Expression of the Rat Liver Haptoglobin Gene is Mediated by Isoforms of C/EBPα, -β and -δ Proteins

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Abstract. CCAAT/enhancer binding proteins (C/EBP) α, -β and -δ play an important role in mediating I interleukin-6 (IL-6) dependent expression of acute-phase protein (APP) genes in liver during acute-phase (AP) response. Based on the presence of type IL-6 responsive element (IL-6 RE) in the rat haptoglobin (Hp) gene promoter we assumed that some C/EBPα, -β and/or -δ isoforms could mediate the expression of this gene during turpentine-induced AP response. By Western immunoblot and Northern blot assays, we found that turpentine treatment of rats led to a coordinate induction of C/EBPβ and -δ as well as repression of C/EBPα isoforms pool levels in rat liver nuclear extracts (NEs) which was preceded by an adequate alteration of their mRNAs expression in liver. Consequently, results of DNA affinity chromatography revealed that affinity of certain C/EBPα isoforms to bind the type I IL-6 RE within the rat Hp gene promoter decreased whereas affinities of certain C/EBPβ isoforms and C/EBPδ were increased and induced, respectively. Our data suggest that turpentine-induced alterations of C/EBPα, -β and -δ pool levels and DNA-binding activities can be regarded as an integral part of activation of the Hp gene expression in the course of AP response.

Key words: Acute-phase response — C/EBPα — C/EBPβ — C/EBPδ — Haptoglobin gene

Abbreviations: C/EBP, CCAAT/enhancer binding protein; AP, acute-phase; APP, acute-phase protein; Hp, haptoglobin; NEs, nuclear extracts; NPs, nucleoproteins; IL-6 RE, interleukin-6 responsive element.

Introduction

C/EBPs are the most abundant family of transcription factors in liver characterized by the presence of basic region, involved in DNA binding adjacent to a leucine

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zipper motif, responsible for homo- and hetero-dimerization (Wedel and Ziegler-Heitbrock 1995). The expression and biological activity of C/EBPs (α, β, δ, γ, ε and ξ) (Cao et al. 1991) is regulated in a tissue/cell-, species- and inflammatory stimuli-specific manner and is modified under physiopathological conditions such as liver regeneration, toxic stimuli and inflammation (Ferrini et al. 2001).

The early phase of inflammation, known as the AP response is characterized by local and systemic activation of cells and generation of soluble mediators of inflammation which mobilize the metabolic response of the whole organism (Baumann and Gauldie 1994). The most significant manifestation of this response is an induction of APP genes expression in liver which occurs through an interaction of specific transcription factors with hormone responsive elements in the promoter and enhancer of target APP genes. In that manner three principal mediators of various APP genes expression, C/EBPα, -β and -δ factors, display specific regulatory functions in response to IL-6 by binding to the functional C/EBP binding motif known as type I IL-6 RE (Ratajczak et al. 1992; Juan et al. 1993; Lee et al. 1993; Nishio et al. 1993; Ray and Ray 1994a). It has been shown that C/EBPα has an indispensable role in preserving an open conformation of APP genes promoters and recruitment of other C/EBPs to them during AP response (Burgess-Beusse and Darlington 1998), while C/EBPβ and -δ are involved not only in initial activation but also maintaince of an induced state of APP genes expression thus allowing later stage of AP response to occur (Poli 1998).

In our previous studies we have identified type I IL6-RE in the rat Hp gene promoter and discovered an involvement of only C/EBPβ isoforms in its turpentine induced gene expression (Grigorov et al. 1998). Hp is considered as one of the major APPs synthesized in rat hepatocytes during AP response due to its pleiotropic activities amoung which the main one is binding of hemoglobin after haemolysis (Kushner and Mackiewicz 1987). The full expression of its gene is mediated by proximal promoter element termed ABC which contains elements responsive to glucocorticoids, IL-1 and IL-6 (Marinković and Baumann 1990). Since turpentine oil causes sterile tissue injury through a confined but strong local inflammation followed by IL-6 mediated systemic AP reaction, here we used this inflammatory stimuli to evaluate the expression and activity of two other principal C/EBP factors, α and δ. Our results imply that changes in the pattern of C/EBPα, -β and -δ expression and DNA-binding activity correlate with the rat Hp gene expression.

