Original Article

Differences between Molecular Mechanisms Involved in the Regulation of Haptoglobin Gene Expression during the Acute Phase Response and Dietary Restriction

(haptoglobin / female rat / dietary restriction / acute phase response / STATs / C/EBPs)

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Abstract. Haptoglobin is a glycoprotein involved in the acute phase response. Previously we reported that haptoglobin gene expression was up-regulated during dietary restriction in young female rats. The present study aimed at determining whether chronic dietary restriction affects haptoglobin blood levels through changing levels and/or activities of IL-6-related transcription factors STAT and C/EBP in the liver as is the case during the acute phase response. To this end, we compared a female Wistar rat model of 50% 6-week-long dietary restriction with the standard laboratory model for the acute phase response induced by turpentine administration. During the turpentine-induced acute phase response, the transitory 5.4-fold increase of rat haptoglobin expression was accompanied by a prominent rise of serum IL-6 concentration and the increased binding of STAT3 and 35kD C/EBPβ/LAP transcription factors to the haptoglobin gene hormone-responsive element. Results obtained after immunoblotting and DNA affinity chromatography (using hormone-responsive element) suggest that the stable 1.7-fold increase of serum haptoglobin level during dietary restriction was the result of increased amounts and activities of constitutive transcription factors C/EBPα and STAT5b, and to a smaller extent of STAT3. When dietary restriction rats were administered turpentine, a 8.7-fold increase in haptoglobin expression was followed by a considerable increase in the amount and hormone-responsive element binding activity of STAT3 but not 35kD C/EBPβ/LAP. We concluded that haptoglobin gene up-regulation during chronic dietary restriction was regulated by different mechanisms than during the acute phase response, and that it depended on the amount(s) and activit(ies) of transcription factor(s) that characterize low-grade inflammatory conditions.

Introduction

Elevation of circulating concentration of haptoglobin (Hp) is a part of the hepatic acute phase (AP) response to a variety of stress stimuli that can range from inflammation and infection to tissue injury (Baumann and Gauldie, 1994; Gabay and Kushner, 1999). The AP response is characterized by initiation of a signal transduction process that causes remarkable changes in hepatic protein levels as a result of up- or down-regulated synthesis and/or activation of liver-enriched transcription factors regulating target genes, including those coding for AP proteins. Because of its pleiotropic anti-inflammatory activities, of which the binding of free haemoglobin serves to prevent haemoglobin-stimulated oxidative tissue damage and prostaglandin synthesis (Mackiewicz et al., 1993; Baumann and Gauldie, 1994), Hp is one of the major AP proteins. The rate of Hp mRNA production and the serum concentration of Hp protein usually increase three- to six-fold during the response to an acute stress stimulus (Marinković and Baumann, 1990; Gabay and Kushner, 1999). The synthesis of Hp, as of other AP proteins, is controlled at the transcriptional level by the action of pro-inflammatory cytokines and the complex interchange that results from a specific assembly of transcription factors with the hormone-responsive elements (HREs) in the promoter of its gene (Marinković and Baumann, 1990; Ševaljević et

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Abbreviations: AL – *ad libitum*, ALT – turpentine-treated AL animals, AP – acute phase, C/EBP – CCAAT/enhancer-binding protein, DR – dietary restriction, DRT – turpentine-treated DR animals, Hp – haptoglobin, HRE – hormone-responsive element, IL – interleukin, LAP – liver activator protein, LIP – liver inhibitory protein, STAT – signal transducer and activator of transcription.

al., 1995). The *Hp* gene is regulated by interleukin (IL)-6, IL-1β and tumour necrosis factor (TNF)-α in rats and mainly by IL-6 in humans (Mackiewicz et al., 1993; Ševaljević et al., 1995). We have previously reported the involvement of signal transducers and activators of transcription (STAT)3 (Grigorov et al., 2000) and STAT5b (Grigorov et al., 2002), as well as of CCAAT/enhancer-binding protein (C/EBP)α and C/EBPβ (Milosavljević et al., 2003) in the regulation of rat *Hp* gene expression. According to these studies, STAT3 is the main factor that is responsible for the induced increased *Hp* gene activity during the AP response, while STAT5b regulates expression under basal conditions. C/EBPα has an indispensable role in preserving an open conformation of AP protein gene promoters and recruitment of other C/EBPs during the AP response (Burgess-Beusse and Darlington, 1998). C/EBPβ is involved not only in the initial activation, but also in the maintenance of an induced state of AP protein gene expression, thus allowing for the later stage of the AP response to occur (Poli, 1998). As transcription factors whose activity is controlled by IL-6, STAT proteins have been shown to regulate expression of the *Hp* gene by binding to the type II IL-6 regulatory element in the HRE (Grigorov et al., 2000, 2002), while members of the C/EBP protein family recognize the type I element (Milosavljević et al., 2003).

