, 2011; doi:10.1152/ajpendo.00168.2011.—Insulin-like growth factor-binding proteins (IGFBPs) play important roles in downregulating IGF activity and growth and development in vertebrates under hypoxic stress. However, the mechanisms of hypoxia regulation of IGFBPs in teleost fishes are unknown. The involvement of reactive oxygen species (ROS) and hypoxia-inducible factors (HIFs) in hypoxia upregulation of IGFBPs in Atlantic croaker were investigated. Three croaker IGFBPs, IGFBP-1, IGFBP-2, and IGFBP-5, were cloned and characterized. Chronic hypoxia exposure [dissolved oxygen (DO): 1.7 mg/l for 2–4 wk] caused significant increases in hepatic and neural IGFBP-1 mRNA expression compared with tissue mRNA levels in fish held under normoxic conditions (6.5 mg DO/l). Moreover, longer-term chronic hypoxia exposure (2–2.7 mg DO/l for 15–20 wk) caused significant increases in mRNA levels of all three IGFBPs in both liver and brain tissues. Hypoxia exposure also markedly increased superoxide radical (O_2^·) production and HIF-1α mRNA and HIF-2α protein expression in croaker livers. Pharmacological treatment with an antioxidant attenuated the hypoxia-induced increases in O_2^· production and HIFα mRNA and protein expression as well as the elevation of IGFBP-1 mRNA levels. These results suggest that the upregulation of IGFBP expression under hypoxia stress is due, in part, to alterations in the antioxidant status, which may involve ROS and HIFα.

Insulin-like growth factor-binding proteins (IGFBPs) comprise a protein family with both high binding affinity for IGFs (designated as IGFBP-1 to IGFBP-6) (19, 33) and low affinity for IGFs [IGFBP-related proteins (IGFBP-rP)] (3, 34). IGFBPs are present mainly in the circulation and extracellular fluids (18, 33) and are also expressed in peripheral tissues involved in growth and reproduction in vertebrates (18). The high-affinity IGFBPs bind to IGF-I and IGF-II peptides to regulate their physiological activities (33), whereas the low-affinity IGFBP-rP appear to exhibit biological effects that are IGF independent (3, 34). In addition, the high-affinity IGFBPs modulate IGF signaling in complex ways that involve inhibiting IGF action by both preventing binding to the IGF receptor and promoting IGF action possibly through aiding its delivery to the receptor (19). These proteins increase the half-life of IGFs by protecting them from degradation and by enhancing their stability (18, 19). Thus the high-affinity IGFBPs are critical components of IGF signaling and play a pivotal role in regulating IGF’s actions.

Most of our current knowledge of the functions of IGFBPs has been obtained from mammalian models, and much less is known about the functions and regulation of IGFBPs in non-mammalian vertebrates, particularly in teleost species (18, 73). The IGFBP genes have been characterized in several teleost fishes: IGFBP-1a, -1b, -2a, -2b, -3, -5a, -5b, -6a, and -6b in zebrafish (11, 14, 32, 72, 76), IGFBP-2 in seabream (20), shi drum (51), and common carp (13), IGFBP-1, -2, -3, and -5 in yellowtail (48), and IGFBP-1 to -6 in rainbow trout (31). Several IGFBPs have also been purified from serum in rainbow trout (2) and chinook and coho salmon (59–61). Evidence suggests that the overall structures of fish IGFBPs and their mammalian counterparts are highly conserved (18, 73).

Hypoxia has profound effects on growth, development, metabolism, and reproduction as well as endocrine functions in aquatic animals (67, 68, 74). Many of these physiological responses to hypoxia are likely controlled by hypoxia-inducible factor (HIF), an oxygen-sensitive transcription factor that regulates the expression of numerous genes involved in adaptation to hypoxia (46, 58). Four HIFα isoforms (HIF-1α, HIF-2α, HIF-3α, and HIF-4α) have been discovered and characterized in vertebrates (23, 39, 52, 71). Among them, HIF-1α and HIF-2α have high sequence homology in their functional domains and have been identified as key transcription factors regulating the genes involved in adaptation to hypoxia (54). Recent studies have also shown that there is a reciprocal relationship between the expression of HIF-1α and HIF-2α proteins in certain cultured cells (9, 54). The HIFα proteins accumulate and associate with HIFβ, an oxygen-insensitive transcription factor, to form a functional transcription complex under hypoxic conditions (58). The active HIFα complex then binds to hypoxia response elements (HRE) on the promoter regions of numerous hypoxia target genes such as erythropoietin, vascular endothelial growth factor, and transforming growth factor, resulting in changes in their rates of transcription (7, 58, 66).

Most, if not all, IGFBPs are hypoxia-inducible transcripts that are also regulated through HIF-1α. Earlier in vitro studies in cultured human cells and in vivo studies in rodents have shown that multiple IGFBP transcripts are elevated in the circulation and tissues under hypoxic conditions (30, 36, 37, 49, 55, 64, 65, 69, 70). Similarly, recent in vivo studies in teleost fishes have demonstrated that hypoxia increases IGFBP-1 mRNA expression (29, 44, 75) and that this upregulation of IGFBP-1 is mediated through HIF-1α (28). In addi-
tion, several lines of evidence indicate that the overexpression of HIF-1α increases IGFBP-1 promoter activity in cultured human cells (65) and interacts with a functional HRE on the IGFBP-1 gene in zebrafish under low-oxygen conditions (30).

