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# STAT3/NF-κB interactions determine the level of haptoglobin expression in male rats exposed to dietary restriction and/or acute phase stimuli

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**Abstract** Haptoglobin is a constitutively expressed protein which is predominantly synthesized in the liver. During the acute-phase (AP) response haptoglobin is upregulated along with other AP proteins. Its upregulation during the AP response is mediated by *cis–trans* interactions between the hormone-responsive element (HRE) residing in the haptoglobin gene and inducible transcription factors STAT3 and C/EBP $\beta$. In male rats that have been subjected to chronic 50% dietary restriction (DR), the basal haptoglobin serum level is decreased. The aim of this study was to characterize the *trans*-acting factor(s) responsible for the reduction of haptoglobin expression in male rats subjected to 50% DR for 6 weeks. Protein-DNA interactions between C/EBP and STAT families of transcription factors and the HRE region of the haptoglobin gene were examined in livers of male rats subjected to DR, as well as during the AP response that was induced by turpentine administration. In DR rats, we observed associations between the HRE and C/EBP$\alpha/\beta$, STAT5b and NF-κB p50, and the absence of interactions between STAT3 and NF-kB p65. Subsequent induction of the AP response in DR rats by turpentine administration elicited a normal, almost 2-fold increase in the serum haptoglobin level that was accompanied by HRE-binding of C/EBP$\beta$, STAT3/5b and NF-kB p65/p50, and the establishment of interaction between STAT3 and NF-κB p65. These results suggest that STAT3 and NF-κB p65 crosstalk plays a central role while C/EBP$\beta$ acquires an accessory role in establishing the level of haptoglobin gene expression in male rats exposed to DR and AP stimuli.

**Keywords** Acute inflammation · Attenuated haptoglobin expression · C/EBPs · Dietary restriction · NF-κB · STATs

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## Introduction

Dietary restriction (DR), is a frequent phenomenon in human and animal populations. DR has been shown to alter drug metabolism, hormonal regulation [1], immune and inflammatory processes [2], including the acute phase (AP) response [3] and other biological processes [4]. Nutritional intervention exerts an important effect on the liver [5]. The liver is the central organ for glucose homeostasis regulation, xenobiotic metabolism and detoxification, steroid hormone biosynthesis and degradation [6]. Liver metabolism dramatically changes during the AP response, a systemic reaction of the organism to disturbances in homeostasis caused by infections, tissue injury, trauma or surgery, neoplastic growth and immunological disorders [7]. Within a few hours after infection, the pattern of protein synthesis by the liver is drastically altered resulting in an increase of some blood proteins, the positive AP proteins [8]. Hepatic mRNA upregulation of those AP proteins is associated with a decrease in synthesis of normal blood proteins, such as transthyretin (formerly called prealbumin), retinol binding protein, cortisol binding globulin, transferrin and albumin, which represent the negative AP proteins [9]. The AP proteins are primarily represented by blood serum proteins, such as different protease inhibitors, coagulation proteins, complement factors and transporters. By controlling the composition of

serum AP proteins, the liver has a major impact on health and homeostasis [6]. Changes in serum protein profiles partly depend on the amount of food intake and muscle catabolism in response to an inadequate nutritional status [9]. During states of inadequate nutrition when catabolic reactions are stimulated, the full positive AP protein response is generally reduced.

Haptoglobin is one of the major positive AP proteins. It exerts a broad range of anti-inflammatory activities, acts indirectly as a bacteriostatic agent and an antioxidant by virtue of its ability to bind free hemoglobin and facilitate its immediate clearance by macrophages [10]. Its elevated synthesis in response to inflammatory stimuli is attributed primarily to the transcriptional induction of its gene. The transcription of the rat haptoglobin gene is regulated by synergistic actions of several interacting DNA-binding nuclear proteins that belong to the C/EBP (CCAAT-enhancer-binding proteins) and STAT (Signal Transducer and Activator of Transcription) families of transcription factors. These proteins assemble on the hormone-responsive promoter element (HRE), also referred to as sequence ABC, that resides from −165 bp to −56 bp relative to the transcription start site [11]. The full expression of haptoglobin gene is mediated by HRE which contains elements responsive to glucocorticoids, interleukin-1 (IL-1) and IL-6 [12].

Previously we established that application of 50% DR for 6 weeks affects the liver inflammatory response in young female and male rats in different ways [13]. We also established that elevated expression of the haptoglobin gene in female rats during chronic DR was regulated by mechanisms that were different from those occurring during the AP response. These were primarily the result of increased amounts and elevated DNA-binding activities of constitutive transcription factors C/EBPα and STAT5b and to a smaller extent of inducible STAT3 [14]. In contrast to female rats, the males displayed a DR-related decrease in haptoglobin serum level that pointed to the existence of gender-related differences in molecular mechanisms that regulate haptoglobin gene activity in response to DR [13]. As the response to a lower dietary intake appears to occur at the transcriptional level, we assumed that lower haptoglobin expression during DR was caused by changes in abundance and/or activities of C/EBPα, C/EBPβ, STAT3 and STAT5b transcription factors, the main transcriptional regulators of haptoglobin gene activity. Hence, we investigated the activity of these transcription factors in the liver of male rats that were subjected to DR, as well as in DR rats that were treated with turpentine in order to induce the AP response. We examined protein-DNA interactions between C/EBP and STAT families of transcription factors with the HRE region of the haptoglobin gene, as well as protein–protein interactions.

