Effects of mercuric chloride on the regulation of expression of the acute phase response components $\alpha_1$-acid glycoprotein and C/EBP transcription factors

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Abstract

We have previously shown that in response to treatment with HgCl$_2$, the adult mouse liver exhibits both transcriptional and translational regulation of the acute phase response genes. In this study we asked whether the heavy metal treatment affects the regulation of the C/EBP transcription factors which play a key role in regulation of the acute phase response gene. Our studies have shown that the AGP gene is transcriptionally activated while transcription of the CCAAT/enhancer-binding trans-activating protein (C/EBP)$\alpha$ gene is slightly down-regulated and that of the C/EBP$\beta$ gene does not respond. Both the C/EBP$\alpha$ and C/EBP$\beta$ mRNAs produce multiple isoforms possibly by alternative translation initiation (ATI) of multiple internal AUG initiation sites. The C/EBP$\beta$ mRNA appears to be stabilized. Although similar regulatory processes occur in response HgCl$_2$ vs. LPS, our data suggest that the translational processes (ATI) are differentially affected. In addition, a major difference lies in the fact that the C/EBP$\beta$ gene is not transcriptionally activated by HgCl$_2$. Our data show decreased binding activity and pool levels of the C/EBP$\alpha$ isoform (p42$^{\alpha}$/EBP$^{\alpha}$) and increased binding activity and pool levels of C/EBP$\beta$ isoform (p35$^{\beta}$/EBP$^{\beta}$) in response to HgCl$_2$. We propose that this isoform may be involved in the regulation of AGP gene expression in response to heavy metals and that there is a significant difference between the HgCl$_2$-mediated and LPS-mediated inflammatory response. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The acute phase response represents the body’s well orchestrated response that mediates cell survival immediately following such pathological insults as infection, myocardial infarction, surgery, burns, neoplastic disease or heavy metal poisoning [1–3]. It has previously been shown that the $\alpha_1$-acid glycoprotein (AGP) gene, a major acute phase reactant, is induced in the liver of mice by heavy metals such as HgCl$_2$, Pb, Cd, Zn, and Cu [3,4]. Additionally, we have shown that heavy metals can induce the expression of an AGP promoter – chloramphenicol acetyltransferase reporter gene [3]. This response to heavy metals does not require the synergistic action of cytokines and glucocorticoids for maximum gene expression [4]. Moreover, the down-regulation of albumin gene expression, a characteristic of the bacterial lipopolysaccharide (LPS)-mediated acute phase response does not occur after treatment with heavy metals. These observations suggest that the hepatic response to heavy metals is not a typical acute phase response mediated by LPS.

The CCAAT/enhancer-binding trans-activating proteins (C/EBP$\alpha$, C/EBP$\beta$, and C/EBP$\delta$) are translated from respective single mRNA species [5] encoded by their corresponding intronless genes. These mRNAs are translated to produce several isoforms proteins that have truncated transcriptional activation domains and are characterized by specific transcriptional regulatory functions [6–8]. These isoforms of C/EBP$\alpha$ and C/EBP$\beta$ have been identified in mouse liver [6,9,10,11] and tissue culture cells including hepatocytes [12,13], HeLa cells [14] and virus-infected cells.
The fact that only one C/EBPα and C/EBPβ mRNA is transcribed from their intronless genes suggests that these isoforms could be produced by the alternative translational initiation (ATI) of several in frame AUG start sites within each mRNA [9,11]. Initiation at these downstream AUGs, would account for the observed multiple isoforms detected by Western or Southwestern analyses, which is consistent with the mechanism of ATI. Formation of C/EBPα and C/EBPβ isoforms has also been attributed to proteolytic cleavage of the high molecular weight isoform [14,16]. It has been proposed that C/EBPα regulates low molecular weight C/EBPβ isoform production through activation of calpain, a proteolytic enzyme that cleaves C/EBPβ [14,16,17]. However, our studies suggest that the 20-kDa isoform is generated by the ATI at an internal AUG initiation site ([11]; Xiong et al., unpublished).

We have previously shown that the 20-kDa isoform of C/EBPβ (p20K;EBPβ) may play a key role in the regulation of AGP gene expression in response to LPS [18,19]. This regulation is mediated by the interaction of the 20-kDa C/EBPβ isoform with the cis-acting acute phase response element (APRE), located in the AGP gene promoter [9,18]. Furthermore, we have shown that the APRE is a compound binding site in which a glucocorticoid response element (GRE) overlaps with the C/EBP binding site. Transient expression analyses have suggested that the interaction of glucocorticoid receptor and C/EBPβ with the APRE is essential for maximal gene expression [19].