Because oxygen is a key component of reactive oxygen species (ROS) such as superoxide anions and peroxides, ROS have been suggested to act as oxygen sensors (15) and be linked to the regulation of HIF-1α under low-oxygen conditions (10, 27, 50). Inhibition of cellular ROS formation decreases the accumulation of HIF-1α protein (27). Moreover, evidence has accumulated that the ROS stabilizes HIF-1α under hypoxic conditions and that this is largely due to inhibition of prolyl hydroxylase enzyme activity (8, 50). On the other hand, HIF-1α levels also increase under nonhypoxic conditions in response to growth factors, cytokines, and insulin (5, 24), many of which also stimulate the generation of ROS. ROS also appear to play an important role in the upregulation of IGFBP-1 mRNA and protein in cultured human cells and IGFBP-1 release under nonhypoxic conditions (38). However, these studies have been conducted with cultured cells in vitro systems, and there is a lack of information on endogenous ROS generation in animal tissues after in vivo exposure to hypoxia and its association with hypoxia-induced changes in IGFBP and HIFα expression.

Therefore, in the present study, hypoxia-induced increases in the expression of IGFBPs in tissues of a teleost species, Atlantic croaker (Micropogonias undulatus), were characterized, as well as the potential mechanisms of IGFBP upregulation. Atlantic croaker (croaker) is a relatively hypoxia-tolerant marine species (6) that inhabits estuaries along the US Atlantic and Gulf of Mexico coasts, which are seasonally hypoxic [dissolved oxygen (DO) <2.0 mg/l], as well as the extensive coastal region in the northern Gulf of Mexico, which is hypoxic throughout the summer (66–68). Chronic exposure to an environmentally realistic hypoxia regime, DO <2.0 mg/l for 15–20 wk, has previously been shown to cause reproductive impairment, ovarian masculinization, endocrine disruption, and upregulation of HIFαs in croaker (67, 68). However, it is not known whether this hypoxia treatment alters IGFBP expression in this species. Therefore, in the present study, the effects of long-term (15–20 wk) and a relatively short period (2–4 wk) of hypoxia exposure on IGFBP expression were investigated. First, three distinct IGFBPs, IGFBP-1, IGFBP-2, and IGFBP-5, were cloned and characterized from croaker liver, and their mRNA expression in liver and brain tissues was examined after 2–4 and 15–20 wk of chronic hypoxia exposure. The patterns of ROS generation and HIFα upregulation were also investigated under the same hypoxic conditions. Treatment with the antioxidant vitamin E prevents ROS production in mammalian cells under hypoxic conditions (45). Therefore, the effects of vitamin E treatment on the ROS, HIFα, and IGFBP responses in croaker tissues to hypoxia exposure were examined. The results show that ROS generation in response to hypoxia and the downstream upregulation of HIFαs and IGFBP mRNAs in croaker tissues were blocked by treatment with vitamin E. Thus, the results of this study provide new insights into the processes of adaptation to hypoxia and the molecular aspects of HIFα and IGFBP regulation in fish tissues during hypoxia stress.

MATERIALS AND METHODS

Chemicals. Oligonucleotides were synthesized by Eurofins MWG Operon (Huntsville, AL). Molecular biology reagents were obtained from Agilent Technologies (La Jolla, CA), Ambion (Austin, TX), Promega (Madison, WI), Invitrogen (Carlsbad, CA), and Roche Diagnostics (Penzberg, Germany). Rabbit anti-actin and goat polyclonal to rabbit IgG (horseradish peroxidase) were from Novus Biologicals (Littleton, CO) and SouthernBiotech (Birmingham, AL), respectively. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

Experimental animals. Young-of-the-year Atlantic croaker [length: 10–11 cm; body weight (BW): 12–18 g] were collected by otter trawl in the vicinity of Port Aransas, TX, by local fishermen in May 2007. Fish were transported to the University of Texas Marine Science Institute and treated with Paracide-F (Argent Chemical, Redmond, WA) at 170 ppm in seawater for 1 h to minimize parasite infections. Fish were then transferred to large indoor tanks (4,727 liters) equipped with a recirculating seawater system (salinity 30–32%) and maintained under control photoperiod (photophase 12 h) and temperature (27°C) conditions. They were fed chopped shrimp daily at the rate of 3.5% BW/day and acclimated to laboratory conditions for ≥1 mo prior to experimentation.