The magnitude and profile of AP proteins in the circulation differ according to the inducing agent employed (Ševaljević et al., 1988; Baumann et al., 2000; Ivanović-Matić et al., 2008), suggesting that the expression of their genes is individually regulated by different patterns and levels of production of specific inflammatory mediators and related intracellular proteins (Grigorov et al., 2000). The diversity of the extent, direction and timing of the changes in AP protein levels under different inflammatory conditions stresses the need for understanding the underlying regulatory mechanisms. In recent years AP proteins have been studied as potential markers of persistent systemic alterations referred to as low-grade inflammation. It is hoped that similar studies will identify immune-associated biomarkers which could be targeted by pharmacologically active compounds aimed at treating various immune-mediated diseases. We have found previously (Grigorov et al., 2004) that when female Wistar rats were exposed to chronic dietary restriction (DR) in the peripubertal period, the observed mild chronic inflammatory condition was associated with a significant decrease of absolute liver weight and increased serum concentrations of Hp, aspartate aminotransferase and alanine aminotransferase. The presence of these enzymes reflect changes in hepatic functioning and their increase in the blood often points to liver damage. The increase of serum Hp level was lower during DR than during acute turpentine administration.

Using a female Wistar rat model of a 50% 6-week-long DR, we attempted to determine whether the elevation of Hp expression during chronic low-grade inflammatory conditions was reflected by changes in protein levels and binding activities of STAT and C/EBP proteins to the HRE of the *Hp* gene. Comparison of the obtained results with those after acute turpentine administration revealed that STAT5b and truncated C/EBPα isoforms are main factors involved in the regulation of *Hp* gene activity in basal and low-grade inflammatory conditions and STAT3 during the AP response. Also, our results revealed that active C/EBPβ only participated in *Hp* gene regulation during acute, transient inflammatory conditions of well-nourished rats.

Material and Methods

Animals and diets

Female Wistar rats were used in this study. The animals were caged individually in a vivarium maintained in a 12-h light/dark cycle at a temperature of 22 ± 2 °C and 50% relative humidity. All rats were free of infection. The animals were fed commercial rat food that was available *ad libitum* (AL) until one month of age, when the rats were divided into two groups of 20 animals each. Group AL continued to receive food AL, whereas the DR group was fed daily during a 6-week period with 50% of the total amount of food that was consumed by the AL group. Daily food intake as well as weekly body weights were monitored. After 6 weeks of food restriction, at 2.5 months of age the mean body weight in the DR group of rats was 110.5 ± 6.6 g (mean ± standard error of mean, SEM) and in the AL group it was 225.2 ± 19.3 g. At this stage of the experiment in 10 animals from the AL and in 10 animals from the DR groups the AP response was induced by a single subcutaneous injection of turpentine oil (1 μl/g of body weight) in the lumbar region. Five rats per group were killed by decapitation 12 h after the turpentine treatment, at the time point at which the maximal increase in transcriptional activity of the rat *Hp* gene is established in response to an acute stimulus (Ševaljević et al., 1989). The remaining five rats per group were killed 24 h after the turpentine treatment, the time point when a maximal increase in serum Hp concentration becomes established (Ševaljević et al., 1989). The relative concentrations of Hp and IL-6 were measured in sera obtained after blood clotting and centrifugation at 5000 x *g* for 15 min. Liver tissue was quickly frozen in liquid nitrogen and stored at -80 ºC for analysis of the protein abundance and DNA binding activities. All animal procedures were approved by the Ethical Animal Care and Use Committee of the Institute for Biological Research, Belgrade, which acts in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85/23, revised 1986).

DNA probe

The DNA affinity column consisted of a nucleotide fragment between -165 and -49 bp from the 5′-flanking region of the rat Hp gene (4): 5′CAAGTATGAAGCAA

GAGCTCAGCTTTGAAAAGGGGTTTGCTTTGT-GGTTACTGGAACAGTCACTGACCTTAGCAAG-GCCGACATTGTGCAAACACAGAAATGGAAGAA AAGGAGGT3′. This fragment, functionally identified as a hormone-responsive element (HRE) of the rat Hp gene, was obtained from Dr. Heinz Baumann (Roswell Park Memorial Institute, Buffalo, NY).

Measurement of Hp and IL-6 level in rat serum

The serum level of Hp was measured by rocket immunoelectrophoresis according to Baumann (1988). Hp was determined using a polyspecific antibody to human Hp (Sigma-Aldrich Inc., Milwaukee, WI) which was cross-reactive with rat Hp. The method is based on the immunoprecipitation between the antigen (Hp) present in the serum and the Hp antibody incorporated in agarose gel at points of their optimal concentration, characterized by the formation of immunoprecipitation peaks or rockets. The relative concentration of Hp was established by quantification of the areas under the respective immunoprecipitation peaks. The values obtained after quantification were expressed as means ± SEM from three separate experiments.

The serum level of IL-6 was determined by Western imunoblot assay as described below.

