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Short communication

The IGFBP-1 mRNA Expression in HepG2 Cells is Affected by Inhibition of Heme Biosynthesis

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Insulin-like growth factor binding protein-1 (IGFBP-1) appears to be an important modular of the insulin growth factor (IGF) bioactivity in metabolic disease and chronic hypoxia. Treatment of desferrioxamine (Dfo), cobalt, or nickel in HepG2 cells stimulated the expression of IGFBP-1 mRNA as hypoxia. However, the presence of ferric ammonium citrate (FAC) in the 1% O_2 decreased the upregulation of the IGFBP-1 mRNA expression. In addition, actinomycin D and cycloheximide abolished the increase in the expression of IGFBP-1 mRNA that was induced by Dfo and transition metals (cobalt and nickel). To obtain further information about the putative oxygen sensor, we postulate that putative heme proteins, responsible for the oxygen-sensing process in HepG2 cells, should be sensitive to hypoxia. The mechanism of these upregulations of the IGFBP-1 mRNA expression by Dfo and transition metals was investigated by treatment with 2 mM of 4,6-dioxoheptanoic acid (DHA), an inhibitor of heme biosynthesis. The results showed that 1% O_2-, Dfo-, cobalt-, or nickel induced IGFBP-1 mRNA expressions in HepG2 cells were all markedly inhibited when the heme synthesis was blocked by DHA. We suggest that the IGFBP-1 mRNA expression in the HepG2 cell is regulated by 1% O_2, Dfo, cobalt, or nickel, implicating the involvement of the putative heme-containing oxygen-sensing molecule.

Keywords: Cobalt, Dfo, DHA, IGFBP-1, Nickel

Introduction

The human insulin-like growth factor-binding protein-1 (IGFBP-1) appears to be an important modulator of insulin growth factor (IGF) bioactivity in metabolic disease (Unterman *et al.*, 1992). Also, IGFBP-1 is primarily expressed by the liver, uterine decidua, and secretory endometrium (Brinkman *et al.*, 1988; Ooi *et al.*, 1990). It is elevated in the circulation and liver in fetuses with hypoxia and the intrauterine growth restriction (IUGR, birth weight <10th percentile for gestational age). It is believed to contribute to IUGR by inhibiting the IGF-mediated fetal growth (Giudice *et al.*, 1995). In humans, IUGR is a leading cause of severe fetal and neonatal morbidity and mortality (Tazuke *et al.*, 1998). Recently, in cultured HepG2 cells, the IGFBP-1 mRNA expression is increased by desferrioxamine (Dfo) (Park *et al.*, 2000), as iron chelator mimic hypoxia. Tazuke *et al.* (1998) identified three consensus sequences for the hypoxia response element (HRE) in intron 1 of the human IGFBP-1 gene, and demonstrated that at least one is hypoxia responsive with regard to the IGFBP-1 gene expression. IGFBP-1 induction by hypoxia is mediated via the hypoxia-inducible factor-1 (HIF-1) (Tazuke *et al.*, 1998), which is important in the response of other hypoxia-inducible genes (Wang and Semenza, 1993). For example, genes whose transcription is activated by HIF-1 include the following: EPO, encoding erythropoietin (Schuster *et al.*, 1989), vascular endothelial growth factor (VEGF) (Levy *et al.*, 1995), glycolytic enzymes (Semenza *et al.*, 1994), and inducible nitric oxide synthetase (Bunn and Poyton, 1996).

Cobaltions, as well as iron chelators, have been hypothesized to bind to the heme portion of the hypoxia sensor and induce HIF-1, as does hypoxia (Bunn and Poyton, 1996). Also, the involvement of an iron-containing heme protein in the hypoxic upregulation of several genes, including EPO (Goldberg *et al.*, 1988), VEGF (Goldberg and Schneider, 1994; Liu *et al.*, 1998), and others (Morita and Kourembanas, 1995), have been determined using similar approaches.

Therefore, we hypothesized that an increase of the IGFBP-1 mRNA expression level in HepG2 cells in response to Dfo, cobalt, and nickel supports the involvement of heme protein, or a similar oxygen sensor in the hypoxia regulation of the IGFBP-1 mRNA expression. To test this hypothesis, we

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quantitated the IGFBP-1 mRNA expression levels in HepG2 cells treated with Dfo, transition metals (cobalt, nickel, or FAC), and DHA that affect the heme biosynthesis. These results suggest that Dfo, cobalt, nickel, FAC, or DHA regulate the IGFBP-1 mRNA expression in HepG2 cell, which implicates the involvement of the putative heme-containing oxygen-sensing molecule.