Experiment 1: effects of 2–4 wk of hypoxia exposure on IGFBP levels. Thirty fish of mixed sex were transferred into eight tanks with recirculating seawater systems (2.025 liters, including biological filter). Four tanks were assigned randomly to normoxic conditions (DO: 6.5 mg/l), and the other four tanks were maintained under hypoxic conditions (1.7 mg DO/l). The DO levels in the hypoxia exposure tanks were lowered by reducing the aeration gradually from 80–100 to 60, 40, and 20% through the air flow system, as described previously (53). Fish in each tank were fed the same amount of chopped shrimp (3.5% BW/day) and were sampled after continuous exposure for 2 and 4 wk to the target DO level (1.7 mg/l). At the end of the experiments fish were euthanized under deep anesthesia using quinaldine sulphonate (20 mg/l), following the guidelines of the University of Texas Austin Animal Care and Use Committee, which approved the experiments. Brain and liver tissues were rapidly excised, frozen in liquid nitrogen and stored at −80°C for up to 1 mo prior to analysis.

Experiment 2: effects of 15–20 wk of hypoxia exposure and a 5-wk recovery period on IGFBP levels. Fish were stocked into nine tanks (30 mixed-sex fish/tank) with a recirculating seawater system and fed chopped shrimp. Three tanks were assigned randomly to normoxic conditions (6.5 mg DO/l), and the other six tanks were maintained under hypoxic conditions. The hypoxia experimental treatments were divided into two subgroups; fish in the first subgroup were sampled after 15 (2.0 mg DO/l) and 20 wk of hypoxia exposure (2.0 mg DO/l for 1st 15 wk and then increased to 2.7 mg DO/l for next 5 wk), and fish in the second subgroup were subjected to normal oxygenation for 5 wk after 15 wk of hypoxia (2.0 mg DO/l) and sampled at the end of the recovery period. At the end of the experiments, fish were anesthetized using quinaldine sulphate, and tissues were rapidly excised and frozen in liquid nitrogen.

Experiment 3: potential mechanisms of IGFBP upregulation during hypoxia stress. Thirty fish of mixed sex were stocked into four tanks and exposed to normoxic (6.5 mg DO/l) and hypoxic (1.7 mg DO/l) conditions. Fish were anesthetized with quinaldine sulphate as described above and injected intraperitoneally with either vehicle (1 mO mg E/g BW). At the end of experiment fish were anesthetized, and the liver tissues were excised and frozen in liquid nitrogen. For measurement of superoxide radical production (O2·−; an index of ROS), fresh liver samples were cut into thin slices (~100 mg).

Rapid amplification of cDNA ends and cloning. Molecular protocols used were similar to those of Rahman and Thomas (53). Briefly, total RNA was extracted from liver tissues using TRI reagent (Sigma-Aldrich) and treated with DNase (Promega) according to the manu-
facturer’s protocol to eliminate genomic DNA. First-strand cDNA synthesis was carried out using the RNA ligase-mediated rapid amplification of cDNA ends system (RACE; Invitrogen). Partial cDNAs of IGFBP-1, IGFBP-2, and IGFBP-5 were obtained by PCR amplification. Primers for amplifying of cDNA fragments were designed to span highly conserved regions of the known sequences of IGFBP-1, -2, and -5 in teleost fishes (Table 1). The PCR products were ligated, cloned into pGEM-T easy vector (Promega), transformed into competent cells (Promega), and sequenced. The full-length sequences of croaker IGFBP-1, -2, and -5 were obtained using 5’- and 3’-RACE amplification kits (Invitrogen), using gene-specific primers (Table 1).

Sequence and phylogenetic analyses. The sequence identities of croaker IGFBPs were verified using the Basic Local Alignment Search Tool program and NCBI database and aligned using the ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2). Multiple sequence alignments were adjusted manually to the regions corresponding to different motif sequences. Phylogenetic analyses were carried out with Cluster X using the neighbor-joining approach according to Tamura et al. (63). The consensus trees were constructed using the MEGA4 software package (http://megasoftware.net). Bootstrap analysis (1,000 replicates) was performed to assess the degree of support for groupings on the tree. Full species names and GenBank accession numbers for the sequences of IGFBP-1 to -6 are listed in the Fig. 2 legend for the tree. Only sequences that included a full coding DNA sequence were used in the analysis.

Tissue distribution of IGFBPs. The expression of croaker IGFBPs mRNAs among different tissues was determined by RT-PCR analysis using gene-specific primers for croaker IGFBPs (Table 1). The quality of each RT reaction was assessed with PCR primers designed to amplify a 249-bp fragment of croaker 18S rRNA (Table 1) to ensure that the PCR products were not the result of genomic DNA contamination.

Northern blot analysis. Total RNA was extracted using Tri reagent, treated with DNase, electrophoresed on a 1% (wt/vol) agarose-formaldehyde gel, and blotted onto a nylon membrane for 2 h. The membrane was then briefly rinsed in running buffer, treated by baking at 80°C for 15 min and incubated overnight at 68°C. The membrane was then washed with low [2× saline-sodium citrate (SSC), 0.1% SDS] and high (0.1× SSC, 0.1% SDS) stringency buffer at 68°C for 15 min and incubated with a DIG-labeled antibody (1:20,000) for 30 min. At the end of incubation, the membrane was washed and exposed to hyperfilm (Amersham Biosciences, Buckinghamshire, UK) to detect specific mRNA signals by the addition of DIG luminescent detection substrate (Roche Diagnostics).