## Materials and methods

### Animals and diets

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).

Male Wistar rats were used in this study. The animals were caged individually and maintained in a 12 h light/dark cycle at 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 1 month of age when the rats were divided into two groups of 10 animals each. Group AL continued to receive food ad libitum whereas the DR group was fed daily during a 6-week period with 50% of the total amount of food consumed by the AL group. The food intake and body weights were monitored daily and weekly, respectively. After 6 weeks of food restriction and at 2.5 months of age, the mean body weight in the DR group was 122 ± 12 g (mean ± standard error of mean, SEM) and in the AL group it was 275 ± 39. In 10 animals from the AL and DR groups (5 animals per group), the AP response was induced by a single subcutaneous injection of turpentine oil (1 μl/g of body weight) in the lumbar region. The injection of turpentine is a well characterized model of non-infectious (sterile) inflammation. It induces local tissue damage (sterile abscess) that is responsible for the development of a systemic acute phase response [15, 16]. Rats were killed by decapitation 12 h after the turpentine treatment, at the time point when a maximal increase in transcriptional activity of the rat haptoglobin gene is established in response to an acute stimulus [17]. The following groups of rats were thus established: ad libitum fed rats (AL), ad libitum fed rats treated with turpentine (ALT), dietary restricted fed rats (DR) and dietary restricted fed rats treated with turpentine (DRT).

### Measurement of haptoglobin in rat serum

The relative concentration of haptoglobin was measured in sera obtained after blood clotting and centrifugation at 5,000×*g* for 15 min. The serum level of haptoglobin was measured by rocket immunoelectrophoresis according to Baumann [18]. Haptoglobin was determined using a polyclonal antibody to human haptoglobin (Sigma-Aldrich Inc) which was cross-reactive with rat haptoglobin. The method is based on the immunoprecipitation between the antigen (haptoglobin) present in the serum and the haptoglobin antibody incorporated in the agarose gel at points of

their optimal concentration and characterised by the formation of immunoprecipitation peaks or rockets. The relative concentration of haptoglobin 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.

### Preparation of liver nuclear extracts

Nuclear extracts were prepared from a pool of five livers from AL, DR and turpentine-treated rats, respectively, following the procedure of Gorski et al. [19]. 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×*g* in a SW 28 rotor (Beckman L7-55) for 30 min at 4°C. 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×*g* in a Beckman Ti 50 rotor for 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×*g* in a Ti 50 rotor for 30 min at 4°C. Nuclear extracts were dialyzed 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.

### 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). The nuclear extracts (20 μg) were subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 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 incubated for an additional 2 h at room temperature in the same buffer containing rabbit polyclonal antibodies specific to STAT3 (H-190), STAT5b (C-17), C/EBPα (14AA), C/EBPβ (C-19), NF-κB p65 (C-20) and NF-κB p50 (H-119) (all were obtained from Santa Cruz Biochemical's Inc.). After washing three times with blotto base buffer containing 1% non-fat dry milk, horseradish peroxidise-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's using the ECL detection system (Amersham Pharmacia Biotech.). Membranes were reprobed according to the supplier′s protocol for membrane reprobing. 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, reblocked and reprobed with another antibody. The blots were scanned and the intensities of the signals were quantified using TotalLab (Phoretix, Newcastle Upon Tyne, England) electrophoresis software (ver. 1.10).

### Immunoprecipitation

Immunoprecipitation was carried out with nuclear extracts (500 μg) that were precleared by incubation with control IgG and Protein A-Sepharose. Supernatants obtained after preclearing were incubated for 2 h on ice with 3 μg of rabbit polyclonal anti-NF-κB antibody (C-20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Protein A-Sepharose-coupled beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added for 2 h at 4°C under constant agitation. The beads were pelleted and washed three times with lysis buffer (50 mM Tris 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% TritonX-100, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin). The immunoprecipitated proteins were resuspended in sample buffer and after boiling they were separated by 12% SDS-PAGE. The obtained immunoprecipitates were analyzed by Western immunobloting.

