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Stabilization of IGFBP-1 mRNA by ethanol in hepatoma cells involves the JNK pathway☆

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***Background/Aims*: Insulin-like growth factor-binding protein-1 (IGFBP-1) modulates cell growth and metabolism in a variety of physiopathological conditions. The aim of this study was to determine the molecular mechanisms involved in IGFBP-1 upregulation by ethanol.**

***Methods*: We studied IGFBP-1 regulation by ethanol at the protein, mRNA and gene promoter levels in the human hepatocarcinoma cell line, HepG2, which does not express significantly ethanol-metabolizing enzymes.**

***Results*: Ethanol (35–150 mM) induced the IGFBP-1 mRNA and protein up to 5-fold in a dose-dependent manner. A similar effect was observed using primary cultures of human hepatocytes. Various inhibitors of ethanol metabolism and the antioxidant *N*-acetylcysteine did not prevent ethanol effects. While ethanol did not modify the IGFBP-1 gene promoter activity, it elicited a 2- to 3-fold increase in IGFBP-1 mRNA half-life and this stabilization required the 5′ and the 3′ untranslated mRNA region. Ethanol triggered a rapid activation of c-Jun N-terminal Kinase (JNK) in HepG2 cells and IGFBP-1 induction was significantly decreased by a specific inhibitor of JNK.**

***Conclusions*: This study reveals a novel pathway of gene regulation by alcohol which involves the activation of JNK and the consequent mRNA stabilization.**

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***Keywords*: IGFBP-1; Ethanol; Gene regulation; JNK; Hepatoma cell line**

1. Introduction

Insulin-like growth factor-binding protein-1 (IGFBP-1) is one of the six soluble binding proteins that binds insulin-like growth factors (IGFs) with high affinity. IGFBP-1 is mainly produced by hepatocytes and secreted in the serum; it is also synthesized by kidney and ovarian granulosa cells and, in pregnant women, by decidualized uterine endometrium [1]. In the serum, IGFBP-1 modulates the bioavailability and the cellular actions of IGFs which are essential factors for cell growth, differentiation and metabolism. IGFBP-1 also exhibits IGF independent effects on cell migration, growth, and death by interacting with cell surface partners [2].

Insulin, *via* inhibition of *IGFBP-1* gene expression, is a major determinant of IGFBP-1 levels in the liver and the serum [1]. But *IGFBP-1* gene expression is induced by severe catabolic conditions observed during liver

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Abbreviations: IGFBP-1, insulin-like growth factor-binding protein-1; JNK, c-Jun N-terminal kinase; qRT-PCR, real time quantitative reverse transcription polymerase chain reaction.

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disease and critical illness [3,4] as well as by a variety of stress conditions such as amino acid depletion, hypoxia and proinflammatory cytokines [5–7]. We have recently shown that IGFBP-1 is also induced by dioxin, an environmental contaminant and upon endoplasmic reticulum stress [8,9]. The biological relevance of high levels of IGFBP-1 has been extensively studied using transgenic technology. A dominant feature of the transgenic lines is the fetal and postnatal growth restrictions [10,11] and alterations of female reproductive functions [12,13]. Some transgenic lines also display impaired glucose homeostasis [14]. Moreover, acute elevation of circulating IGFBP-1 in rats leads to decreased protein synthesis in specific muscle tissues [15]. Furthermore, knockdown of IGFBP-1 in Zebrafish significantly alleviated the hypoxia-induced growth retardation and developmental delay [16]. These data suggested that IGFBP-1 induction may be a conserved physiological mechanism to restrict growth process under stress conditions in order to preserve the energy for survival functions.

Interestingly, several metabolic and growth effects of elevated IGFBP-1 levels such as fetal growth deficiency and erosion of lean body mass are reminiscent of the toxic consequences observed during chronic alcohol abuse [17,18]. This led to the hypothesis that IGFBP-1 induction during alcohol intoxication could mediate some of the effects of alcohol. *In vivo* studies showed that acute and chronic ethanol intoxication in humans and animals resulted in increased IGFBP-1 circulating levels. In humans, ingestion of moderate amounts of alcohol by healthy individuals resulted in an acute and potent increase in serum IGFBP-1 and a delayed and less powerful decline in serum IGF-I [19,20]. In rats, chronic and acute ethanol intoxication upregulated both serum IGFBP-1 and IGFBP-1 mRNA in the liver and to a lesser extent in the kidney [21,22]. Studies by Kumar et al. in mice suggested that the induction of hepatic IGFBP-1 by ethanol is partially mediated by increased levels of tumor necrosis factor-alpha (TNF-α) [21].