Quantitative real-time PCR analysis. Quantitative real-time PCR (qRT-PCR) was performed to determine the relative expression of IGFBP-1, -2, -5 and HIF-1α mRNAs using one step of Brilliant SYBR Green qRT-PCR Master Mix (Agilent Technologies). Each transcript level was normalized on the basis of the quantification of croaker 18S rRNA. Gene-specific primers for croaker IGFBPs (Table 1) and HIF-1α (sense 5’-AGACCGAGGATGAAACCA-3’ and antisense 5’-GCCCCAGTGAAACCTGGT-3’ primers, 52) were used for amplification of croaker IGFBPs and HIF-1α mRNA. The qRT-PCR data were converted into threshold cycle (C_T) values. The analysis of relative mRNA expression was performed using the 2^(-ΔΔC_T) method (41).

Table 1. Primers used for cloning and mRNA expression of croaker IGFBP-1, IGFBP-2, and IGFBP-5 cDNAs

<table>
<thead>
<tr>
<th>Primers Used</th>
<th>Sequence for IGFBP-1</th>
<th>Sequence for IGFBP-2</th>
<th>Sequence for IGFBP-5</th>
</tr>
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<tbody>
<tr>
<td>For partial sequence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSP Forward</td>
<td>5’-CGTGYWCCHCAGGAGMRGCTG-3’</td>
<td>5’-CGTGYWCCHCAGGAGMRGCTG-3’</td>
<td>5’-CGTGYWCCHCAGGAGMRGCTG-3’</td>
</tr>
<tr>
<td>PSP Reverse</td>
<td>5’-ATTCGGACGAGGAGACACACCC-3’</td>
<td>5’-GCGTGYWCCHCAGGAGMRGCTG-3’</td>
<td>5’-GCGTGYWCCHCAGGAGMRGCTG-3’</td>
</tr>
<tr>
<td>For 5’RACE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSP 5’-1</td>
<td>5’-CTGGCGTCTCCCTGATCTTCTGAGGAC-3’</td>
<td>5’-CTGGCGTCTCCCTGATCTTCTGAGGAC-3’</td>
<td>5’-CTGGCGTCTCCCTGATCTTCTGAGGAC-3’</td>
</tr>
<tr>
<td>GSP 5’-2</td>
<td>5’-CTGGCGTCTCCCTGATCTTCTGAGGAC-3’</td>
<td>5’-CTGGCGTCTCCCTGATCTTCTGAGGAC-3’</td>
<td>5’-CTGGCGTCTCCCTGATCTTCTGAGGAC-3’</td>
</tr>
<tr>
<td>For 3’RACE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSP 3’-1</td>
<td>5’-AACGACAGTCCTGTCACCCCTCTG-3’</td>
<td>5’-AACGACAGTCCTGTCACCCCTCTG-3’</td>
<td>5’-AACGACAGTCCTGTCACCCCTCTG-3’</td>
</tr>
<tr>
<td>GSP 3’-2</td>
<td>5’-AACGACAGTCCTGTCACCCCTCTG-3’</td>
<td>5’-AACGACAGTCCTGTCACCCCTCTG-3’</td>
<td>5’-AACGACAGTCCTGTCACCCCTCTG-3’</td>
</tr>
</tbody>
</table>

In RT-PCR for tissue distribution, probe synthesis for Northern blot analysis, and real-time qRT-PCR for mRNA expression

| Forward | 5’-AACGACAGTCCTGTCACCCCTCTG-3’ | 5’-AACGACAGTCCTGTCACCCCTCTG-3’ | 5’-AACGACAGTCCTGTCACCCCTCTG-3’ |
| Reverse | 5’-AACGACAGTCCTGTCACCCCTCTG-3’ | 5’-AACGACAGTCCTGTCACCCCTCTG-3’ | 5’-AACGACAGTCCTGTCACCCCTCTG-3’ |

IGFBP, IGFBP-binding protein; PSP, partial sequence primer; GSP, gene-specific primer; RACE, RNA ligase-mediated rapid amplification of cDNA ends; qRT-PCR, quantitative reverse transcription-polymerase chain reaction. In the primer sequences, K = G or T, N = A, C, G, or T; M = A or C; R = A or G; S = C or G; W = A or T; Y = C or T.

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resolved on a 10% SDS-PAGE gel, transferred onto a nitro-blot PVDF membrane, and blocked with 5% nonfat milk in PBS-T (50 mM Tris, 100 mM NaCl, and 0.1% Tween 20, pH 7.4) for 1 h. Membranes were rinsed with PBS-T buffer, probed with primary HIF-2α antibody (dilution: 1:1,000; Novus Biologicals) overnight at 4°C, washed with PBS-T, and incubated for 1 h with a goat polyclonal to rabbit IgG (horseradish peroxidase) secondary antibody (1: 10,000; Southern-Biotech). The blots were then exposed to X-ray film to visualize the specific protein band by addition of chemiluminescent substrate (Pierce, Rockford, IL) and photographed on hyperfilm (Amersham Biosciences). The negatives were scanned using a scanner, and the intensities of HIF-2α protein bands were estimated using ImageJ software (http://rsbweb.nih.gov) to quantify protein expression. An ~45-kDa band of actin protein was used as an internal control for normalization of HIF-2α protein expression.