### DNA affinity chromatography

The DNA affinity column contained a nucleotide fragment between −165 and −49 bp from the 5′ flanking region of the rat haptoglobin gene [12]. The fragment, functionally identified as a HRE, was obtained from Dr Heinz Baumann from the Roswell Park Memorial Institute, Buffalo, NY. A DNA affinity column with the rat haptoglobin gene HRE was prepared according to Kadonaga and Tjian [20]. The HRE fragment was annealed and ligated to obtain oligomers and then covalently coupled to CNBr-activated Sepharose CL-2B (Amersham-Pharmacia Biotech, Uppsala, Sweden). The HRE affinity resin was equilibrated in a 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. Equal amounts of nuclear extracts (8 mg) were mixed with competitor salmon sperm DNA and incubated for 10 min at room temperature. The protein-DNA mixture was then applied to the HRE affinity column and after 30 min, HRE-bound nucleoproteins were

eluted with dialysis buffer containing 1 M KCl. Total amounts of eluted proteins were subjected to SDS-PAGE and analyzed by Western immunobloting.

### Statistical analysis

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

## Results

### Differential haptoglobin expression during DR and the turpentine-induced AP response

Using rocket immunoelectrophoresis (Fig. 1) we assessed the haptoglobin serum levels. In the ALT rats, the serum haptoglobin level was increased 1.8-fold ($P < 0.05$) with respect to matched controls (the AL group). The DR rats were characterized by a decrease in basal haptoglobin level by 47% ($P < 0.05$) compared to AL rats. At 12 h after turpentine administration, the DRT rats displayed a nearly 1.5-fold increase in serum haptoglobin level compared to AL rats.

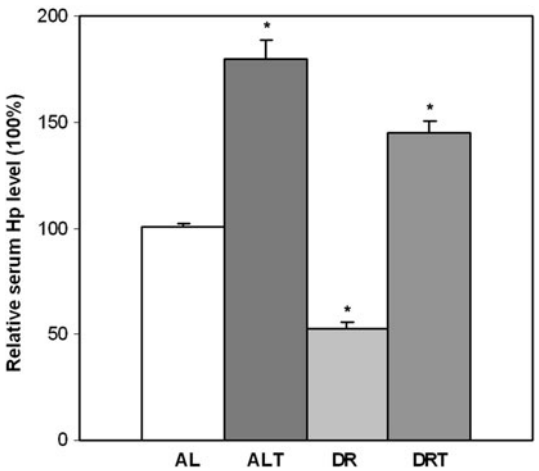


**Fig. 1** Relative haptoglobin levels in the sera of rats exposed to ad libitum feeding and dietary restriction before and after induction of the AP response by turpentine administration. The relative levels of haptoglobin in the serum were determined by rocket immunoelectrophoresis with an anti-haptoglobin antibody as described in "Materials and methods" section. Changes in serum haptoglobin levels were expressed as percentages relative the control values that were taken as 100%. AL—male rats allowed to feed ad libitum; ALT—AL rats that were administered turpentine (1 µl/g of body weight); DR—male rats fed with an amount of chow equivalent to 50% of the food intake of AL rats; DRT—DR rats treated with the same dose of turpentine. The values are expressed as means ± SEM, from three separate experiments, $n = 5$ in each experimental group. *$P < 0.05$ compared to AL rats

### Changes in abundance of constitutive and inducible members of STAT and C/EBP trans-acting factors in liver nuclear extracts related to DR and the AP response

The C/EBP isoforms are products of differential translational initiation at multiple AUG sites within the single C/EBP mRNA [21]. Transcriptional regulation of target genes by C/EBP depends on the relative levels of active and dominant negative isoforms [22]. Western immunoblot analysis with anti-C/EBPα antibody revealed the presence of multiple C/EBPα isoforms, i.e. 45, 42, 38, 35 and 30 kD proteins in the samples prepared from AL, ALT, DR and DRT rats (Fig. 2a). Full-length 45 kD C/EBPα protein exhibits a more efficient transcriptional activity than the truncated 30 kD form [23]. In ALT rats the levels of the 45, 42, 38, 35 and 30 kD isoforms decreased by 63, 72, 89, 84 and 43%, respectively. DR rats did not exhibit any discernible changes in abundance of the 45, 42 and 30 kD C/EBPα isoforms, while the 38 and 35 kD isoforms decreased in abundance by 55 and 65%, respectively. In DRT rats all C/EBPα isoforms decreased to the levels observed in the ALT group. C/EBP*β* was also represented by multiple isoforms, i.e. 42, 35, 32 and 20 kD proteins (Fig. 2b). The AP response was accompanied by increased abundance of all isoforms, in particular of the 35 and 20 kD proteins (1.88- and 1.73-fold increases, respectively). The 35 kD isoform, referred to as LAP or liver-enriched activator protein, is a major C/EBP*β* isoform and a powerful *trans*-activator [23], while the 20 kD isoform or LIP (liver-enriched inhibitory protein) mostly attenuates transcription [24–26]. DR rats were characterized by a different profile of changes in C/EBP*β* isoforms compared to the AL group. The 35 kD isoform exhibited the most prominent increase (2.25-fold), while the 32 and 20 kD isoforms decreased (by 50 and 40%, respectively). In the DRT group, increase of the 42 and 35 kD isoforms (3.40- and 4.35-fold, respectively), and decrease of the 32 and 20 kD isoforms (by 88 and 46%, respectively) were detected.