Preparation of liver nuclear extracts

Nuclear extracts were prepared from a pool of five livers from AL, DR or turpentine-treated rats, respectively, following the procedure of Gorski et al. (1986). The livers were homogenized in buffer containing 2 M sucrose, 10 mM HEPES pH 7.6, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM spermidine, 1 mM PMSF, 1 mM DTT and 10% glycerol. After filtration of the homogenate through two layers of cheesecloth, the nuclei were pelleted by centrifugation at 72,000 x *g*, in an SW 28 rotor (Beckman L7-55, Beckman Instruments, Palo Alto, CA) for 30 min at 4 ºC. The pelleted nuclei were resuspended in lysis buffer (10 mM HEPES pH 7.6, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol). To precipitate the chromatin, $(NH_4)_2SO_4$, pH 7.9, was added slowly with constant stirring to a final concentration of 0.36 M. Chromatin was sedimented by centrifugation (82,000 x *g*, Ti 50 rotor, 60 min, 4 °C). The nucleoproteins were precipitated from the supernatant after the addition of crystallized $(NH_4)_2SO_4$ to a final concentration of 2.6 M and sedimented by centrifugation at 82,000 x *g* in a Ti 50 rotor, for 30 min at 4 °C. Nuclear extracts were dialysed overnight against 25 mM Hepes pH 7.6, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT and 10% glycerol, resuspended in dialysis buffer and frozen in small aliquots at -80 °C.

DNA affinity chromatography

DNA affinity column with the HRE element of the rat *Hp* gene linked to a CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) was prepared according to Kadonaga and Tjian (1986). The HRE affinity column was equilibrated in Bio-Rad-Econo-Column with dialysis buffer (25 mM Hepes pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) containing 0.1 M KCl. Nuclear extracts (8 mg) in the dialysis buffer were mixed with denatured salmon sperm DNA for 10 min at room temperature. The protein-DNA mixture was then applied to the HRE affinity column and incubated for 30 min at 4 °C. HRE-bearing nucleoproteins were eluted with dialysis buffer containing 1.0 M KCl. Aliquots of the eluates were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 9% and 12% gels in a BIO-RAD Mini-PROTEAN II electrophoresis cell, and subsequently examined by Western immunoblot analysis.

Western immunoblot analysis

The Western immunoblot assay was performed according to the procedure recommended by the supplier of the ECL Western analysis kit RPN 21 (Amersham Pharmacia Biotech). Serum samples (10 µl) and nuclear extracts (20 µg) were subjected to 9% SDS-PAGE for STATs analysis or 12% SDS-PAGE for IL-6 and C/EBPs, and electrophoretically transferred to PVDF membranes (Hybond-P, Amersham Pharmacia Biotech). The membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in blotto base buffer (0.1% Tween 20, 20 mM Tris-HCl pH 7.6, 137 mM NaCl) and then incubated for an additional 2 h at room temperature in the same buffer containing rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) specific to STAT3 (H-190), C/EBPα (14AA), or C/EBPβ (C-19), mouse monoclonal STAT5b (G-2) and goat polyclonal IL-6 antibody (R-19). After washing three times with blotto base buffer containing 1% non-fat dry milk, horseradish peroxidase-conjugated secondary antibody was applied for 1 h at room temperature. Membranes were washed extensively in blotto base buffer and antibody binding was detected on X-ray film by enhanced chemiluminescence using the ECL detection system (Amersham Pharmacia Biotech). Membranes were reprobed according to the supplier's re-probing protocol. The membranes were incubated in 2% SDS, 100 mM β-mercaptoethanol and 62.5 mM Tris-HCl pH 6.8, for 30 min at 50 °C and then rinsed three times in blotto base buffer, re-blocked and re-probed with another antibody. The blots were scanned, analysed and the intensities of the signals were quantified using TotalLab (Phoretix, Newcastle Upon Tyne, England) electrophoresis software (v 1.10).

Statistical analysis

Differences between the experimental groups were tested using non-parametric Mann-Whitney's U-test (Statistica v. 5.0, StatSoft, Tulsa, OK). $P < 0.05$ was considered the threshold level for significance.

Results

The expression of Hp in 2.5 months old female Wistar rats, as determined by the relative change of concen-