Statistical analysis. All of the experimental results were analyzed by one-way ANOVA and Fisher’s protected least-significant difference test for multiple comparisons and Student’s t-test for unpaired comparisons. A probability of \( P < 0.05 \) was considered statistically significant.

RESULTS

Molecular characterization of croaker IGFBP-1, IGFBP-2, and IGFBP-5 cDNAs. The full-length croaker IGFBP-1 cDNA consists of 1,264 bp nucleotides with an open reading frame (ORF) encoding a protein with 243 amino acid residues (GenBank accession no. HQ738525) and a predicted molecular weight of \( \sim 25.8 \) kDa (Fig. 1). The protein contains 17 cysteine residues clustered in the NH2- and COOH-terminal regions. The deduced amino acid sequence of croaker IGFBP-1 shows high identity with that of zebrafish (66%), common carp (52%), and yellowtail (52%) IGFBP-1s. It has 42 and 40% identity to high identity with that of zebrafish (66%), common carp (52%), and yellowtail (52%) IGFBP-1s. It has 42 and 40% identity to yellowtail, grouper, seabream, zebrafish, common carp, and rainbow trout, respectively. It also exhibits 41–46% sequence identity with higher vertebrate IGFBP-2s (e.g., chicken, mouse, and human). Similar to other vertebrates, the GCGCCXXC, CWCV, and RGD motifs are present in the croaker IGFBP-2 protein (Supplemental Fig. S1B).

The croaker IGFBP-5 cDNA is 1,325 bp nucleotides in length with an ORF encoding 258 amino acids (HQ738527; Fig. 1). The mature protein has an estimated molecular mass of \( \sim 28.5 \) kDa and is characterized by the presence of 18 cysteine residues. The deduced amino acid sequence of croaker IGFBP-5 shows high sequence identities with those of yellowtail (89%), salmon (86%), rainbow trout (84%), zebrafish (70%), rainbow smelt (64%), mouse (53%), and human (51%) IGFBP-5s, whereas its sequence identity to chicken IGFBP-5 is low at 37%. Like other teleosts, the croaker IGFBP-5 protein contains a heparin-binding motif (FKRKQCKP) as well as two potential nuclear localization signals (RKGFFKRR and PSRGRKR) in its COOH-terminal domain (Supplemental Fig. S1C).

A phylogenetic tree of the deduced amino acid sequences of IGFBPs was constructed to determine the evolutionary relationships of croaker IGFBP-1, -2, and -5 to those of other vertebrate IGFBPs. The results show that croaker IGFBP-1, -2, and -5 cDNAs align with teleost IGFBPs and are more closely related to the nonmammalian IGFBP-1, -2, and -5 clades than to the corresponding tetrapod IGFBPs (Fig. 2).

IGFBP mRNA expression by RT-PCR and Northern blot analyses. The tissue distribution patterns of croaker IGFBP-1, -2, and -5 transcripts were examined by RT-PCR. The IGFBP-1 transcript showed higher expression in the liver than in other tissues (Fig. 3A). Moderate IGFBP-1 mRNA levels were observed in the brain, eye, heart, spleen, and testis, whereas the transcript was weakly expressed in gill, intestine, kidney, and ovary. The IGFBP-2 transcript was also most abundant in liver. Relatively low IGFBP-2 expression was observed in brain, eye, spleen, and testis (Fig. 3B). The IGFBP-2 transcript was not detected in the gill, heart, kidney, or muscle, whereas a weak signal was apparent in the ovary. The IGFBP-5 transcript was ubiquitously expressed, with relatively high transcript levels in all of the tissues examined (Fig. 3C). Equal amplification of the templates in the reactions for all of the tissues was confirmed with croaker 18S rRNA (Fig. 3D). No PCR products were detected in the negative reactions.

We carried out Northern blot analysis to determine the molecular size of croaker IGFBP transcripts. As shown in Fig. 3E, single hybridization bands of ~1.5, 1.7, and 1.5 kb were detected for IGFBP-1, IGFBP-2, and IGFBP-5, respectively, in liver tissue, which corresponded to the predicted size ranges of the cloned full-length croaker IGFBP cDNAs.
Effects of 2–4 and 15–20 wk of hypoxia exposure on IGFBP regulation and condition factor. IGFBP-1, -2, and -5 mRNA levels in croaker liver and brain were quantified by qRT-PCR using croaker 18S rRNA as an internal control and are expressed as relative values per 100 ng total RNA (Figs. 4 and 5). IGFBP-1 mRNA levels were significantly increased 10- to 15-fold in liver and three- to sixfold in brain after 2 and 4 wk of hypoxia exposure (1.7 mg DO/l) compared with normoxic controls (Fig. 4, A and B), whereas IGFBP-2 and -5 mRNA levels in hypoxia-exposed fish were similar to control values (Fig. 4, C–F).