Those *in vivo* studies clearly documented a positive effect of ethanol on IGFBP-1 expression but the molecular mechanisms of this regulation remained elusive. In particular, it was still unclear whether the induction of IGFBP-1 could result from a direct cellular effect of ethanol rather than from a general systemic effect and whether ethanol metabolism could contribute to such an effect. In order to decipher the mechanisms of human IGFBP-1 gene induction by ethanol, we characterized this regulation in the human hepatoma cell line HepG2 which does not metabolize ethanol significantly. Indeed, two main enzymes responsible for oxidative metabolism of ethanol, alcohol dehydrogenase (ADH) and microsomal cytochrome P450 2E1 (CYP2E1) display very low activity in HepG2 cells compared to hepatocytes [23,24]; this property is in fact useful to study the effects of ethanol that are not mediated by its oxidative metabolism.

We show here that ethanol induces *IGFBP-1* gene expression in HepG2 cells by increasing its mRNA half-life and that oxidative metabolism of ethanol is not involved in this regulation. We also demonstrate that the direct effect of ethanol on IGFBP-1 is mediated by the activation of c-Jun N-terminal kinase (JNK), a pathway known to be induced by stress, proinflammatory stimuli, mitogenic factors and to play various roles in hepatic functions and liver injury [25,26].

2. Materials and methods

2.1. Chemicals

Chemical products were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France), Calbiochem (VWR International SA, Fontenay Sous Bois, France) and Ethyl Alcohol Absolute RPE from Carlo Erba Reactifs SA (Val de Reuil, France). Oligonucleotides were obtained from Operon Biotechnologies (Cologne, Germany).

2.2. Cell culture, treatment and cell viability

Human hepatocarcinoma cell line, HepG2, was cultured at 37 °C in humidified atmosphere at 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM, Gibco) complemented with 10% fetal bovine serum, non-essential amino acids (Gibco), 200 UI/ml penicillin, 50 μg/ml streptomycin (Sigma) and 500 mg/ml Fungizone (Squibb). Ethanol treatment of HepG2 cells proceeded as follows: the day after plating (1×10^6 cells/25 cm^2 flask), DMEM was replaced by William's E medium with or without ethanol. Diallyl sulfide, cyanamide, 4-methyl-pyrazole and *N*-acetylcysteine (Sigma) were added 2 h prior to ethanol treatment. SB 202190 and PD 98059 (Sigma) and JNK inhibitor 1 (L)-Form (Calbiochem) were added 3 h prior to ethanol treatment. Human hepatocytes (Biopredic) were cultured in William's E medium as described by Guguen-Guillouzo et al. [27].

Cell viability was tested using the Celltiter 96 Aqueous One Solution Cell Proliferation assay, MTS (Promega), according to the manufacturer's instructions using 30,000 HepG2 cells/well of 96-well plate.

2.3. Cellular and cell medium proteins

Culture medium of HepG2 cells containing secreted IGFBP-1 was saved and whole cell extracts were prepared. Cells were lysed with RIPA (50 mM Tris–HCl, pH 7.9, 1% NP40, 150 mM NaCl, 0.1% SDS, 10% glycerol) supplemented with protease inhibitors (Complete Mini, EDTA free, Roche Diagnostic) and 100 mM NaF, 17.5 mM β-glycerophosphate, 10 mM tetra-sodium pyrophosphate. Following sonication and centrifugation, protein content in the lysate preparations was determined using the BCA protein assay reagent (Pierce, Rockford, IL).

2.4. Western blots

Each sample (40 μg of cellular protein or 20 μg of cell medium protein) in 60 mM Tris, pH 6.8, 2% SDS, 10% glycerol and 0.16% pyronin was run on polyacrylamide gels. Blots were incubated for 2 h at room temperature in phosphate buffer saline, 0.1% Tween 20, 0.2% I-Block™ (Tropix), then overnight with antibodies directed against IGFBP-1 (Mediagnost, 1/5000); JNK/SAPK (pT183/pY185) Phospho-specific (BD Transduction Laboratories 1/1000); Pan-JNK (SAPK/JNK Cell Signaling Technology, 1/1000); β-actin (Santa Cruz Biotechnology, 1/5000).

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