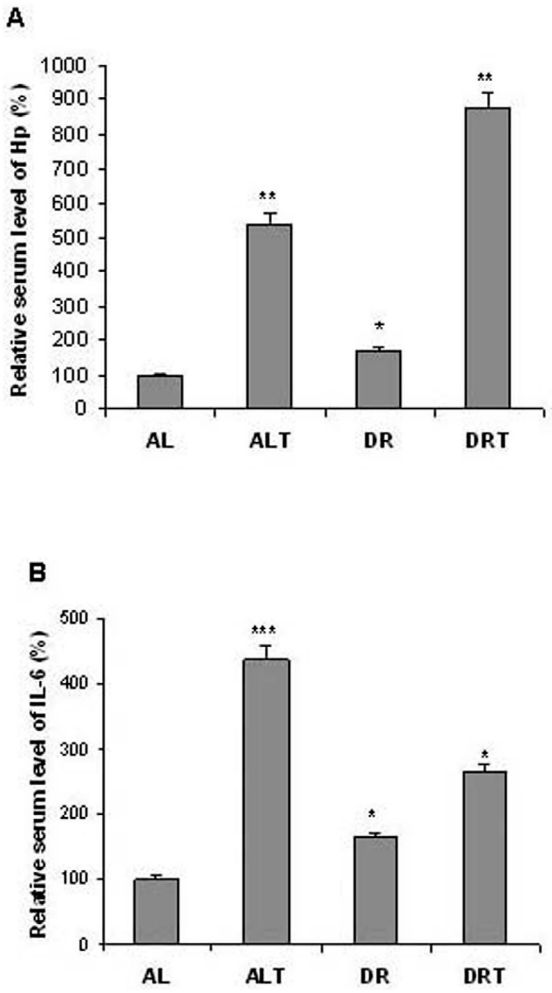

Fig. 1. Effects of dietary restriction and/or turpentine treatment on serum levels of Hp and IL-6 in female Wistar rats. The relative concentrations of Hp in the serum were determined by crossed immunoelectrophoresis (**A**). Serum levels of IL-6 were determined by the Western immunoblot assay (**B**). Changes in serum Hp and IL-6 levels were expressed as percentages of the initial control values taken as 100 %. The data represent three separate experiments performed with sera from five rats per group. AL, female rats fed *ad libitum*; DR, female rats fed with an amount of chow equivalent to 50 % of the normal food intake; ALT, *ad libitum* fed rats treated with turpentine (1 μl/g of body weight); DRT, dietary restricted rats treated with the same dose of turpentine. The values are expressed as means ± SEM from three separate experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with AL fed rats.

tration in rat sera after DR and/or turpentine treatment, was examined by rocket immunoelectrophoresis (Fig. 1A). The DR rats were characterized by a 72% ($P < 0.05$) increase in basal Hp compared with the level measured in AL rats. When the DR rats were treated with turpentine (DRT), the increase in serum Hp concentration 24 h after treatment was 8.7-fold ($P < 0.01$) while in the corresponding AL rats after the same treatment (ALT) it was 5.4-fold ($P < 0.01$). The data imply that liver cells were probably sensitized by factor(s) induced by food restriction which made them more susceptible to the action of the second inflammatory stressor, turpentine. This could be due to the action of inflammatory mediators, such as IL-6, which play a central role in regulating transcription of the *Hp* gene (Marinković and Baumann, 1990). Since the circulating levels of IL-6 are directly and closely correlated with the severity of inflammatory response, we compared relative serum levels of Hp and IL-6 and revealed similar changes in relation to DR and turpentine treatment (Fig. 1). According to Western immunoblot analysis (Fig. 1B), the serum IL-6 levels in DR rats were elevated by about 63% ($P < 0.05$) compared with the level in AL rats. Turpentine administration led to 4.3-fold ($P < 0.001$) increase of IL-6 concentration in AL rats and a 2.4-fold ($P < 0.05$) increase in the DR group.

STAT3 is an indispensable transcription factor for IL-6-mediated intracellular signalling in the liver. After activation, STAT3 plays an important role in inducing or modulating transcription of multiple genes whose products are primarily anti-inflammatory, such as the gene encoding Hp (Gabay and Kushner, 1999; Grigorov et al., 2000). To compare the changes in abundance of STAT3 during the AP response and after chronic DR, we determined its nuclear protein level in the livers of the DR group of rats before and 12 h after turpentine treatment. In AL rats, anti-STAT3 antibody (Fig. 2A) recognized a low level of the constitutively active 91kD STAT3 isoform, which increased 12-fold ($P < 0.05$) in response to turpentine (ALT). The 2.4-fold increase ($P < 0.01$) in the 91 kD STAT3 in the nuclear extracts after DR was significantly potentiated (22.6-fold, $P < 0.05$) after turpentine treatment (DRT) compared to AL rats.

In accordance with our observation that nuclear abundance of STAT3 and its binding activity for the *Hp* gene HRE is related to STAT5b export from the nucleus (Grigorov et al., 2002), we determined the protein pool levels of STAT5b during acute and DR-related inflammatory conditions. After re-probing of the membrane described in Fig. 2A with anti-STAT3 antibody, STAT5b was shown to be an abundant nuclear transcription factor in the liver nuclei of AL female rats. DR rats displayed a 1.7-fold lower amount of 92 kD STAT5b ($P < 0.05$) compared to AL rats and a 1.3-fold higher amount than the turpentine-treated AL group (ALT). Turpentine treatment led to a decrease of STAT5b in the nuclear extracts from both AL (2.2-fold, $P < 0.05$) and DR groups, which was more marked in the DR rats (8.8-fold, $P < 0.05$). These results imply that the respective levels of nuclear STAT3 and STAT5b affect different expression levels of *Hp* gene during the AP response and DR. Fig. 2B shows higher abundance of STAT5b than STAT3 in normal and DR-related conditions and the opposite situation after acute turpentine treatment. This finding suggests that STAT5b is involved in the regulation of *Hp* gene expression in the basal state and during chronic low-level inflammation, while STAT3 regulates