Metallothionein genes are transcriptionally induced in several species by heavy metals through the interaction of trans-acting factors with corresponding cis-acting metal response elements (MRE) [20,21]. The consensus DNA sequence within the APRE that binds to the C/EBP proteins is similar to that of the MRE consensus DNA sequence [4]. In this study, we examine whether members of the C/EBP family of transcription factors are affected by heavy metals in mice, i.e., whether the exposure to heavy metal has any effect on the formation of C/EBPα and C/EBPβ isoforms.

2. Materials and methods

2.1. Animals

Male, 4-months-old Balb/C mice (19–20 g) were purchased from Charles River Breeding Laboratory. They were maintained under 12-h light–dark cycles and provided free access to food and water. Pseudomonas-derived bacterial LPS (Sigma, 1 mg/kg bw) or HgCl₂ (0.5 mg/kg bw) were injected intraperitoneally (IP) in 0.1 ml pyrogen free saline. The animals were sacrificed 3 h after injection and livers were excised and extracted for further analysis as described below.

2.2. Nuclear protein preparation

A detailed description of the preparation of liver nuclear extracts from 4-month-old normal, HgCl₂- and LPS-treated mice has been described [22]. Five mice were included in each group and liver nuclear extracts were isolated from each mouse. Protein concentrations were determined by the method of Bradford [23]. The nuclear extracts from COS-1 cells were prepared as follows: COS-1 cell cultures, approximately 70% confluent, are rinsed three times with 10 ml ice cold TBS buffer (25 mM Tris, pH 7.4; 5 mM KCl; 0.8% NaCl). The attached cells were then scraped off the culture dish in 10 ml ice cold TBS buffer. The cell suspension was transferred to a 15-ml centrifuge tube and spun at 4°C for 5 min in a J6-B centrifuge. The supernatant was discarded and the cells were suspended in 400 ml of buffer A (10 mM HEPES, pH 7.9; 10 mM KCl; 1 mM DTT; 0.1 mM EGTA; 1 mM PMSF; 1 µg/ml each of antipain, chymostatin, leupeptin and pepstatin; 50 mM sodium fluoride; 10 mM sodium pyrophosphate; 1 mM sodium orthovanadate; 10 µM sodium molybdate and 20 mM β-glycerol phosphate). The cell suspension was transferred to a microcentrifuge tube and incubated on ice for 15–30 min after which, 20 ml of NP-40 (10% solution) was added. The microfuge tube was vortexed vigorously for 30 s and centrifuged for 30 s. The supernatant was transferred to a new tube and stored for use as the cytoplasmic protein fraction. The pellet was resuspended in 100 ml buffer B (buffer A with 0.4 M KCl) by vortexing for 30 s. The fraction was centrifuged for 5 min in the cold room. The supernatant was removed and used as nuclear extract.

2.3. Electrophoretic mobility shift (EMSA) and super shift assays

EMSAs were performed as described by Alam et al [18]. The APRE, corresponding to the C/EBP binding of the AGP promoter (5′-GAACATTTTGCCGAAGACATTTTC-CCAAG-3′) and its complementary strand were used as a probe for EMSA or Southwestern blot analysis after labeling with [γ32P]ATP by T4 polynucleotide kinase [24]. The oligonucleotide 5′-AGTCCTGTACAGAAT-3′ (C/EBP consensus sequence is underlined) that corresponds to the C/EBP binding site of the mouse serum amyloid A (SAA) gene [25] was used for competition experiments. Equal amounts of the two complementary strands were heated at 95°C for 10 min in STE buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, and 1 mM EDTA) and allowed to anneal by slowly cooling to room temperature (RT). For supershift assays C/EBPα, C/EBPβ and C/EBPδ-specific antibodies were preincubated for 20 min with nuclear extracts before adding the probe. The DNA–protein complexes were resolved by electrophoresis in 6% non-de-
naturing polyacrylamide gels in 0.5×TBE, 25 mM Tris base, 25 mM boric acid, 0.5 mM EDTA.