Materials and Methods

Animals and treatment

AP response was elicited in two-months-old male Wistar rats by a single subcutaneos injection of turpentine oil (1 μl/g of body weight) in the lumbal region. The animals were sacrificied 12 h later when maximal transcription of the rat Hp gene was established (Ševaljevic et al. 1989). Although the injection *per se* was not observed to cause AP-related changes, the control animals were routinely injected

with pyrogen-free saline. The animals were kept at constant temperature, humidity and dark/light intervals.

DNA probes

The hormone responsive element of the rat Hp gene known as ABC element (spanning from −176 to −56 bp relative to the transcription initiation site), subcloned into Hinc II site of pUC13, was obtained from Dr. Baumann (Roswell Park Memorial Institute, Buffalo, NY, USA). Plasmids containing C/EBPα, -β and -δ cDNAs were kindly donated by Dr. McKnight (UT South Western Medical Center, Dallas, Texas, USA). cDNA inserts were isolated from each plasmid by digesting pEMBL$_{19}$-C/EBPα with HindIII, Bluescript KS$^+$-C/EBPβ and Bluescript KS$^+$-C/EBPδ with EcoRI-HindIII and then labeled by a random priming technique using [α-^{32}P]dCTP (Amersham Pharmacia Biotech., UK).

Preparation of rat liver nuclear extracts

Nuclear extracts were prepared from the pool of five livers from control and 12 h turpentine-treated rats following the procedure elaborated by Gorski et al. (1986). Protein concentration in NEs was determined by the method of Bradford (1976). Aliquots of NEs were frozen and stored at −70 °C.

RNA isolation and Northern blot analysis

Total RNA was isolated from livers of control and 12 h turpentine-treated rats by using RNAeasy Mini Kit 74104 (Qiagen, USA). RNAs (20 μg) were loaded on 1.2 % agarose-2.2 mol/l formaldehyde gel and transferred onto a nylon membrane (Hybond N, Amersham Pharmacia Biotech., UK). To confirm equivalent RNA loading and transfer, membranes were rinsed with 0.04 % methylene blue and photographed. Membranes with equivalent amounts of RNA were hybridized with specific random-primed ^{32}P-labelled cDNA probe (10^6 cpm/ml) according to the membrane manufacturer's instructions. The levels of C/EBPα, -β and -δ mRNAs in the livers of 12 h turpentine-treated rats were normalised to that in the control livers (100 %) by densitometry.

DNA affinity chromatography

DNA affinity column with ABC element of the rat Hp gene linked to a CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech., UK) was prepared according to Kadonaga and Tjian (1986). ABC affinity column was equilibrated in Bio-Rad-Econo-Column with the dialysis buffer (25 mmol/l HEPES, pH 7.9; 12.5 mmol/l $MgCl_2$; 1 mmol/l dithiothreitol; 20 % glycerol) containing 0.1 mol/l KCl. Liver NEs (8 mg) resolved in the dialysis buffer was mixed with denaturated salmon sperm DNA and allowed to incubate for 10 min at room temperature. The protein-DNA mixture was then applied to the ABC affinity column and incubated for 30 min at 4 °C. ABC-bearing NPs were eluated with dialysis buffer containing 1.0 mol/l KCl. Aliquots of the eluates were analysed by 12 % SDS-PAGE in BIO-RAD Mini-PROTEAN II electrophoresis cell and subjected to subsequent Western immunoblot assay.

Western immunoblot assay

Equal amounts of rat liver NEs and NPs eluated from ABC affinity column were separated by 12 % SDS-PAGE according to Laemmli (1970), transferred to Hybond-P membranes (Amersham Pharmacia Biotech., UK) and probed with rabbit polyclonal antibodies (Santa Cruz Biochemicals Inc., USA) specific to C/EBPα (14AA, dilution 1 : 1000), C/EBPβ (C-19, dilution 1 : 1000) and C/EBPδ (C-22, dilution 1 : 750) Western immunoblot assay was performed according to the procedure recommended by the supplier of the enhanced chemiluminescence (ECL) Western analysis kit RPN 21 (Amersham Pharmacia Biotech., UK). Blot images were scanned, analyzed and the intensities of the signals were quantitated using Molecular Dynamics Image Quant Software Program. Signals were normalized to those of the control (100 %). Every experiment was performed in triplicate. Each membrane was consecutively reprobed with all three anti-C/EBP antibodies according to the supplier's reprobing protocol.