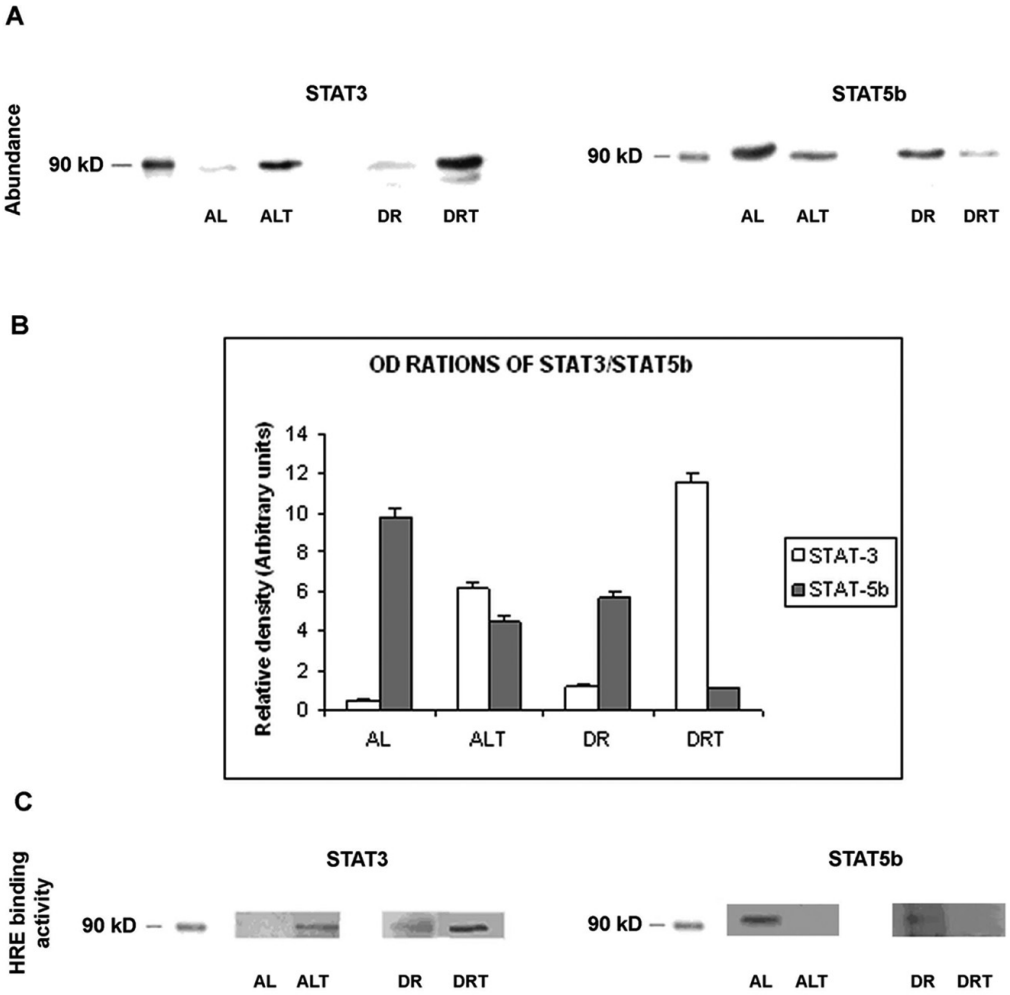

Fig. 2. Changes in STAT3 and STAT5b levels and HRE-binding activities in liver nuclei in relation to dietary restriction and/or turpentine treatment

Liver nuclear extracts were directly blotted with STAT3 or STAT5b antibody (**A**) or subjected to DNA affinity chromatography (using HRE) and sequential Western immunoblotting with STAT3 and STAT5b antibody (**C**). The intensity of bands on directly blotted membranes was quantified by densitometry and presented proportionately (**B**). Data represent three separate experiments performed with liver nuclear extract pooled from five rats. AL, female rats fed *ad libitum*; DR, female rats fed with an amount of chow equivalent to 50% of the normal food intake; ALT, *ad libitum* fed rats treated with turpentine (1 μl/g of body weight); DRT, dietary restricted rats treated with the same dose of turpentine.

Hp gene activity predominantly after acute stress stimuli. This was confirmed by DNA affinity chromatography. Nuclear extracts were passed through a Sepharose resin coupled with the *Hp* gene HRE. The HRE-specific binding proteins were purified and the presence of STAT3 and STAT5b assessed by Western immunoblotting (Fig. 2C). Of the HRE-binding nuclear proteins in AL rats, only binding of STAT5b was detected. In contrast to the basal state, acute stress after turpentine administration (ALT) was followed only by STAT3 binding. Of the HRE-binding nuclear proteins in DR rats, the binding of both the inducible 91kD STAT3 isoform and STAT5b were detected. These results suggest that *Hp* gene expression was probably attenuated under conditions that involved STAT5b activity. Like the ALT group, the HRE-binding nuclear proteins from DRT rats were enriched with the STAT3 isoform, the amounts of which were significantly higher than in ALT rats. Considering the 8.7-fold increase of the serum Hp level in DR rats treated with turpentine, this result pointed to an essential role of STAT3 in the establishment of the level of *Hp* gene activity.