Materials and Methods

Chemicals Actinomycin D, cycloheximide, 4, 6-dioxoheptanoic acid (DHA), cobalt, nickel, ferric ammonium citrate (FAC), as a source of iron and desferrioxamine (Dfo) were purchased from the Sigma Chemical Co. (St. Louis, USA). The Dig-labeling Kit, restriction enzyme, and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals (Germany). The RPMI 1640 medium, fetal bovine serum, Dulbeccos phosphate buffered saline (PBS), and penicillin-streptomycin were purchased from GibcoBRL (Grand Island, USA). All other chemicals used in the RNA work were prepared in diethyl pyrocarbonate (DEPC)-treated water.

Cell culture and culture conditions to assess the cellular response to hypoxia The HepG2 cell, a human hepatoblastoma cell line, was grown in a RPMI 1640 medium (Gibco-BRL) that was supplemented with 10% heat-inactivated fetal bovine serum at 37°C in 5% CO_2. At the initiation of each experiment, the cells were resuspended in the medium at a density of 2.5×10^5 cells/ml. For hypoxic conditions, the cells were placed in airtight chambers (NuAire, USA), which were flushed with a 5% carbon dioxide/95% nitrogen mixture until the oxygen concentration was 1%. The HepG2 cells were stimulated with Dfo, cobalt, or nickel at a final concentration of 100 μM, 250 μM, and 100 μM, respectively. In some experiments, the cells were treated with various concentrations (5, 25, 50, and 100 μg/ml) of FAC as a source of iron. When used, actinomycin D and cycloheximide were added to the final concentration of 1 μg/ml, 50 μg/ml, respectively, for 2 h before exposure to 1% O_2, Dfo, cobalt, or nickel. Also, the inhibition of heme synthesis was studied by incubating the cells with DHA (2 mM) for 24 h before exposure to 24 h of 1% O_2, Dfo, cobalt, or nickel.

Preparation of cDNA probes The differentially displayed cDNA fragment (299 bp) of human IGFBP-1 was cloned (Park *et al.*, 2000) into a pBluescript plasmid vector, as described previously (Klimkait, 2000). For Northern analysis, 50 ng of the probe was labeled with digoxigenin (DIG)-11-dUTP (Boehringer-Mannheim), and used for the random primed labeling reactions of the cDNA probes.

Preparation of total RNA Total cellular RNA was extracted from HepG2 cells using a rapid ice-cold lysis buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, and 0.5% Sarkosyl) (Chomczynski and Sacchi, 1987). Total cellular RNA in the aqueous phase was precipitated with cold isopropyl alcohol and washed in 70% ethanol. The resulting RNAs were redissolved in DEPC- treated water. Isolated RNA samples were quantified by a spectrophotometric analysis at 260 nm, and ethidium bromide staining of 18S and 28S ribosomal RNA bands was performed to confirm RNA integrity.

Northern blot analysis Total cellular RNA (approximately 20 μg/lane) was denatured in formaldehyde, electrophoresed on an 1% agarose gel that contained 2.2 M formaldehyde (Engler-Blum *et al.*, 1993) and a trace amount of ethidium bromide. It was then transferred to positively charged nylon membranes (Schleicher & Schull, Dassel) using the Turboblotter system (Schleicher & Schull, Dassel), according to the manufacturers instructions, and crosslinked by UV irradiation. Prehybridization was carried out at 68°C for 1 h in a solution containing 0.25 M Na_2HPO_4 [pH 7.2], 1 mM EDTA, 20% SDS, and a 0.5% blocking reagent (Boehringer-Mannheim). Hybridization was carried out at 68°C for 12 hr with one of the DIG-labeled IGFBP-1 probes in the prehybridization solution. After hybridization, the membranes were washed three times for 20 min at 65°C with a washing buffer (20 mM Na_2HPO_4, 1 mM EDTA, and 1% SDS). IGFBP-1 mRNA was detected using the DIG luminescent detection kit (Boehringer-Mannheim), according to the manufacturer's instructions. The intensity of the blots was normalized with 18S ribosomal RNA on ethidium bromide staining (Lee *et al.*, 2001). The band intensity was quantified by scanning the X-ray film with the Fluor-S™ MultiImager (Bio-Rad, Hercules, USA).

Statistical analysis All data are expressed as mean ± SD of the duplicate experiments. A statistical analysis was performed with ANOVA. It compared the differences between groups with $P < 0.05$ that were considered significant.