2.4. Southwestern analysis of liver nuclear proteins

Nuclear extracts (30 µg) isolated from livers of 4-month-old normal, HgCl₂-, or LPS-treated mice were electrophoretically separated on 12% SDS–polyacrylamide gels as described [9]. The proteins were then electrotransferred to 0.45–µM pore Westram polyvinylidene difluoride (PVDF) membranes (Schleicher and Schuell) in a buffer containing 25 mM Tris–HCl, 192 mM glycine, and 20% methanol, pH 8.3 [26]. Southwestern analysis was performed by a modification of a previously described procedure [27]. Our modification of this procedure has been described in detail [9].

2.5. Western blot (immunoblot) analysis of nuclear extracts

Twenty micrograms of liver nuclear extract was boiled for 5 min at 100°C, subjected to SDS-PAGE on a 12% polyacrylamide gel [27,28], and transferred to Westram PVDF membranes. The filters were then immunostained according to the procedure recommended by the supplier of the ECL Western analysis kit (Amersham). The Western blots were incubated at RT for 1 h in TBS (25 mM Tris, 137 mM NaCl, 5 mM KCl) containing 5% non-fat dry milk (Bio-Rad, Hercules, CA, USA) and 0.05% Tween 20 (Sigma). The blots were then incubated for 2 h in blocking buffer containing antiserum. Antisera specific to C/EBPα, C/EBPβ and C/EBPδ [9,29,30] were diluted 1:10 000. To remove unbound primary antibody, the filters were washed three times in TBS containing 0.05% Tween 20 for 10 min at RT. The blots were then incubated in washing buffer containing [125]I-labeled protein A (Amersham; specific activity, 30 µCi/mg) for 2 h at RT. After four 15-min washes in washing buffer at RT, each damp blot was placed between two sheets of plastic wrap, and the wrapped blots were placed over sheets of X-ray film in photographic cassettes with intensifying screens. After autoradiography (~80°C for 2 days), the signals were quantified with a scanning densitometer.

2.6. Isolation and Northern analysis of RNA

Total RNA was isolated from the livers of 4-month-old mice according to the method previously described [9,31]. For Northern blotting, RNA samples were resolved by electrophoresis through a 1.4% formaldehyde-agarose de-naturing gel buffered with 0.02 M [3-(N-morpholino)-propanesulfonic acid] and 1 mM EDTA (pH 7.4). The RNAs were transferred overnight from gels to Nitroplus membranes (Schleicher and Schuell, Keene, NH, USA) in the presence of 20×SSC (1×SSC is 0.15 M NaCl plus 0.015M Na-citrate) and baked for 2 h at 80°C under a vacuum. Preparation of 32P-labeled C/EBPα and C/EBPβ cDNA probes and hybridization conditions for Northern analysis have been described [9]. Upon completion of hybridization, filters were exposed to X-ray film. The relative amounts of C/EBP mRNAs were determined by densitometric analysis of Northern blot autoradiograms with an Applied Imaging densitometer with Lynx 5.1 densitometry software.

2.7. Nuclear run-on transcription analysis

The preparation of mouse liver nuclei and conditions for nuclear run-on transcription has been described [9]. Upon completion of hybridization dried filters were exposed to X-ray film. The intensity of hybridization signals was quantitated using the Applied Imaging densitometer with Lynx 5.1 densitometry software.

2.8. Cell culture and transfection of C/EBP expression vectors in COS cells

COS-1 cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% bovine calf serum and seeded at a density of 10⁶ cells per 100-mm dish 24 h before transfection. Transfections with 5 mg of the pCMV-C/EBPβ plasmid DNA was performed as previously described using the DNA–calcium phosphate coprecipitation method [3,9]. For normalization purposes SV-βgal (Promega) plasmid was cotransfected to the COS-1 cells [3]. Different concentrations of HgCl₂ were added to COS-1 cells 24 h after transfection and nuclear extracts were prepared 24 h later as described previously [33]. The protein concentrations of the nuclear extracts were determined according to the method of Bradford [23].