Longer-term (15 and 20 wk) exposure to hypoxia caused significant increases in liver (~4-fold) and brain (~2-fold) IGFBP-1 mRNA levels (Fig. 5, A and B). In the recovery experiment, IGFBP-1 mRNA levels in the hypoxia-exposed fish (2 mg DO/l for 15 wk) had returned to basal control values within 5 wk of exposure to normoxic conditions (Fig. 5, A and B). IGFBP-2 and IGFBP-5 mRNA levels in croaker livers were significantly elevated after exposure to 20 wk of hypoxia (Fig. 5, C and E), whereas IGFBP-2 and -5 mRNA levels in brains were elevated after both 15 and 20 wk of hypoxia exposure (Fig. 5, D and F). Both IGFBP-2 and -5 mRNA levels in livers and brains declined in the hypoxia-exposed fish after the DO in the tanks was restored to normal levels (6.5 mg DO/l) and were not significantly different from controls (Fig. 5, C–F).

We calculated condition factor \( K = 100 \times W/L^3 \) \( W \) = weight of fish in grams, \( L \) = length of fish in millimeters (35)) to investigate whether 2–4 or 15–20 wk of hypoxic conditions affect fish growth. We found no significant difference in condition factor after 2–4 wk of hypoxia exposure (Fig. 6A), whereas longer hypoxia exposure (15 and 20 wk) significantly decreased condition factors (Fig. 6B). In the recovery experiment, the condition factor had returned to control values within 5 wk of exposure to normoxic conditions (Fig. 6B).

Interactive effects of hypoxia and an antioxidant on regulation of ROS, HIF\( \alpha \)s, and IGFBPs. To investigate the possible interactive effects of an antioxidant with hypoxia on regulation of ROS, HIF\( \alpha \), and IGFBP, we first measured ROS production...
HIF-2 levels were increased twofold in hypoxia-exposed fish compared with normoxic controls, and this hypoxic effect was completely abolished by treatment with vitamin E (Fig. 7E). However, there were no significant changes in IGFBP-2 or IGFBP-5 mRNA levels after this period of hypoxia exposure or after treatment with the antioxidant (Fig. 7F).

**DISCUSSION**

In this report we describe the molecular characterization and hypoxia-induced expression of three IGFBP isoforms, IGFBP-1, IGFBP-2, and IGFBP-5, from a marine perciform teleost, Atlantic croaker. The croaker IGFBP-1, -2, and -5 cDNAs possess the conserved functional domains and characteristic motifs of vertebrate IGFBP proteins, which suggest they have roles similar to other IGFBPs in the regulation of IGF activity and growth and development. We show for the first time in an aquatic species that chronic hypoxia exposure causes upregulation of IGFBP-2 and IGFBP-5 mRNAs in the liver in addition to its well-known action to upregulate IGFBP-1 mRNA. In addition, the present findings extend our knowledge of hypoxia upregulation of IGFBPs in fish to the most hypoxia-sensitive organ, the brain. Interestingly, the expression patterns and time courses of upregulation of the various IGFBPs in the brain and liver were similar, with IGFBP-1 mRNA expression upregulated after 2–4 wk hypoxia exposure, whereas IGFBP-2 and IGFBP-5 mRNAs were increased only after longer-term (15–20 wk) hypoxia exposure. In addition, we demonstrate that the elevation of IGFBP-1 mRNA expression is accompanied by increases in ROS production, HIF-1α mRNA expression, and HIF-2α protein levels in liver tissues. Moreover, the observation that pharmacological treatment with an antioxidant, vitamin E, attenuated the hypoxia-induced increases in hepatic ROS production, HIF-1α mRNA expression, and HIF-2α protein levels as well as IGFBP-1 mRNA levels suggests that these changes are mediated through alterations in the oxidative status in the liver. Taken together, these results suggest an involvement of a ROS-HIF pathway in the regulation of IGFBP-1 during hypoxia stress. To our knowledge, this is the first evidence for a role of oxidative status and interactions of ROS and HIFαs in IGFBP-1 regulation in any aquatic vertebrate during hypoxic stress.

The three distinct IGFBP isoforms cloned from croaker in the present study, IGFBP-1, -2, and -5, have presumed NH2-terminal, COOH-terminal, and L domains containing functional motifs characteristic of IGFBPs (33). The NH2- and COOH-terminal domains are highly conserved, whereas the L domain varies within the IGFBP family (Fig. 1). As predicted, the croaker IGFBPs show higher amino acid sequence identities to teleost IGFBPs (IGFBP-1: 40–42%; IGFBP-2: 41–46%; and IGFBP-5: 37–53%). The phylogenetic analysis confirms that the croaker IGFBP-1, IGFBP-2, and IGFBP-5 isoforms are more closely related to teleost than mammalian IGFBPs. However, despite their overall structural similarity, there are several
structural differences between mammalian and teleost IGFBP proteins. In the COOH-terminal region, an important RGD motif found in mammalian IGFBP-1 and -5 proteins that is involved in cell-to-cell interactions and intracellular matrix association (19) is not present in croaker and other teleost IGFBP-1 and -5 proteins. The binding of mammalian IGFBP-1s to \( \alpha_5 \beta_1 \) integrin occurs through its RGD motif, resulting in stimulation of cell migration (16, 26). Interestingly, the RGD motif is present in all vertebrate IGFBP-2s, which suggests that the acquisition of RGD motif in IGFBP-1 may have occurred after the divergence of teleosts from other vertebrates (31, 44).