Results and Discussion

Effect of exposure to Dfo and transition metals (cobalt, nickel, or FAC) on the IGFBP-1 mRNA expression To investigate the effects of Dfo and transition metals (cobalt and nickel) on the levels of IGFBP-1 mRNA expression, the HepG2 cells were incubated at a state of normoxia (21% oxygen), including Dfo (0~100 μM), cobalt (0~200 μM), or nickel (0~1000 μM) for 24 h. The total RNA was isolated and hybridized to the IGFBP-1 cDNA probe by Northern blotting. As shown in Fig. 1~Fig. 3, Dfo (100 μM), cobalt (100 μM), or nickel (250 μM) treatment increased (5-fold, 3.2-fold, and 4.2-fold, respectively) the expression of IGFBP-1 mRNA in comparison to the untreated cells. However, treatment of cells with cobalt or nickel at concentrations greater than 100 μM or 250 μM, respectively, failed to further enhance the IGFBP-1 mRNA expression. However, it caused the inhibitions of upregulated IGFBP-1 mRNA. Also, the cells were exposed to media containing various concentrations of FAC (5, 25, 50, 100 μg/ml) for 12 h at 1% O_2 condition and the IGFBP-1 mRNA expression was analyzed. The FAC dose-response analysis revealed that compared with untreated cells, the IGFBP-1 mRNA expression was markedly decreased (1.3-fold) in FAC-treated cells (over 5 μg/ml concentration), then decreased below the baseline (20% O_2) expression at 100 μg/

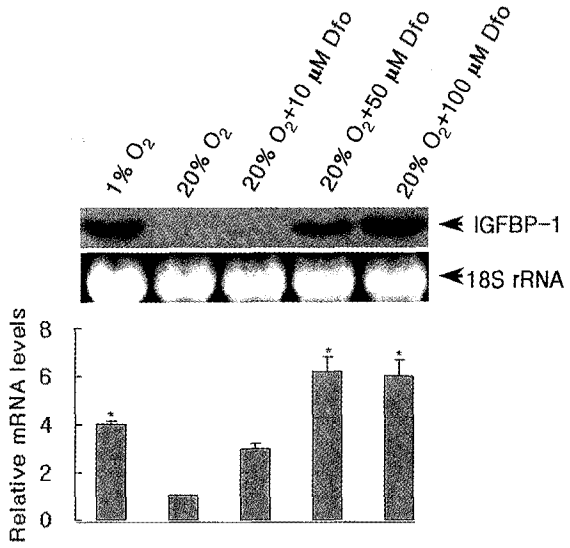

Fig. 1. Dfo stimulates a dose-dependent increase in the IGFBP-1 mRNA expression. Dfo stimulation resulted in a significant, dose-dependent increase in the IGFBP-1 expression that was significantly different from 20% O_2 at 50 μM, 100 μM (*P<0.01).

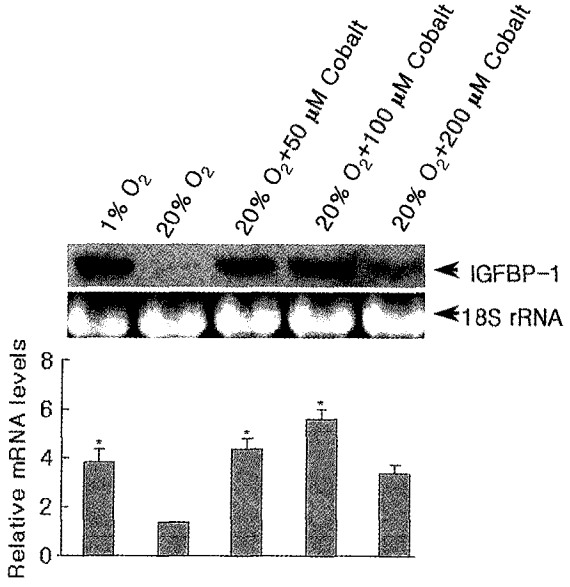

Fig. 2. Cobalt stimulates a dose-dependent increase in the IGFBP-1 mRNA expression. Cobalt stimulation resulted in a dose-dependent increase in the IGFBP-1 mRNA expression, beginning at 50 μM and peaking in expression to >3.2-fold at 100 μM (*P<0.01), then returning to decrease the expression at 200 μM.