Results and Discussion

Several independent studies showed that C/EBPα, -β and -δ are differentially expressed in response to various types of inflammatory stimuli (Hsieh et al. 1998; An et al. 1996; Gilpin et al. 1996). Thus, to examine whether and in what way turpentine oil causes changes in the expression level of C/EBPα, -β and -δ genes, we performed Northern blot hybridization of total RNA from the livers of control (Fig. 1A, lane 0 h) and 12 h turpentine-treated (Fig. 1A, lane 12 h) rats with adequate cDNA probes. Turpentine treatment resulted in decrease in C/EBPα mRNA level for 14 % (Fig. 1B) which is consistent with the observed reduction of C/EBPα mRNA in Hep3B liver cells following cytokine treatment (Juan et al. 1993). At the same time, the levels of C/EBPβ and -δ mRNAs increased for 53 % and 144 % in relation to the control, respectively (Fig. 1B) which corroborates the fact that an expression of C/EBPβ and -δ mRNAs is low in normal liver tissue but is markedly up-regulated by IL-6 during AP response (Alam et al. 1992; Ramji et al. 1993).

Both C/EBPα and C/EBPβ mRNAs were found to code for several smaller protein isoforms with distinct regulatory and physiological functions (Descombes and Schibler 1991; Lin et al. 1993; Ossipow et al. 1993; An et al. 1996; Hsieh et al. 1998). Thus, to identify which isoform(s) of C/EBPα and -δ are present in rat liver NEs in addition to C/EBPβ, we performed Western immunoblot assay with rabbit polyclonal anti-C/EBPα, -β and -δ antibodies.

Anti-C/EBPα antibody recognized six different isoforms of C/EBPα protein with the molecular masses ranging from 14 to 42 kD in the liver NEs of control rats (Fig. 2A, lane 1). Their amount decreased significantly in the NEs of turpentine-treated rats (Fig. 2A, lane 2) suggesting that they may not play a major role in the AP response. In order to establish whether any of these C/EBPα isoforms exhibit an affinity to bind the ABC element of the rat Hp gene, we purified ABC-binding NPs from the ABC affinity column and analized them with by anti-C/EBPα an-

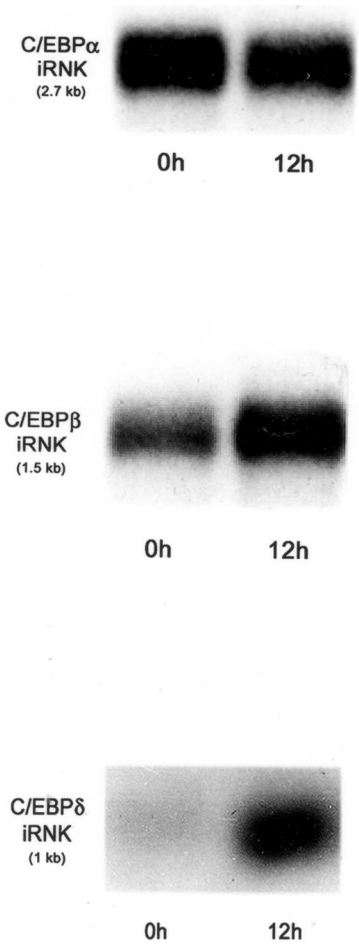

Figure 1. C/EBPα, -β and -δ mRNA levels in rat liver in response to turpentine-treatment. Total RNA (20 μg) isolated from livers of control (lane 0 h) and 12 h turpentine-treated (lane 12 h) rats were subjected to 1.2% agarose-2.2 mol/l formaldehyde gels, transferred to nylon membranes and hybridized with [α-^{32}P]dCTP labelled C/EBPα, -β and -δ cDNA probes. The sizes of the individual mRNAs in kb are displayed on the left. The signals on these autoradiograms were quantified as described in Material and Methods and obtained relative C/EBPα, -β and -δ mRNA levels are shown on the graph.

tibody. Under basal conditions, only 42 kD-, 30 kD- and 20 kD-C/EBPα isoforms displayed strong affinity to bind the ABC element (Fig. 2B, lane 1). Turpentine treatment of rats was followed by a very weak binding of only 42 kD-C/EBPα isoform for ABC element (Fig. 2B, lane 2). 42 kD- and 30 kD-C/EBPα isoforms were reported to display similar DNA-binding activities for the mouse α1-acid glycoprotein (α1-AGP) promoter after bacterial lipopolysaccharide (LPS) treatment (An et al. 1996).