STAT3 is the main mediator of IL-6 type cytokine signaling. It exists in two isoform: the full-lenght STAT3α and the truncated STAT3*β*, which lacks the C-terminal activation domain and is generally considered a dominant negative form [27]. In the nuclear extract prepared from AL rats, a low level of the constitutively active 91 kD STAT3 isoform was observed (Fig. 3a), and in ALT rats it increased considerably (10.6-fold). In the DR and DRT groups, the 91 kD STAT3 isoform was significantly increased (8.4- and 13.75-fold, respectively). The level of

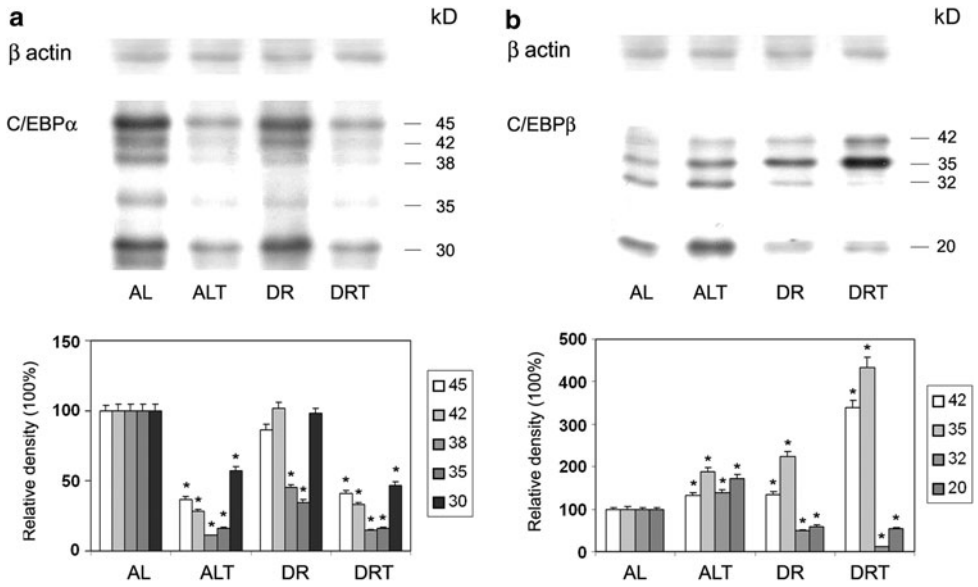


**Fig. 2** Immunoblot analysis of rat liver nuclear extract proteins with anti-C/EBPα (**a**) and anti-C/EBPβ (**b**) antibodies. Proteins (20 μg) were separated by SDS-PAGE, transferred to PVDF membranes and probed with anti-C/EBPα and anti-C/EBPβ antibodies. The antigen–antibody complexes were visualized by the ECL detection system. AL—male rats allowed to feed ad libitum; ALT—AL rats that were administered turpentine (1 μl/g of body weight); DR—male rats fed with an amount of chow equivalent to 50% of the food intake of AL rats; DRT—DR rats treated with the same dose of turpentine. The immunoblots were quantified with TotalLab (Phoretix) electrophoresis software (ver. 1.10). Data are expressed as mean ± SEM, $n = 5$/group. $^*P \leq 0.05$ compared to AL rats. The molecular weights of C/EBP isoforms are indicated