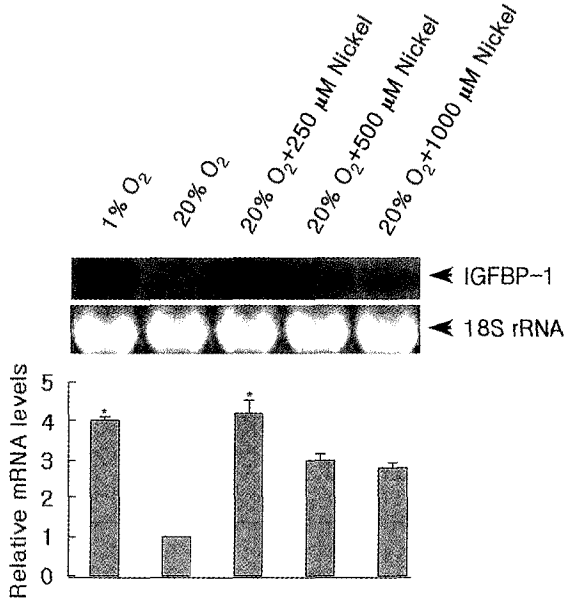

Fig. 3. Nickel stimulates a dose-dependent increase in the IGFBP-1 mRNA expression. Nickel stimulation resulted in a significant, dose-dependent increase in the IGFBP-1 expression that was significantly different from 20% O_2 at 250 μM (*P<0.01).

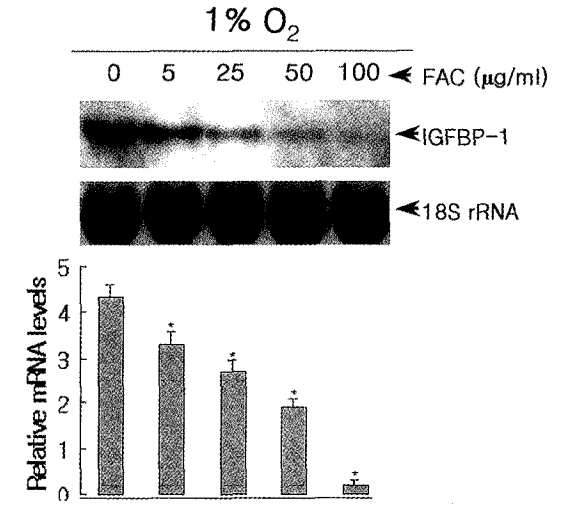

Fig. 4. FAC caused a dose-dependent decrease in the IGFBP-1 mRNA expression. The IGFBP-1 mRNA expression were markedly decreased (1.3-fold) in the FAC-treated cell (over 5 μg/ml concentration), and then decreased below the baseline (20% O_2) expression at 100 μg/ml (*P<0.05)

ml (Fig. 4). The expression of the IGFBP-1 mRNA by agents may be explained by the substitution of cobalt or nickel for ferrous iron in the porphyrin ring, thereby locking the heme protein in the deoxy conformation, just as in low oxygen tension (Goldberg *et al.*, 1988; Goldberg *et al.*, 1991).

Actinomycin D or cycloheximide inhibits IGFBP-1 mRNA up-regulation by Dfo and transition metals (cobalt and nickel) To investigate the mechanisms of IGFBP-1 mRNA upregulation in response to Dfo or transition metals (cobalt and nickel), the HepG2 cells were pretreated with actinomycin D (1 μg/ml), an inhibitor of transcription, and cycloheximide (50 μg/ml), an inhibitor of translation, for 2 h before exposure to each stimulus. The results showed that treatment with actinomycin D completely abolished the