By using the advantage of increased sensitivity of ECL compared to that of radioactive detection system, two more C/EBPβ isoforms were identified at the 42 kD and 32 kD positions besides the previously discovered 20 kD- and 35 kD-C/EBPβ ones (Grigorov et al. 1998). Apart from 42 kD-C/EBPβ, pool level of other C/EBPβ isoforms increased after turpenine treatment (Fig. 3A, lane 2) in

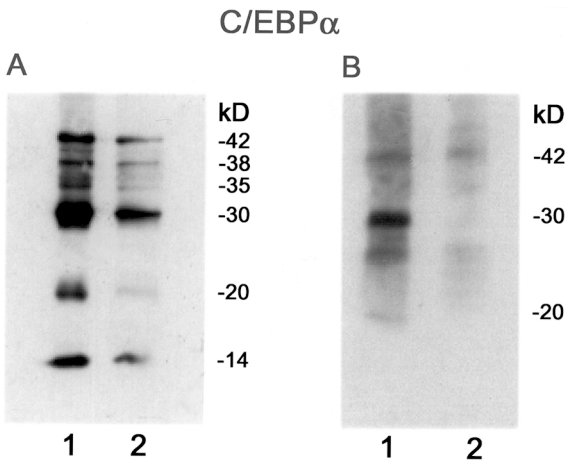

Figure 2. Western immunoblot analysis of the levels of C/EBPα isoforms in the rat liver nuclear extracts in response to turpentine treatment. Liver nuclear extracts (A) and nucleoproteins eluated from ABC-bearing affinity column (B) were subjected to 12% SDS-PAGE and subsequent ECL-Western immunoblot analysis with anti-C/EBPα antibody as described in Material and Methods. Lanes 1 and 2 represent nuclear extracts (A) or ABC-binding nucleoproteins (B) from the livers of control and turpentine-treated rats. Molecular masses of C/EBPα isoforms in kD are shown on the right.

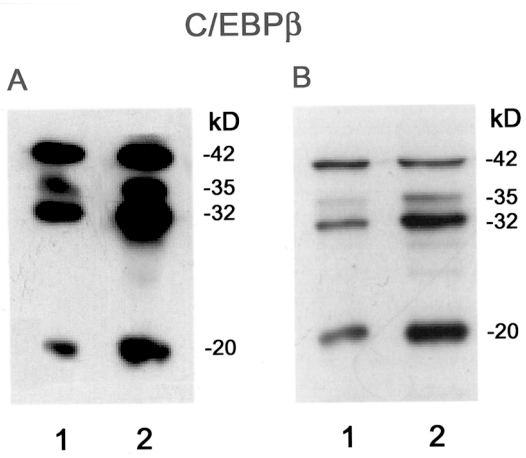

Figure 3. Western immunoblot analysis of the levels of C/EBPβ isoforms in the rat liver nuclear extracts in response to turpentine treatment. Lanes 1 and 2 represent nuclear extracts (A) or ABC-binding nucleoproteins (B) from the livers of control and turpentine-treated rats. Molecular masses of C/EBPβ isoforms in kD are shown on the right.

relation to the control (Fig. 3A, lane 1). Although all four C/EBPβ isoforms bound ABC element of the rat Hp gene under basal conditions (Fig. 3B, lane 1), their affinity increased after turpentine treatment with the exception of 42 kD-C/EBPβ (Fig. 3B, lane 2).