2.9. Antisera

Polyclonal antibodies to C/EBPα, C/EBPβ and C/EBPδ were prepared against specific oligopeptides [5,29,30]. For anti-C/EBPα the oligopeptide, AGPHPDRLRTGGGGG [29], which is immediately adjacent to the DNA binding site; for anti-C/EBPβ the oligopeptide, LRNLFKQLPEP-LLASAGHC [5], which is at the C-terminus; and for anti-C/EBPδ the oligopeptide, LRQFFKKLPSPPFLPPTGA-DCR [5,30], which is at the C-terminus were used. The oligopeptide CWMEYANFYEPDC, was used to prepare the N-terminal anti-C/EBPβ antibody. Antisera specific for the Flag tag sequences inserted into pCMV-C/EBPβ expression vector were purchased from Eastman Kodak (Rochester, NY, USA).

2.10. Statistical analysis

Statistical analysis was done using the two-tailed Student’s t-test, and statistical significance was accepted with values of P < 0.05.
3. Results

3.1. Effect of HgCl₂ on C/EBP DNA-binding activity

Our previous studies have shown that IP injection of heavy metals induces a subset of acute phase reactant genes, e.g., AGP, SAA and C-reactive protein (CRP) [3, 4]. Since these genes are also induced by LPS and their activation is mediated by C/EBP transcription factors, we asked whether the response of AGP to heavy metals such as HgCl₂ might be mediated by the interaction of C/EBPs with the APRE of the AGP promoter. Therefore, we used the same APRE and nuclear extracts from fresh mouse livers isolated 3 h post HgCl₂ (0.5 mg/kg bw) or LPS (1 mg/kg bw) injection to perform EMSA. The doses of HgCl₂ and LPS used were previously found to induce similar levels of hepatic AGP mRNA [3]. The results in Fig. 1A confirm that four major DNA–protein complexes (C1–C4) are formed in normal livers. Competition analysis using an oligonucleotide that contains the C/EBP binding site of the mouse SAA gene suggests that the DNA–protein complexes formed with the AGP APRE contain C/EBP proteins (Fig. 1A). The data in Fig. 1B show the changes in levels of DNA–protein complexes in response to HgCl₂ as compared to LPS treatment. In the LPS-treated livers the relative amount of the C1 and C2 complexes decreases while the C4 DNA–protein complex increased significantly [9, 11, 19] (Fig. 1B). In HgCl₂-treated livers the relative amount of the C1 DNA–protein complex decreased while the relative amounts of the C3 and C4 complexes increased. To identify the C/EBPs in the C1–C4 complexes we performed supershift analysis using specific antibodies to C/EBPα, C/EBPβ, and C/EBPδ (Fig. 1C). The supershift data indicate that the C1 and C2 complexes contain C/EBPα, the C3 complex contains both C/EBPα and C/EBPβ, and the C4 complex contains C/EBPβ [9, 19]. No C/EBPδ supershifted complex was observed in
HgCl$_2$ treated livers suggesting that the expression of this isoform may not be induced by HgCl$_2$ treatment. These results suggest that there are quantitative changes in the binding activity of the C/EBP$_{\alpha}$ and C/EBP$_{\beta}$ isoforms in response to HgCl$_2$ or LPS treatment.

3.2. Identification of C/EBP proteins that bind to the AGP promoter APRE

To identify the apparent molecular weight and DNA-binding activity of C/EBP isoforms we performed Southwestern analysis using the same nuclear extracts and the AGP gene APRE oligonucleotide as probe [4,9]. The data in Fig. 2 show major protein bands with molecular weights of 42, 35, and 30 kDa and a minor band at 38 kDa that bind the AGP APRE. It has been previously shown that the 42-, 38-, and 30-kDa bands are formed by C/EBP$_{\alpha}$, the 35-kDa band is formed by C/EBP$_{\beta}$ and the 20-kDa band is formed by both C/EBP$_{\alpha}$ and C/EBP$_{\beta}$ [6,7,9,30,34]. Both HgCl$_2$ and LPS treatment significantly decreased the relative amount of the p42$^{\alpha}$/C/EBP$_{\alpha}$. However, HgCl$_2$ results in a significant increase of the p35$^{\alpha}$/C/EBP$_{\alpha}$ band while LPS mediates a significant increase of the p20$^{\alpha}$/C/EBP$_{\alpha}$-$\beta$/C/EBP$_{\beta}$ band [9]. The above results clearly show significant quantitative changes in pool levels of C/EBP$_{\alpha}$ and C/EBP$_{\beta}$ isoforms to the APRE binding activity in response to both HgCl$_2$ and LPS.