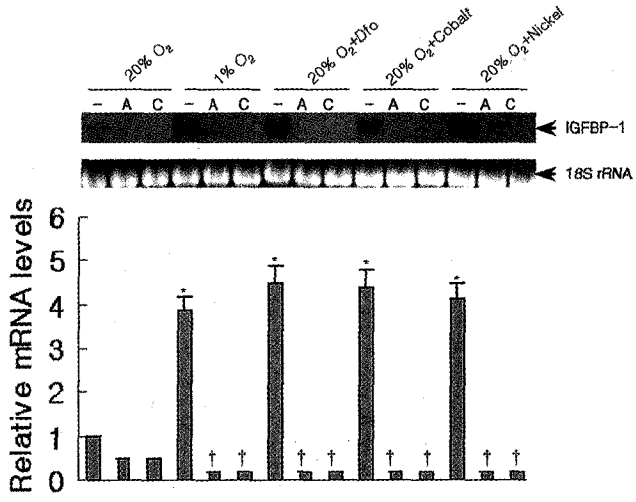

Fig. 5. Effect of actinomycin-D (A) and cycloheximide (C) pretreatment on IGFBP-1 induction by 1% O_2, Dfo, cobalt, and nickel. Hypoxia (*lane* 4), Dfo (*lane* 7), cobalt (*lane* 10), and nickel (*lane* 13) all increased the IGFBP-1 mRNA expression (*P<0.05), compared with 20% O_2 (*lane* 1). In contrast, with the addition of actinomycin-D and cycloheximide, hypoxia (*lane* 5, 6), Dfo (*lane* 8, 9), cobalt (*lane* 11, 12), and nickel (*lane* 14, 15), each case demonstrated a dramatic decrease in the expression of IGFBP-1 mRNA (P<0.01, respectively)

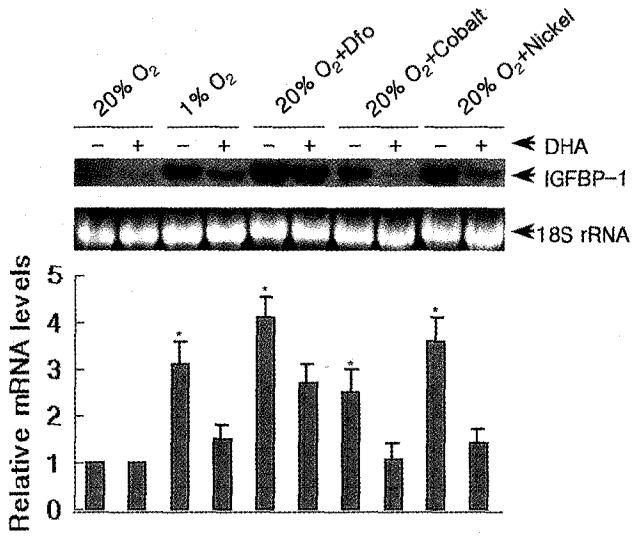

Fig. 6. Effects of 4,6-dioxoheptanoic acid (DHA) on 1% O_2, Dfo, cobalt, and nickel-induced IGFBP-1 mRNA expression by HepG2 cells. Cells were grown as stated previously, but were incubated for 24 h with 2 mM DHA before exposure to 1% O_2, 100 μM Dfo, 100 μM cobalt, and 250 μM nickel for additional 24 h (*P<0.05).

upregulation of IGFBP-1 mRNA (Fig. 5), suggesting that active RNA transcription was required for the increased levels of IGFBP-1 mRNA secondary to 1% O_2, Dfo, cobalt, or nickel. Similarly, the cycloheximide treatment completely abolished the response (Fig. 5). These results suggest that de novo protein synthesis was required for the upregulation of the IGFBP-1 mRNA expression in the HepG2 cell in response to 1% O_2, Dfo, cobalt, or nickel.

Effect of a heme synthesis in the hypoxic regulation of IGFBP-1 mRNA expression To determine whether the regulation of IGFBP-1 mRNA is mediated by the heme protein as an oxygen sensor, the HepG2 cells were incubated for 24 h in media containing DHA (2 mM), an inhibitor of aminolevulinate dehydratase (Tschudy *et al.*, 1981), which is the key enzyme in the heme synthetic pathway. The HepG2 cells were then grown either in 1% O_2, or in the presence of Dfo (100 μM), cobalt (100 μM), or nickel (250 μM). A Northern blot analysis revealed that the DHA caused about a 2.1-fold decrease in 1% O_2 induced IGFBP-1 mRNA expression, as well as about a 1.5-fold decrease in Dfo induced, about a 2.5-fold decrease in cobalt induced, and approximately a 2.5-fold decrease in nickel induced IGFBP-1 mRNA expression (Fig. 6). These results suggest that the heme synthesis that is blocked by DHA treatment possibly impairs an oxygen sensing mechanism in HepG2 cells, and subsequently blocks the IGFBP-1 mRNA expression that is induced by 1% O_2, Dfo, cobalt, or nickel. Based on these experiments, we suggest that the ability of heme protein to regulate the IGFBP-1 mRNA expression is assessed through the use of Dfo, cobalt, or nickel, which have been shown to mimic hypoxia by preventing the binding of oxygen to heme groups (Bunn and Poyton, 1996), or by blocking heme biosynthesis (Goldberg *et al.*, 1988). In conclusion, we suggest that the putative heme protein might be involved in the regulation of the IGFBP-1 mRNA expression.

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