In a recent study we demonstrated by Western immunoblotting that there are multiple C/EBPα and C/EBPβ isoforms in control rat liver nuclear extracts and that the pool levels of these isoforms are altered by turpentine treatment (Milosavljević et al., 2003). Results from Western immunoblot analyses and DNA affinity chromatography shown in Fig. 3 indicate that there are significant qualitative and quantitative differences in the abundance and binding activity of C/EBP proteins during the AP response and DR. As expected, anti-C/EBPα antibody recognized multiple C/EBPα isoforms, with molecular masses that ranged from 27 to 45 kD in the liver from nuclear extracts of 2.5-month-old AL female rats (Fig. 3A). This is in agreement with the finding that

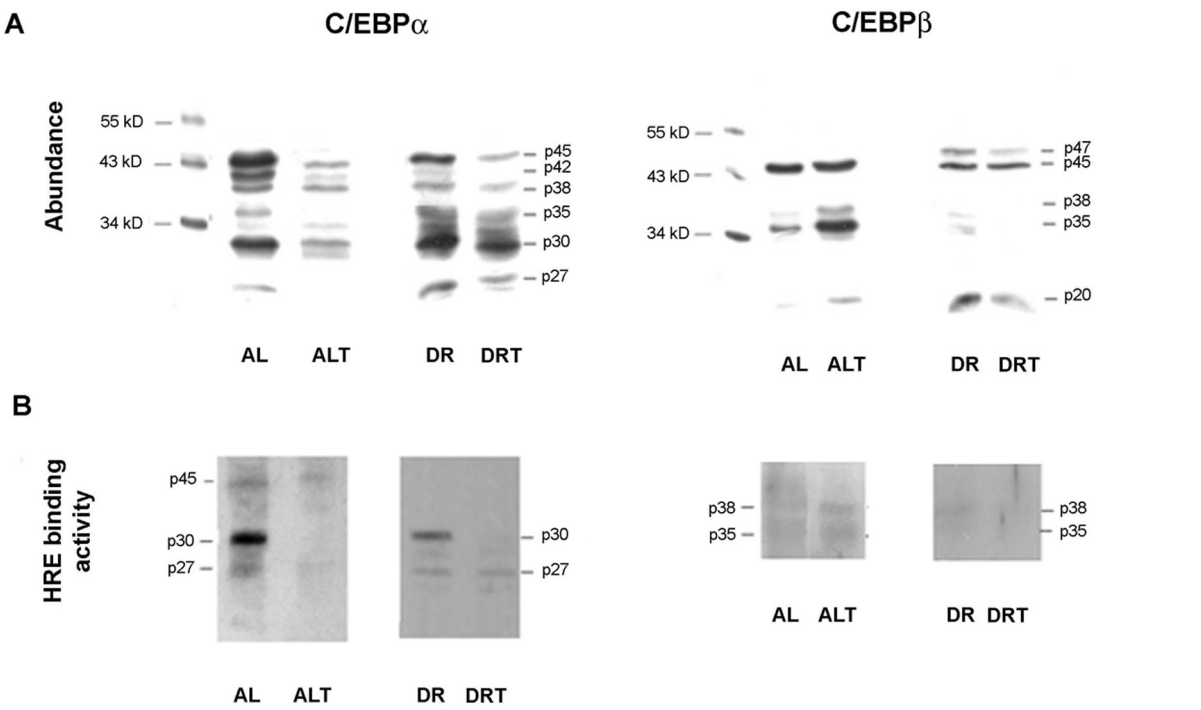

Fig. 3. Changes in rat liver nuclear abundance (A) and HRE-binding activities (B) of C/EBPα and C/EBPβ isoforms in relation to dietary restriction and/or turpentine treatment

Liver nuclear extracts were directly blotted with C/EBPα or C/EBPβ antibody (**A**) or subjected to DNA affinity chromatography (using HRE) and sequential Western immunoblotting with C/EBPα and C/EBPβ antibody (**B**). The data represent three separate experiments performed with liver nuclear extracts that were pooled from five rats. AL, female rats fed *ad libitum*; DR, female rats fed with an amount of chow equivalent to 50% of the normal food intake; ALT, *ad libitum* fed rats treated with turpentine (1 μl/g of body weight); DRT, dietary restricted rats treated with the same dose of turpentine.

C/EBPα mRNA coded for several protein isoforms with distinct regulatory and physiological functions. The 45 kD (p45) is fully functional, while the 30 kD (p30) protein lacks the major transcriptional portion of the protein (Ossipow et al., 1993; Hsieh et al., 1998). In the ALT rats after 12 h, the C/EBPα isoforms were down-regulated relative to the controls, whereas the 35 kD (p35) and 27 kD (p27) isoforms were not detected. The opposite situation was observed in the DR group where, apart from the 38 kD, 42 kD and 45 kD C/EBPα isoforms, which were slightly less apparent, the abundance of C/EBPα isoforms between 27 and 35 kD was greater than in the AL sample. The 27 kD, 30 kD and 35 kD C/EBPα isoforms increased 3.5- ($P < 0.05$), 1.5- ($P < 0.01$) and 14.8-fold ($P < 0.01$), respectively. Treatment of DR rats with turpentine down-regulated the expression of C/EBPα isoforms after 12 h as in the AL group; however, the abundance of 30 kD, 35 kD and 27 kD C/EBPα isoforms was still higher than in AL rats.