The finding that croaker IGFBP-1 mRNA is highly expressed in liver but has relatively low expression in other tissues is consistent with previous results in other teleost species (31, 44, 48, 60). The pattern of IGFBP-1 expression in various tissues suggests that it is synthesized locally and modulates the local action of IGFs. The high expression of croaker IGFBP-2 mRNA in the liver and lower levels in the brain and other tissues, some of which show no detectable expression, has also been observed in other teleost species (20, 31, 48). Similar to the observations in other vertebrates, the IGFBP-5 mRNA was highly expressed in all croaker peripheral tissues examined (22, 31, 48). The different tissue distributions of the three croaker IGFBPs mRNA suggests that they have different physiological functions in these tissues to regulate the local actions of IGFs.

The observation that single hybridization bands at \( 1.5 \), \( 1.7 \), and \( 1.5 \) kb were obtained by Northern blot analyses of croaker IGFBP-1, -2 and -5 mRNAs, respectively, suggests that all of them are present in single transcripts. Similarly to croaker, single \( 1.5 \)-kb IGFBP-1 transcripts were found in zebrafish (44) and rat (47), and single IGFBP-2 transcripts

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**Fig. 4.** Effects of 2 and 4 wk of hypoxia (HYP) exposure (1.7 mg DO/l) on IGFBP-1, IGFBP-2, and IGFBP-5 mRNA expression in livers (A, C, and E) and brains (B, D, and F) and 18S rRNA expression in liver (G) and brain (H) of Atlantic croaker. Note: exposure duration refers only to the period where fish were exposed to target DO; fish were previously exposed to declining DO for additional 2-day adjustment period. Each value represents the mean ± SE (n = 7–8). Student’s t-test; *P < 0.05 and **P < 0.01, significant differences from normoxic controls (CTL). Ct, threshold cycle.
(1.8 kb) were detected in zebrafish (17) and chicken (57). On the other hand, two IGFBP-2 transcripts (~2.2–2.5 and ~1.3–1.5 kb) have been detected in seabream (20) and common carp (12), and in the rat a minor IGFBP-2 transcript at ~2.4 kb was detected in several tissues in addition to a major transcript at ~1.6 kb (42). It is not known whether the two IGFBP-2 transcripts found in these species are the result of alternative splicing. Recently, the existence of duplicate IGFBP-2 and -5 genes and IGFBP-2a and -2b and IGFBP-5a and -5b cDNAs has been demonstrated in zebrafish and chinook salmon, respectively (14, 62, 76). Therefore, the possibility exists that a second transcript of IGFBP-2 and -5 may be present in teleosts that underwent an additional duplication event early in the teleost lineage (1). However, the detection of only single transcripts of the croaker IGFBPs in the present study by Northern blot analysis suggests that the functions of the IGF system are predominantly regulated by only one isoform of each of these IGFBPs in this species.

Fig. 5. Effects of longer-term (15 and 20 wk) hypoxia exposure on IGFBP-1, IGFBP-2, and IGFBP-5 mRNA expression in liver (A, C, and E) and brain (B, D, and F) and 18S rRNA expression in liver (G) and brain (H). Fish were exposed to 2.0 mg DO/l for 15 wk with or without an additional 5 wk of exposure to moderate hypoxia (2.7 mg DO/l) or normoxia (recovery). Note: exposure duration refers only to period where fish were exposed to target DO (2 mg/l); fish were previously exposed to declining DO for additional 2-day adjustment period. Each value represents the mean ± SE (n = 7–8). Student’s t-test; *P < 0.05, **P < 0.01, and ***P < 0.001, significant differences from normoxic controls. Exp, exposure; rec, recovery; REC, recovery group fish.
Although hypoxia has been shown to increase the expression of multiple IGFBP mRNAs (IGFBP-1, IGFBP-2, and IGFBP-5) in mammalian tissues (40, 49, 64), the present study is the first to demonstrate upregulation of all of these IGFBP mRNAs by hypoxia in an aquatic species. Previous studies in adult teleosts had demonstrated only upregulation of hepatic IGFBP-1 mRNA after hypoxia exposure (21, 44, 75). The finding that IGFBP-1 mRNA levels in both croaker liver and brain tissues Fig. 6. A and B: effects of 2 and 4 wk of hypoxia exposure (1.7 mg DO/l), longer-term hypoxia exposure (15 and 20 wk), and 5 wk of recovery under normoxic conditions on croaker condition factors. Condition factors were calculated from the $K = 100 \times W/L^3$ (30), where $W =$ weight of fish in grams and $L =$ length of fish in millimeters. Each value represents the mean ± SE ($n = 20–30$). Student’s $t$-test; *$P < 0.05$ and **$P < 0.01$, significant differences from normoxic controls.