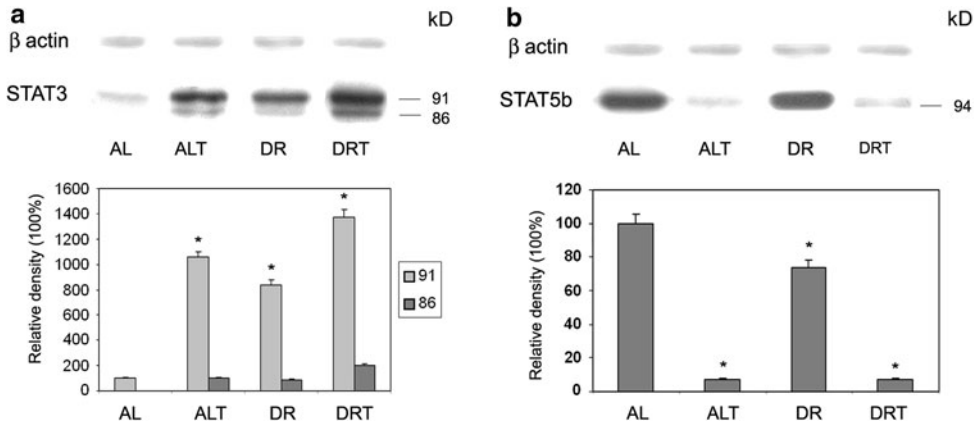


**Fig. 3** Immunoblot analysis of rat liver nuclear extract proteins with anti-STAT3 (**a**) and anti-STAT5b (**b**) antibodies. Proteins (20 μg) were separated by SDS-PAGE, transferred to PVDF membranes and probed with anti-STAT3 and anti-STAT5b antibodies. The antigen–antibody complexes were visualized by the ECL detection system. AL—male rats allowed to feed ad libitum; ALT—AL rats that were administered turpentine (1 μl/g of body weight); DR—male rats fed with an amount of chow equivalent to 50% of the food intake of AL rats; DRT—DR rats treated with the same dose of turpentine. The immunoblots were quantified with TotalLab (Phoretix) electrophoresis software (ver. 1.10). Data are expressed as mean ± SEM, $n = 5$/group. $^*P \leq 0.05$ compared to AL rats. The molecular weights of STATs isoforms are indicated

STAT5b was highest in the nuclear extract prepared from AL rats (Fig. 3b), and in the ALT group it was greatly decreased (14.3-fold). The DR affected slightly the level of STAT5b, thus in DR rats it was 25% lower than in the AL rats. In DRT rats STAT5b decreased to the level observed in the ALT group.

## Binding activities of C/EBPα, C/EBPβ, STAT3 and STAT5b isoforms for the haptoglobin gene HRE during DR and the AP response

The arrangement of putative C/EBPα/β and STAT3/5b consensus binding sites (C/EBP- T/(T/G)NNG(A/C/T)

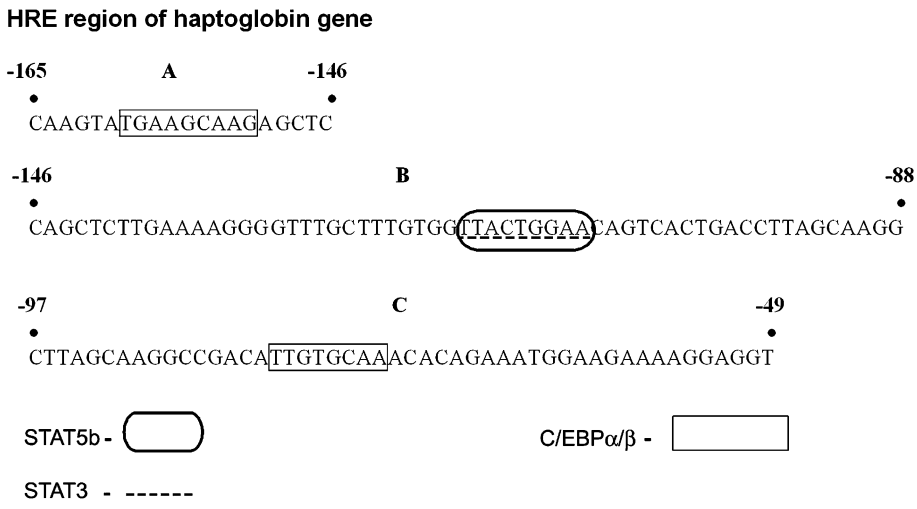


**Fig. 4** Nucleotide sequence of the HRE/ABC element of the haptoglobin gene. The nucleotide sequence in the −165/−49 bp region of the haptoglobin gene was established previously [12]. The C/EBP$\alpha/\beta$, STAT3/5b consensus binding sites are as indicated; C/EBP-T/(T/G)NNG(A/C/T)AA(T/G), STAT-TTN$_5$/N$_6$AA

AA(T/G), [28]; STAT-TTN$_5$AA, [29]) within the HRE region (−165/−49 bp) of the haptoglobin gene is presented in Fig. 4. C/EBP$\alpha/\beta$ binding sites lie within regions A (−159/−151) and C (−81/−74) of the HRE. The STAT3 and/or STAT5b regulatory motif 5′-TTACTGGAA-3′ is located at −117 to −109 bp within region B. The participation of transcription factors in haptoglobin gene transcriptional regulation was examined by DNA affinity chromatography of nuclear extracts that were prepared from AL and DR rats before and after turpentine treatment and subsequent Western immunoblot analysis (Fig. 5). Of the HRE-bound C/EBP proteins present in the nuclear extract that was prepared from AL rats, only the binding of the full-length 45 kD C/EBP$\alpha$ isoform was detected. ALT rats displayed HRE binding of the 45 kD C/EBP$\alpha$ and inducible 35 kD C/EBP$\beta$ isoforms. The HRE-binding nuclear proteins from DR rats were enriched with 45 and 30 kD C/EBP$\alpha$ isoforms and with the 35 kD C/EBP$\beta$ isoform. The nuclear extract from DRT rats was characterized by a 35 kD C/EBP$\beta$ HRE-binding protein, while no HRE binding activity of C/EBP$\alpha$ was detected. The nuclear extract from AL animals was enriched with a STAT5b HRE-binding protein. Turpentine-induced upregulation of the haptoglobin gene (ALT and DRT rats) was associated by the binding of STAT3 as well as STAT5b while DR was accompanied only by STAT5b binding.