3.3. Identification of C/EBP isoforms that are induced by HgCl$_2$ and LPS

Previous studies have indicated that LPS treatment stimulates the formation of specific C/EBP$_{\alpha}$ and C/EBP$_{\beta}$ isoforms [9]. In these experiments we performed Western analysis to compare the C/EBP isoforms produced by livers of HgCl$_2$ or LPS-treated mice. Our results presented in Fig. 3A,B show that the specific anti-C/EBP$_{\alpha}$ antibody detects two main bands with molecular weights of 42 and 30 kDa. Treatment with HgCl$_2$ results in a significant reduction of the 42-kDa C/EBP$_{\alpha}$ pool levels while the relative intensity of the abundant 30-kDa and low abundance 20-kDa isoforms do not change significantly. The LPS-treated liver samples show decreased 42- and 30-kDa isoform pool levels and increased 20-kDa isoform pool levels [9,11]. At the same time, the data also show that HgCl$_2$ results in an increase of the pool levels of the 35-kDa C/EBP$_{\beta}$ isoform and a slight increase in the levels of the p20$^{\beta}$/C/EBP$_{\beta}$ isoform (Fig. 3C,D). Conversely, in response to LPS there is a dramatic increase in the level of the 20-kDa C/EBP$_{\beta}$ isoform and a decrease of the pool level of the 35-kDa C/EBP$_{\beta}$ isoform. These data suggest that there are specific, quantitative differences in C/EBP isoforms in response to HgCl$_2$ and LPS treatment.

3.4. Effect of HgCl$_2$ on hepatic C/EBP$_{\alpha}$ and C/EBP$_{\beta}$ mRNA levels

The EMSA, Southwestern and Western analyses reveal that HgCl$_2$ causes significant changes in the DNA-binding activity and protein pool levels of C/EBPs. To examine whether HgCl$_2$ affects C/EBP mRNA levels, mice were injected with HgCl$_2$ (0.5 mg/kg bw) and at various times post treatment, total hepatic RNA was isolated and subjected to Northern analysis. The HgCl$_2$ treatment results in reduction of C/EBP$_{\alpha}$ mRNA levels 3–12 h post treatment (Fig. 4A). C/EBP$^{\beta}$ mRNA levels increased in HgCl$_2$-treated mice by 3 h post treatment. The responses of the C/EBP$_{\alpha}$ and C/EBP$_{\beta}$ mRNA levels to HgCl$_2$ are similar to those observed in response to LPS [32]. These data suggest that the decreased C/EBP$_{\alpha}$ protein pool levels may be due to decreased C/EBP$_{\alpha}$ mRNA levels and the increased C/EBP$_{\beta}$ protein pool levels may be due to increased C/EBP$_{\beta}$ mRNA levels. The data also indicate that HgCl$_2$ may affect either the rate of transcription or stability of the C/EBP mRNA.

3.5. Run-on transcription analysis in the liver of HgCl$_2$- and LPS-treated mice

To examine whether the HgCl$_2$-mediated decrease in
C/EBPα mRNA levels and increase in C/EBPβ mRNA levels are due to transcriptional regulation, mice were injected with HgCl₂ (0.5 mg/kg bw) or LPS (1 mg/kg bw) and 3 h later their liver nuclei were isolated and subjected to nuclear run-on transcription analysis. Fig. 4B shows that HgCl₂ and LPS reduce the transcription rate of C/EBPα to the same level. On the other hand, the heavy metal has no effect on the transcriptional rate of the C/EBPβ gene whereas LPS treatment increases the transcriptional rate of the C/EBPβ gene by 4-5-fold [9,32]. In addition, the AGP gene transcriptional rate was increased by 2-3-fold in response to HgCl₂ injection and 4-5-fold in response to LPS. These results indicate that HgCl₂ has a slight inhibitory effect on C/EBPα gene expression while it has no effect on C/EBPβ gene expression. Thus, the increase of C/EBPβ mRNA levels is most likely due to an increase in stability of C/EBPβ mRNA. Finally, the data indicate that HgCl₂ activates transcription of the AGP gene. Thus, HgCl₂ activates differential regulatory events, i.e., slight down-regulation of C/EBPα and no effect on C/EBPβ transcription, a stimulation of AGP transcription and a possible stabilization of C/EBPβ mRNA.