Fig. 7. Effects of 4 wk of hypoxia exposure (1.7 mg DO/l) and antioxidant (AOX: vitamin E) treatment on superoxide radical (SOR) production (A), hypoxia-inducible factor (HIF)-1α mRNA levels (B), nuclear HIF-2α protein expression (C), relative HIF-2α protein levels (D), and IGFBP mRNA levels (E and F) in croaker livers. Each value represents the mean ± SE ($n = 10–12$). Significant differences identified with a multiple range test, Fisher’s protected least significant difference test, are indicated by different letters ($P < 0.05$).
were increased severalfold after 2–4 wk of hypoxia exposure, whereas IGFBP-2 and IGFBP-5 mRNA levels were unaltered after these exposure periods, suggests that IGFBP-1 is the major IGFBP gene involved in early adaptation to hypoxia stress in this species. The present results are consistent with the suggestion that IGFBP-1 plays an important role in regulating growth and development under hypoxic stress (29). Significant impairment of the condition factor, an indicator of growth (35), was seen in croaker after 15 wk of hypoxia stress, prior to any changes in the hepatic expression of the other IGFBP genes. However, the data showing that IGFBP-2 and -5 mRNA levels were increased in croaker brains after this period of exposure to hypoxia stress indicate a potential role for these IGFBPs in regulating growth through neural pathways. Several reports indicate that IGFBP-2 is a negative regulator of growth (17), and hypoxia has been shown to upregulate IGFBP-5 mRNA levels in rat brains (40). Several mammalian studies suggest that IGFBPs may also have important neuroprotective functions by preventing apoptosis during hypoxia-induced ischmic brain injury (4, 55). One potential function identified in the current study is protection against hypoxia-induced oxidant stress. However, additional studies will be required to determine whether IGFBPs have neuroprotective functions in aquatic vertebrates and their mechanisms of action during hypoxia stress.

One of the most interesting findings of the present study is that the hypoxia-induced increase in hepatic IGFBP-1 mRNA levels is accompanied by marked increases in hepatic ROS production. As predicted, this hypoxia exposure regime also caused increased in hepatic HIF-1α mRNA and nuclear HIF-2α protein levels. The observation that the increases in ROS production, IGFBP-1 mRNA, and HIF-1α mRNA were blocked by administration of an antioxidant suggests that they are upregulated by a common mechanism during hypoxia stress. To our knowledge, this is the first report that suggests a potential in vivo interaction of ROS and HIFαs in the regulation of IGFBPs under hypoxic stress in aquatic vertebrates. A recent in vitro study showed that HIF-1α mediates hypoxia-induced IGFBP-1 gene expression in zebrafish liver cells by selectively interacting with hypoxia response elements (30). Moreover, several mammalian studies have shown that hypoxia induces cellular ROS production and that elevated ROS levels result in increases in HIF-1α protein levels in cultured human hepatoma (HepG2) cells (10, 50). In addition, Lang et al. (38) demonstrated that exogenous H2O2, an index of ROS generation, increases IGFBP-1 mRNA expression, protein levels, and IGFBP release in cultured human HepG2 cells. On the other hand, the application of an antioxidant eliminates the hypoxic generation of ROS and reduces HIF-1α protein levels in human hepatoblastoma cells (56). Furthermore, Martin et al. (43) demonstrated that hypoxia significantly reduced concentrations of α-tocopherol, a form of vitamin E, and increased H2O2 levels in human endothelial cells. Collectively, these data, along with our findings, suggest that hypoxia increases ROS production, which in turn leads to the elevation of IGFBP-1 transcript via HIFα activation. In addition, they indicate that antioxidant treatment inhibits the generation of ROS, resulting in a loss of HIFα transcriptional activity, which leads to the suppression of IGFBP-1 upregulation.

In conclusion, three distinct IGFBP transcripts, IGFBP-1, IGFBP-2, and IGFBP-5, were characterized from a marine teleost, Atlantic croaker. Structural analyses of croaker IGFBPs strongly suggest that the three isoforms identified are members of the IGFBP family. Furthermore, we demonstrated for the first time in an aquatic vertebrate that 2–4 and 15–20 wk of hypoxia increase the expression of IGFBP-1, -2, and -5 mRNAs in two different intact organs, the brain and liver, suggesting that IGFBPs are hypoxia-inducible transcripts. Moreover, our in vivo experiments provide the first clear evidence that hepatic ROS generation is physiologically important to IGFBP-1 regulation via HIFα activation during hypoxic stress. The molecular mechanism of ROS and HIFα regulation proposed in this study provides valuable insights into the possible functions and potential role of IGFBP during hypoxic stress. Finally, the finding that the pattern of IGFBP mRNA upregulation in croaker tissues is dependent upon the duration of hypoxia exposure and is reversed after the fish are exposed to normoxic conditions suggests that measurement of IGFBP mRNA levels may be a useful biomarker of environmental hypoxia exposure.

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DISCLOSURES
The authors have no conflicts of interest to declare.

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