## STAT3-NF-$\kappa$B p65 interactions as a determinant of STAT3 binding to the HRE and haptoglobin gene expression

Protein–protein interactions are an important aspect of gene transcription by STAT proteins. The coordinated activation of STAT3 and NF-$\kappa$B by mediators of the AP response appear to be important for the cooperative activation [30] or attenuation [31] of certain AP protein gene promoters.

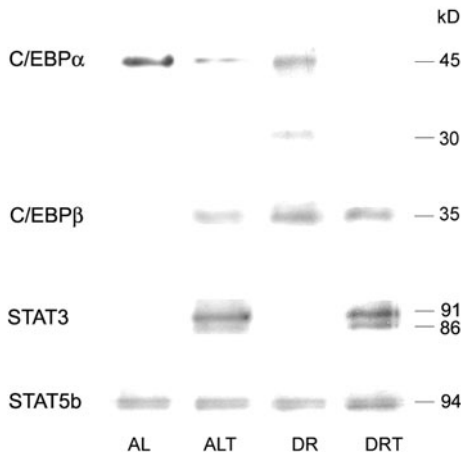


**Fig. 5** Immunoblot analysis of nuclear proteins with anti-C/EBP$\alpha$, anti-C/EBP$\beta$, anti-STAT3 and anti-STAT5b antibodies after DNA-affinity chromatography. Equal quantities (8 mg) of nuclear proteins were applied to the DNA affinity column and eluted with 1 M KCl. Total amounts of eluted proteins were separated by SDS-PAGE, transferred to PVDF membranes and probed with anti-C/EBP$\alpha$, anti-C/EBP$\beta$, anti-STAT3 and anti-STAT5b antibodies. AL—male rats allowed to feed ad libitum; ALT—AL rats that were administered turpentine (1 µl/g of body weight); DR—male rats fed with an amount of chow equivalent to 50% of the food intake of AL rats; DRT—DR rats treated with the same dose of turpentine. The molecular weights of C/EBP and STAT isoforms are indicated

Western immunoblot analysis revealed a 3.3-fold increase in p65 NF-$\kappa$B abundance in ALT rats that was nearly the same as measured in DR rats (Fig. 6a). In DRT rats, p65 was 3.8-fold increased (relative to AL rats). The p50 pool was increased 1.4-fold in ALT rats while in DR rats it was unaffected. In DRT rats, p50 increased to the level detected in the ALT group. Although a sequence homologous to the NF-$\kappa$B binding site was not identified, Western immunoblot analysis of eluted nuclear proteins from the HRE affinity column revealed the presence of p65 and p50 NF-$\kappa$B proteins in the HRE-bound nuclear protein fractions obtained from ALT and

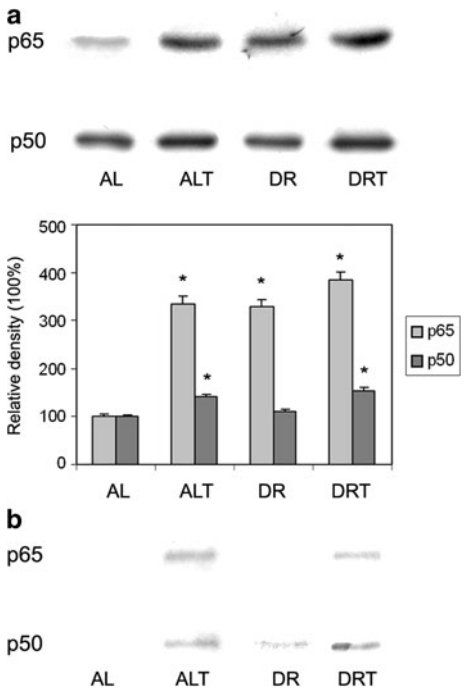


**Fig. 6** Changes in nuclear abundance and HRE-binding activities of p65 and p50 NF-κB relative to turpentine treatment and dietary restriction. Liver nuclear extracts were **a** blotted with anti-p65 and anti-p50 NF-κB antibodies (the results of quantification of the immunoblots using TotalLab (Phoretix) electrophoresis software (ver. 1.10) are presented in the *graphs* and data are expressed as mean ± SEM, $n = 5$/group, $^*P \leq 0.05$ compared to AL rats), and **b** subjected to DNA affinity chromatography using HRE, followed by Western immunoblotting with anti-p65 and anti-p50 NF-κB antibodies. AL—male rats allowed to feed ad libitum; ALT—AL rats that were administered turpentine (1 μl/g of body weight); DR—male rats fed with an amount of chow equivalent to 50% of the food intake of AL rats; DRT—DR rats treated with the same dose of turpentine