3.6. Effect of HgCl₂ on the formation of C/EBPβ isoforms in COS-1 cells transfected with C/EBPβ expression vector.

We have previously demonstrated that the wild-type
expression vector, pCMV-C/EBPβ transfected into COS-1 cells produces the 40-kDa, 35-kDa and 20-kDa C/EBPβ isoforms [11]. The data in Fig. 4 indicate that HgCl2 does not affect the transcription of C/EBPβ gene and that the increase in 35-kDa isoform is a translational event. To further investigate whether HgCl2 affects C/EBPβ isoform production, pCMV-C/EBPβ-transfected COS-1 cells were treated with varying concentrations of HgCl2. The nuclear extracts were then subjected to Western analysis to detect the pool levels of the C/EBP isoforms. The data in Fig. 5A,B show that HgCl2 treatment results in an increased production of the 40-kDa, 35-kDa and 20-kDa C/EBPβ isoforms (Fig. 5A,B). The same samples were subjected to Western analysis using an N-terminal C/EBPβ-specific antibody that could detect a potential degradation product(s) [9,11]. The data in Fig. 4C,D show that the antibody detects only the 35-kDa and 40-kDa C/EBPβ isoforms. The above data suggest that the C/EBPβ isoforms are stimulated by HgCl2 treatment. Whether this is due to stabilization of the mRNA or stimulation of translation remains to be determined.

4. Discussion

In this study we have shown that in response to HgCl2 treatment the adult mouse liver exhibits both transcriptional and translational regulation of components of the acute phase response. The mouse AGP gene, for example, is transcriptionally activated while the C/EBPα gene is slightly down-regulated and the C/EBPβ gene does not respond. Both the C/EBPα and C/EBPβ mRNAs produce specific protein isoforms possibly by AT1, and the C/EBPβ

Fig. 4. Effect of HgCl2 and LPS on mRNA pool levels and transcription of the C/EBPα, C/EBPβ AGP genes. (A) Northern analysis showing the pool levels of C/EBPα and C/EBPβ mRNA in normal and HgCl2-treated mouse livers. RNA (10 μg) was resolved on formaldehyde denaturing gel, transferred to nitrocellulose and then hybridized using C/EBPα and C/EBPβ cDNAs labeled in vitro with [32P]dCTP by random priming reaction. (B) The histogram shows the effect of HgCl2 and LPS on transcription of the C/EBPα, C/EBPβ and AGP genes as indicated by nuclear run-on analysis. Nuclei were prepared as described in Section 2 and nuclear run-on transcription was performed immediately with 5×107 nuclei in the presence of [γ-32P]UTP at 30°C for 45 min. The nascent RNA was purified by phenol extraction and ethanol precipitation. A total of 106 cpm of labeled RNA per assay was hybridized to 5 μg of C/EBPα, C/EBPβ and AGP cDNAs immobilized on nitrocellulose filters [9]. After hybridization the washed, dried filters were exposed to X-ray film. The signals on the autoradiograms were scanned and each value was normalized according to the β-galactosidase assay (see [3]).
mRNA appears to be stabilized. Although similar responses occur with LPS, a major difference lies in the fact that the C/EBPβ gene is transcriptionally activated by LPS. The EMSA data show that HgCl₂-treated nuclear proteins exhibit decreased binding activity of the C/EBPα isoforms and increased C/EBPβ isoform binding activity. This pattern of changes in binding activity is indicative of transcriptional activation of AGP by LPS [9,11]. Thus, the decrease of p42C/EBPα and increase of p35C/EBPβ pool levels and binding activities in response to HgCl₂ is in agreement with transcriptional activation of AGP.

Our previous studies have shown that the LPS-mediated activation of AGP gene involves the synergistic action of both p20C/EBPβ and glucocorticoid receptor whereas the HgCl₂-mediated activation does not involve the glucocorticoid receptor [3,19]. In the present studies, the gel shift and protein pool level data suggest that p35C/EBPβ may play a major role in HgCl₂-mediated activation of AGP. This may represent a significant difference between the HgCl₂- and LPS-mediated mechanisms of activation of the AGP gene since the former requires p35C/EBPβ whereas the latter requires the synergistic action of C/EBPβ (p20C/EBPβ) and glucocorticoid receptor [11].