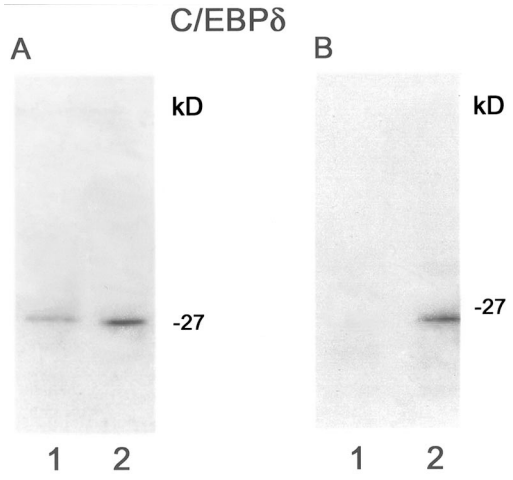

Figure 4. Western immunoblot analysis of the levels of C/EBPδ isoforms in the rat liver nuclear extracts in response to turpentine treatment. Lanes 1 and 2 represent nuclear extracts (A) or ABC-binding nucleoproteins (B) from the livers of control and turpentine-treated rats. Molecular mass of C/EBPδ isoform in kD is shown on the right.

Anti-C/EBPδ antibody detected one protein with molecular mass of 27 kD in NEs of untreated rats (Fig. 4A, lane 1) pool level of which increased after turpentine treatment (Fig. 4A, lane 2). In contrast to this, 27 kD-C/EBPδ bound ABC element of the rat Hp gene only after induction of AP response (Fig. 4B, lane 2). Similar induction of C/EBPδ DNA-binding activity was also observed on promoters of several other APPs in response to different inflammatory stimuli (Juan et al. 1993; Ramji et al. 1993; Poli 1998; Rabek et al. 1998). Such a difference in behaviour of C/EBPδ compared to C/EBPα and C/EBPβ is thought to rely on its higher affinity for C/EBP binding sites either through stabilization by additional factors (Rabek et al. 1998) or structural modification. Studies on the regulation of the α1-AGP and the serum amyloid A (SAA) genes during the AP response have shown that dephosphorylation of C/EBPδ resulted in an inhibition of its DNA binding activity (Ray and Ray 1994a; Poli 1998; Ramji and Foka 2002). This implies a possible role of phosphorylation in turpentine-dependent induction of C/EBPδ DNA binding activity for the rat Hp gene promoter.

Here observed AP-related down-regulation of C/EBPα and up-regulation of C/EBPβ and -δ genes expression in rat liver implicates that a switch of certain C/EBPα with adequate C/EBPβ and -δ isoforms occurs on the Hp gene promoter. At present, the reported *in vitro* binding activities of C/EBPα, -β and -δ are nearly identical but the variety of their isoforms as well as their potential for heterodimer formation could provide a large repertoire of transcription factors with complex *in vivo* regulatory features. Reprobing of each membrane with anti-C/EBPα and -β antibodies (not shown) and their consequent comparative analysis presented in this study revealed that both antibodies recognized two NPs with molecular masses of 20 and 42 kD. During interactions of different C/EBPs with various AP proteins genes promoters at basal conditions, the majority of DNA-protein complexes were

shown to be formed with various C/EBPα homodimers and C/EBPα-C/EBPβ heterodimers (Ossipow et al. 1993; Poli 1998). Accordingly, ABC element probably forms complexes with 20 kD- and 42 kD-C/EBPα homodimers and C/EBPα-C/EBPβ heterodimers containing these two C/EBPα isoforms at basal conditions. Considering that an amount of complexes containing C/EBPα significantly declines upon an induction of AP response due to their replacement by C/EBPβ and C/EBPδ (Alam et al. 1993; Chen and Liao 1993; Juan et al. 1993; Ramji et al. 1993; Ray and Ray 1994b; Poli 1998), our results also implied that a replacement of 20 kD- and 42 kD-C/EBPα with the adequate C/EBPβ isoforms would result in an overall increase in the C/EBPβ : C/EBPα ratio after turpentine-treatment. Similar increase was observed to be age-associated in control mice livers and due to changes in either protein levels or DNA-binding activities of C/EBPα and C/EBPβ for the α1-AGP promoter (Hsieh et al. 1998). Since 20 kD-C/EBPβ is a dominant negative inhibitor able to inhibit transcriptional activities of other C/EBPs (Descombes and Schibler 1991; Welm et al. 1999), it is likely that an increase in its protein level and DNA-binding activity in rat liver following turpentine treatment causes a decline of 20 kD-C/EBPα homodimers and heterodimers and consequent inhibition of its transactivation potential. Finally, we can conclude that the level of the rat Hp gene expression in liver is probably determined by a fine balance between certain C/EBPα on one side and C/EBPβ and -δ isoforms on the other.

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