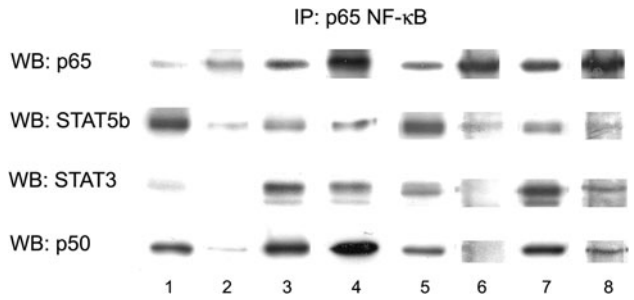


**Fig. 7** Co-immunoprecipitation of p65 NF-κB from nuclear extracts from AL and DR rats, before and after turpentine administration. Immunoprecipitation was performed with precleared nuclear extracts using p65 NF-κB antibody (as described in "Materials and methods" section). Co-immunoprecipitates were analyzed with anti-STAT5b, anti-STAT3 and anti-p50 NF-κB antibodies. 1—nuclear extract from liver of male rats allowed to feed ad libitum (AL); 2—immunoprecipitates from AL rats; 3—nuclear extract from liver of male rats that were administered turpentine (ALT); 4—immunoprecipitates from ALT rats; 5—nuclear extract from liver of male rats fed with an amount of chow equivalent to 50% of the food intake of AL rats (DR); 6—immunoprecipitates from DR rats; 7—nuclear extract from liver of DR male rats treated with turpentine (DRT); 8—immunoprecipitates from DRT rats

DRT rats, and a slight presence of only p50 NF-κB in the sample obtained from DR rats (Fig. 6b). The possibility that NF-κB interacted with the HRE region gene via protein–protein interactions with STAT3 and/or STAT5b was examined by co-immunoprecipitation. Experiments were performed with an antibody raised against NF-κB p65. Analysis of the obtained immunoprecipitates (Fig. 7) with anti-STAT3 and anti-STAT5b antibodies showed that STAT5b coimmunoprecipitated with p65 NF-κB under all examined conditions, i.e. in AL, DR, ALT and DRT rats. STAT3 and p50 co-immunoprecipitated with p65-NF-κB in ALT and DRT rats, suggesting that dimerization of p65 NF-κB with the p50 subunit and its interaction with STAT3 was functionally connected only to haptoglobin gene upregulation.

## Discussion

A general depression of hepatic protein synthesis occurs during starvation [9]. Reduction or abrogation of the synthesis of positive AP proteins was demonstrated during protein-malnutrition and anorexia. Lyoumi et al. [3] showed that a protein-deficient diet increased $\alpha_2$-macroglobulin expression but did not affect the expression of the prominent positive AP protein $\alpha_1$-acid glycoprotein in the rat. It can be concluded that different nutritional interventions affect individual AP protein expression. Our results show that a 50% reduction of food intake caused a decrease in the serum haptoglobin level in male rats. However, these rats exhibited an appropriate increase in the haptoglobin serum level after turpentine induction of the AP response, suggesting that DR suppresses constitutive haptoglobin expression in the liver but not the capacity for haptoglobin upregulation in response to a challenge with an external acute stress stimulus. Since changes in haptoglobin serum levels reflect changes in transcriptional activity of the haptoglobin gene in hepatocytes, we investigated the influence of a decreased protein and calorie intake on the nuclear profiles/levels of C/EBP and STAT families of transcription factors whose interchange determines the transcriptional activity of the haptoglobin gene in the liver.

The C/EBP proteins are important regulators of liver metabolism and functions that mediate between nutrients and hormones and gene expression [32]. The majority of the DNA–protein complexes that have been identified on promoters of several liver AP genes in the physiological state contain C/EBPα homodimers and C/EBPα/C/EBPβ heterodimers [23]. After acute stress, the number of complexes containing C/EBPα is reduced and replaced by C/EBPβ and C/EBPβ/δ homo- and heterodimers [33]. It was suggested that the ratio of these isoforms determines

whether transcriptional activity of target genes is enhanced or suppressed. Our results showed that DR was accompanied by HRE binding of the 45 C/EBPα and 35 kD C/EBPβ proteins and the truncated 30 kD C/EBPα isoform. Turpentine treatment of DR rats was accompanied by HRE binding of the 35 kD C/EBPβ isoform and absence of C/EBPα binding (Fig. 5). Our results suggest that the reduction of haptoglobin synthesis that accompanied reduced food intake was partly due to an alteration of the C/EBPα/C/EBPβ ratio on the HRE of the haptoglobin gene. The main HRE-binding C/EBP proteins in female rats that were subjected to the same type of DR as applied herein were the truncated 30 and 27 kD C/EBPα isoforms [14] while no binding of the 35 kD C/EBPβ polypeptide was detected. Since females exposed to DR are characterized by the elevated expression of the haptoglobin gene [14], these results suggest that C/EBPα and C/EBPβ probably assumed the role of accessory factors that mediated between DR and the transcriptional activity of the haptoglobin gene. An accessory role of C/EBPα and C/EBPβ in mediating between the effects of nutrients and hormones and the rate of transcription of the PEPCK gene has been described [34].