We detected multiple C/EBPα and C/EBPβ isoforms in the livers of normal, HgCl₂- or LPS-treated mice. Similar C/EBP isoforms have been detected in rat liver [29,30], HepG2 cells [6], preadipocytes [12], and adipocytes [7] as well as in transient transfected COS-1 cells [11]. It has been proposed that these isoforms are the products of ATI of multiple AUG sites within the single C/EBPα or C/EBPβ mRNA [6,7,9,13,15]. The data presented in this study are consistent with an ATI hypothesis. There is a difference in the patterns of C/EBPα and C/EBPβ isoform production mediated by HgCl₂ compared to LPS induction [9,35]. We propose that HgCl₂ may activate the mechanism(s) that suppress initiation at the p42C/EBPα and p20C/EBPβ AUGs, but does not affect initiation at the p35C/EBPβ AUG. Thus, the responses to HgCl₂ and LPS exhibit significant differences; in response to LPS, the p30C/EBPα, p20C/EBPβ, and p20C/EBPα are formed whereas with HgCl₂, formation at the 20-kDa isoform of both C/EBPα and C/EBPβ is not significant. These data suggest that the translational processes are differentially affected by HgCl₂ vs. LPS. Several stress factors such as heat shock, heavy metals and oxidants, that induce the synthesis of various stress proteins have been shown to affect the protein synthesis machinery by modulating translation initiation factors [36–38].

Although both HgCl₂ and LPS increase hepatic C/EBPβ mRNA levels, HgCl₂ does not appear to increase the transcriptional rate of the C/EBPβ gene. Thus, the increase in C/EBPβ mRNA in response to HgCl₂ in the liver is most likely attributed to mRNA stabilization. Northern analysis, nuclear run-on (transcription), Western and Southern analyses revealed that both HgCl₂ and LPS cause a decrease in the transcriptional activity of C/EBPα gene. A reduction in C/EBPα gene activity occurs in response to multiple stress stimuli, i.e., LPS, heat shock and heavy metals. We propose that C/EBPα may function as a repressor of acute phase response genes and that the down-regulation of p42C/EBPα may be an important factor in the activation of multiple stress response genes. At the same time, the dramatic increase of hepatic C/EBPβ mRNA and protein levels also correlate with activation of stress response genes.

Differential effects of C/EBPβ isoforms on regulation of transcription have been reported. The p35C/EBPβ directs transcription when it binds to region D of the albumin promoter and acts as a powerful transactivator of the gene [10]. On the other hand, p20C/EBPβ acts as a repressor when it binds to region D of the albumin promoter [10]. This correlates well with the LPS-mediated increase in p20C/EBPβ and the corresponding down-regulation of the albumin gene during the acute phase response. On the other hand, HgCl₂ does not increase formation of the p20C/EBPβ and does not down-regulate albumin synthesis suggesting that this is not a typical acute phase response [3]. However, a comparison of the level of induction of AGP gene transcription by both HgCl₂ and LPS suggests that the transactivation by p20C/EBPβ and glucocorticoid receptor (GR) is more efficient than the transactivation by p35C/EBPβ alone. This difference may be due to a synergism by the combined activity of GR-p20C/EBPβ isoform complex [18].

It has been shown that the mouse heme oxygenase-1 (HO-1) gene, also a stress gene, is induced by heavy metals such as cadmium in Hepa cells [39]. Transient expression analysis has shown that sequences in the HO-1 promoter that bind the Activator Protein 1 (AP-1) and C/EBP transcription factors are required for maximal expression of a reporter gene in response to a heavy metal even though the promoter of this gene has several MRE sequences [39,40]. We have previously shown by transient expression analysis using HepG2 cells that HgCl₂ can drive an AGP promoter (from −595 to +1) reporter gene (CAT) in response to HgCl₂ [3]. However, since there are no AP-1 binding sequences in the AGP gene promoter, we propose that the AGP C/EBP (APRE) binding site plays a major role in the heavy-metal-mediated activation of AGP expression. However, we cannot exclude the possibility that other regions located in the AGP gene promoter may act synergistically with C/EBPs during the HgCl₂-mediated AGP gene induction.

In conclusion, our studies have shown that HgCl₂ treatment causes a post-transcriptional increase of C/EBPβ mRNA in the mouse liver, and subsequent preferential translation of the p35C/EBPβ isoform. We propose that this isoform may be involved in the regulation of AGP gene expression in response to heavy metals, and that there is a significant difference between the LPS-mediated and HgCl₂-mediated inflammatory response.
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