C/EBPβ mRNA gives rise to three different protein products with estimated molecular masses of 45 kD, 35 kD and 20 kD (Ossipow et al., 1993; Hsieh et al., 1998). The 45 kD and 35 kD C/EBPβ isoforms are transcriptional activators, while the 20 kD isoform lacks the N-terminal transactivation domain and is a repressor of transcription referred to as liver inhibitory protein (LIP). The anti-C/EBPβ antibody revealed extensive changes in the C/EBPβ expression profile in DR rats (Fig. 3B). The abundance of 45 kD (p45) isoform was similar in AL and ALT rats, whereas in DR rats this isoform appeared as a 45–47 kD doublet that was down-regulated. The amount of the 35 kD C/EBPβ (p35), also known as liver activator protein or LAP (Ossipow et al., 1993), as well as that of a 38 kD (p38) isoform, increased significantly ($P < 0.01$) after the turpentine treatment of AL rats (7.2- and 3.6-fold, respectively), while they were strongly down-regulated in the nuclear extracts of DR rats and were not detected in the DRT group. Turpentine treatment of AL rats increased 7.2-fold ($P < 0.01$) the abundance of a truncated 20 kD C/EBPβ (p20) isoform, while dietary restriction was followed by 36-fold increase ($P < 0.01$). Nuclear extracts from DRT rats maintained a high level of the 20 kD C/EBPβ isoform, which was 16.5-fold and 9.5-fold higher that in the AL and ALT rats, respectively.

DNA affinity chromatography performed with the *Hp* gene HRE demonstrated specific binding of C/EBPα and C/EBP/β during the AP response and chronic DR. Figure 3 shows that in ALT rats the main HRE-binding C/EBP proteins were full-length 45 kD C/EBPα and 35 kD and 38 kD C/EBPβ isoforms. The truncated 30 kD and 27 kD C/EBPα bound HRE under basal and DR conditions. Their binding was higher in the basal than in the DR state. No HRE-bound 30 kD C/EBPα and C/EBPβ isoforms were observed after the turpentine treatment of the DR group of rats.

Discussion

The aim of this study was to improve our knowledge of the regulatory mechanisms underlying hepatic *Hp* gene expression during different stress conditions. It was shown that nutritional and metabolic signals play an important role in the control of gene expression (Towle, 1995). However, little is known about the nutritional-dependent transcriptional regulation and the signal transduction pathways that mediate it in mammals. By studying the DR-related regulation of the *Hp* gene, we wanted to gain insight into the fine-tuned regulation of gene activity, in which alterations in the amount and activation potential of one or more transcription factors induce specific changes in gene expression.

Our analysis was focused on the investigation of differences between the molecular mechanisms involved in transient transcriptional stimulation of the *Hp* gene during the turpentine-induced AP response and permanent *Hp* gene activation after six weeks of 50% DR in young female rats. The AP response is a limited beneficial response that includes changes in the concentration of plasma AP proteins due to changes in their production by hepatocytes. IL-6 is the main stimulator of Hp production and other anti-inflammatory AP proteins in response to varied stimuli. Its levels reflect the intensity of the inflammation (Gabay and Kushner, 1999). It is critical in controlling the extent of the local and systemic acute inflammatory responses (Xing et al., 1998) and is also important in the development of chronic inflammation (Kaplanski et al., 2003). A higher level of IL-6 in the serum was observed in food-restricted female rats than in AL rats, which was, however, lower than in the turpentine-treated groups. This result is not consistent with results obtained by researchers in the field of ageing and type 2 diabetes (Spaulding et al., 1997; Seo et al., 2006; Ugochukwu and Figgers, 2006), where DR either down-regulates or does not affect IL-6 expression. The differences between the experimental models in the present study may result, at least in part, from the use of healthy peripubertal rats whose hormonal status and energy metabolism differ from that in adults. According to Mascarucci et al. (2002), the observed differences could arise from age-related dysregulation of cytokine production and activity. On the other hand, during severe food restriction the organism's normal energy mobilization and metabolism are altered and the energy reserves (mainly tissue protein, lipids and carbohydrates) are catabolized and oxidized to supply the energy required for metabolism (Du and Mai, 2004). Therefore, 50% DR in healthy young adult rats probably exerts profound effects on the organism's physiological state and on the tissue oxidative status. Our results demonstrated a significant increase in the concentration of Hp due to DR. The antioxidant status of several organs, such as the liver, is increased by diet restriction (Kari et al., 1997). Since Hp is an antioxidant (Friedrich et al., 1995), this increase suggests that oxidative damage to body tissues has to be prevented. This is in accordance with the study of Ugochukwu and Figgers (2006), who observed that caloric restriction increased serum Hp level in diabetic rats.