Increased transcription of the haptoglobin gene in hepatic cells during the turpentine-induced AP response was primarily regulated by activated STAT3 which is referred to as an AP response factor [35]. This was accompanied by a lower abundance of STAT5b and its decreased binding to the HRE of the haptoglobin gene. This finding indicates that STAT5b is involved in the regulation of rat haptoglobin gene activity only in the basal state [26]. The results presented in this work revealed that DR increased STAT3 nuclear abundance and did not affect the level of STAT5b (Fig. 3). However, in spite of the increased abundance of STAT3, it did not exhibit binding to the HRE during DR (Fig. 5). We propose that the failure of STAT3 to bind to the HRE during DR was the key event that downregulated the haptoglobin gene. Previously, we observed that the DR-related increase in haptoglobin serum level in female rats was associated with a greater abundance of STAT5b than STAT3, although both proteins exhibited HRE-binding activities [14]. This result confirmed the requirement of STAT3 binding to the HRE for haptoglobin gene upregulation. By competing for a common site within the B region of the haptoglobin gene HRE (Fig. 4), STAT3 and STAT5b could fine-tune haptoglobin gene expression. Hence, the occupancy of the haptoglobin gene HRE with STAT5b probably prevented STAT3 binding during DR. In line with this assumption is the finding that after turpentine treatment of DR rats, the lower amount of STAT5b and appearance of STAT3 HRE binding correlated with haptoglobin upregulation. The change in STAT3 binding to the HRE could be related to its interaction with other transcription factor(s) shown to participate in STAT-regulated transcription [36].

The synergistic interaction of NF-κB with transcription factors such as STAT is required to achieve a purposeful induction of a particular gene [37]. The most prevalent activated form of NF-κB is a heterodimer consisting of 50 kD (p50) and 65 kD (p65) subunits with *trans*-activating and promoter-binding properties [38]. While NF-κB binding sites within the HRE region of the haptoglobin gene were not observed, HRE-affinity chromatography revealed the presence of the p65 NF-κB protein among the HRE-bound nuclear proteins from turpentine-treated AL and DR rats (ALT and DRT, respectively, in Fig. 6b). Together, these results suggest that NF-κB proteins associated with the HRE region through protein–protein interactions. Since STAT3 co-immunoprecipitated with p65 under AP conditions in both AL and DR rats, we assume that p65 participates in the stimulation of haptoglobin gene transcription via interaction with HRE-bound STAT3. Hagihara et al. [30] demonstrated that a complex that includes STAT3, p65 NF-κB and p300 was essential for the synergistic induction of the serum amyloid A gene by IL-1 and IL-6 [39]. Haptoglobin is an AP protein whose synthesis is regulated by the synergistic action of IL-1β and IL-6 [12]. This suggests that one of the functions of p65 NF-κB in the activation of the haptoglobin gene during the AP response could be to recruit and maintain STAT3 bound to its consensus binding site in the HRE of the haptoglobin gene. In this case, p65 NF-κB would act as a co-activator. This is supported by the absence of STAT3 binding to the haptoglobin gene HRE when no interaction between STAT3 and p65 NF-κB was detected. These results imply that down-regulation of the haptoglobin gene in male rats during chronic DR could also be related to interruption of STAT3 functioning through modulation of its interaction with p65 NF-κB. p65 NF-κB is a potent transcriptional activator whereas complexes containing p50 homodimers are thought to operate as active repressors of transcription [40]. In unstimulated or resting cells, p50 homodimers predominantly bind DNA [41]. Western immunoblot analysis of eluted nuclear proteins from the HRE affinity column revealed low amounts of p50 NF-κB in DR rats, pointing to its involvement in haptoglobin gene downregulation.

To conclude, this study shows that exposure of male rats to long-term DR recruited C/EBPα, C/EBPβ, STAT5b and p50 on the haptoglobin HRE. The resulting changes in C/EBPα/C/EBPβ isoform ratios, as well as loss of STAT3 activity were probably responsible for haptoglobin downregulation during DR. The results provide evidence that DR prevent STAT3 functioning possibly through modulation of its activity with p65 NF-κB interactions. Turpentine treatment of DR rats was accompanied by increased

abundances and activities of C/EBP$\beta$, NF-$\kappa$B p65 and STAT3, and the establishment of STAT3-p65 NF-$\kappa$B interactions which correlates with haptoglobin upregulation and positive AP responsiveness. Thus, STAT3/NF-$\kappa$B crosstalk has been identified as a regulatory step in modulation of haptoglobin expression during DR and/or the AP response in male rats. Our previous finding that in female rats DR-related haptoglobin upregulation was accompanied by the induction of STAT3 activity points to its role in gender-related differences with regard to the response of haptoglobin and inflammatory status provoked by DR. These findings constitute an advance in our knowledge of the mechanisms and signal transduction pathways that mediate DR-dependent transcriptional regulation of AP gene expression.

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