The correlation between serum levels of IL-6 and Hp under conditions of DR suggests the involvement of the IL-6 signal transduction pathway in the regulation of *Hp* gene expression during low chronic inflammatory condition, as generally observed during the AP response. Maximal transcriptional induction of the rat *Hp* gene in the liver that is observed at 12 h of exposure to turpentine (Ševaljević et al., 1989) was shown to be primarily directed by a few positive IL-6 regulatory elements and their cognate binding transcription factors – 35 kD C/EBPβ/LAP and STAT3 (Grigorov et al., 2000, 2002). STAT3 serves as a potent transactivator in the regulation of the rat *Hp* gene during the turpentine-induced AP response and it replaces constitutive STAT5b bound to the same consensus binding sites (Grigorov et al., 2002). Therefore, STAT5b was proposed to be involved in the regulation of rat *Hp* gene activity in the normal physiological state. However, Ripperger et al. (1995) described STAT5b binding to the IL-6RE II site in the livers of intact rats as well as after the induction of the AP response by complete Freund's adjuvant. These results suggest that STAT5b responsiveness depends on the type of inflammatory agent and the activated signalling pathway. The results presented in this work point to the dependence of STAT5b responsiveness on the type of stimulus and its intensity. According to the serum levels of IL-6 and Hp we concluded that 50% DR induced low-grade inflammation in young female rats. The intensity of the stimulus obviously influenced the balance between STAT5b and inducible STAT3, and thereby affected the expression levels of the *Hp* gene. According to Figs. 1A and 2, the high expression of Hp after the turpentine treatment of either AL or DR rats was accompanied by strong STAT3 expression and HRE-binding activity. The DR-related Hp level was associated with a greater abundance of STAT5b than activated STAT3 and HRE-binding activities of both proteins. This finding indicates that the transactivating potential of STAT3-STAT5b combinations is different than that of the individual factors. Due to this cooperativity, a minor alteration in the amounts of these proteins could have a significant effect on their transactivation potential on the promoter.

The strong IL-6-initiated signal during the early phase of the hepatic AP response is primarly conveyed to the AP protein genes by STAT3 (Heinrich et al., 1998) and in the later phase of the AP response by 35 kD C/EBPβ, whose activity is induced by STAT3 (Niehof et al., 2001). During the turpentine-induced AP response, the C/EBPα abundance normally present in quiescent hepatocytes decreases and is transiently replaced by induced C/EBPβ activity (Milosavljević et al., 2003). Contrary to these results, we showed that besides 43 kD C/EBPα isoform, the expression of 30 kD and 27 kD C/EBPα as well as the truncated 20 kD C/EBPβ/LIP isoform was elevated during chronic DR,

while the expression of the 35 kD C/EBPβ/LAP isoform was abrogated. The 20 kD C/EBPβ/LIP isoform was proposed to function as a dominant negative regulator of C/EBPα and the 35 kD C/EBPβ/LAP expression during the AP response. Under DR conditions, 20 kD C/EBPβ/LIP affects C/EBPα to a lesser extent. It should be noted that C/EBPα autoregulates its own expression (Legraverend et al., 1993). Also, activated STAT3 can augment the transcriptional activity of this protein (Numata et al., 2005). We propose that during adaptation to reduction of food intake, the continual presence of increased amounts of IL-6 could maintain STAT3 activation, which is sufficient to activate C/EBPα as well as the *Hp* gene. Therefore, STAT3 could be a factor that sensitizes liver cells and makes them susceptible to the additional action of turpentine, thus allowing greater production of Hp. Studies with C/EBPα knockout mice indicate that it mediates the effects of nutrients and hormones on hepatocyte-specific genes that are important for energy homeostasis (Legraverend et al., 1993; Roesler, 2001; Numata et al., 2005). The *Hp* gene is also a recognized target for C/EBPα transactivation as well as for 20 kD C/EBP/LIP, which has a moderately inhibitory effect on the basal Hp level (Natsuka et al., 1991). Since over-expression of either C/EBPα or 35 kD C/EBPβ/LAP caused upregulation of Hp in human hepatoma cells (Jover et al., 2002), we propose that the DR-related increase in C/EBPα maintains enhanced expression of Hp in combination with STAT3 and STAT5b activity. This increase is lower than during the AP response, probably due to the activity of STAT5b and absence of 35 kD C/EBPβ/LAP activity. The mechanism by which the 35kD C/EBPβ/LAP isoform was abrogated is not understood. LAP and LIP C/EBPβ isoforms are translated from the same mRNA by alternative translation initiation on multiple AUG codons (Poli, 1998; Ossipow et al., 1993). It is possible that during DR the relative frequency of initiation of translation is changed and that translational induction of the 20 kD C/EBPβ/LIP is the primary event.

We concluded that elevation of *Hp* gene expression during chronic DR in young female rats is accomplished through different regulatory mechanisms than during the AP response. This could be result of integrative regulatory effects of hormones, notably of IL-6, and of nutritional signals which induce changes in transcription factors that are characteristic for low-grade inflammatory conditions.

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