Trabajo 6

Effect of dietary zinc deficiency on brain metallothionein-I and MT-III mRNA levels during stress and inflammation

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Effect of dietary zinc deficiency on brain metallothionein-I and -III mRNA levels during stress and inflammation

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Abstract

Zinc is an essential heavy metal for the normal function of the central nervous system (CNS), but the knowledge of its metabolism and functions is scarce. In this report we have studied the effect of a zinc deficient diet on the regulation of brain metallothioneins (MTs). In situ hybridization analysis revealed that brain MT-I induction by restraint stress was significantly blunted in some but not all brain areas in the mice fed the zinc deficient diet compared to normally fed mice. In contrast, brain MT-I induction by the administration of bacterial lipopolysaccharide (LPS) was not significantly lower in the mice fed the zinc deficient diet. In contrast to MT-I, MT-III mRNA levels were minimally affected by either stress or LPS. Yet, significant decreasing effects of the zinc deficient diet were observed in areas such as the neocortex, CA1–CA3 neuronal layer and dentate gyrus of the hippocampus, and the Purkinje neuronal layer of the cerebellum. These results demonstrate that dietary zinc deficiency impairs the response of brain MTs during both stress and LPS-elicited inflammatory response in a highly specific manner. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Metallothionein I; -II and -III; Stress; Endotoxin; Zinc deficiency

1. Introduction

The transition metal zinc is an essential metal for normal development and function of biological systems, including the central nervous system (CNS) (Choi and Koh, 1998; Cousins, 1985; Cuajungco and Lees, 1997; Frederickson et al., 1984; Vallee, 1988, 1993). In addition to being an essential catalytic or structural element of many proteins, zinc also seems to be important for neural activity and for the control of neuronal death, and indeed this metal has been related to several human neurodegenerative diseases (Choi and Koh, 1998; Cuajungco and Lees, 1997; Frederickson et al., 1984). However, much remains to be understood about its metabolism, roles and association with metal binding proteins in the CNS. Metallothioneins (MTs) are a family of small, cysteine-rich, metal-binding proteins (Kagi and Schaffer, 1988) that could have an essential role in the metabolism of zinc in the CNS. There are four isoforms in rodents; MT-I and MT-II are expressed virtually in all tissues, whereas MT-III (or growth inhibitory factor) and MT-IV are two tissue specific isoforms which are mainly localized in the brain (Choudhuri et al., 1995; Uchida et al., 1991; Palmer et al., 1992) and stratified squamous epithelia (Quaife et al., 1994), respectively.

The intracerebral expression of MT-I+II is clearly upregulated during pathological conditions induced by trauma (Penkowa and Moos, 1995; Penkowa et al., 1999a,c), immobilization stress (Hidalgo et al., 1990), kainic acid-induced seizures (Dalton et al., 1995; Zheng et al., 1995), excitotoxic NMDA cortex damage (Acarin et al., 1999b; Hidalgo et al., 1997b), and administration of 6-aminonicotinamide (Penkowa et al., 1997, 1999b). Furthermore, MT-I+II expression is...
increased in the myelin-deficient jimpy mouse (Vela et al., 1997) and in several human adult neurodegenerative disorders such as Alzheimer’s disease (AD) and Pick’s disease (Duguid et al., 1989; Nakajima and Suzuki, 1995), and amyotrophic lateral sclerosis (ALS) (Sillevis Smitt et al., 1992), as well as in aging (Suzuki et al., 1992) and after brain ischemia (Neal et al., 1996). MT-III was discovered unexpectedly as a factor decreased in AD and initially was named growth inhibitory factor due to its inhibitory effect upon nerve cell growth/survival in vitro (Uchida et al., 1991). However, the down-regulation of MT-III during AD has not been thoroughly confirmed (Amoureux et al., 1997; Carrasco et al., 1999; Erickson et al., 1994). A number of animal models have shown that MT-III mRNA and/or protein levels are significantly altered during CNS damage (Acarin et al., 1999a; Anezaki et al., 1995; Carrasco et al., 1999; Hozumi et al., 1995, 1996; Inuzuka et al., 1996; Penkowa et al., 1999b,c; Yuguchi et al., 1995a,b). Taken together, these studies strongly suggest that MTs are important proteins in the brain for coping with the tissue damage caused by a wide array of factors and diseases. It has been demonstrated indeed that MT-III is important for coping with kainic acid-induced neuronal damage in the CA3 hippocampal field (Erickson et al., 1997), and that MT-I+II are essential for a normal wound healing and neuronal survival after traumatic brain injury (Penkowa et al., 1999a).

The factors involved in the control of the MT genes in these neurodegenerative diseases and traumatic brain injury models are poorly known. However, a number of studies suggest that cytokines could be especially relevant since inflammatory reactions are often associated to those processes (Ghirnikar et al., 1998; Hopkins and Rothwell, 1995; McIntosh et al., 1998; Rothwell and Hopkins, 1995) and several cytokines have been involved in the control of MTs in different tissues (Bell et al., 1987; Cousins and Leinart, 1988; De et al., 1990; Liu et al., 1991; Sato et al., 1992) and cell lines (Bauer et al., 1993; Friedman and Stark, 1985; Karin et al., 1985; Schroeder and Cousins, 1990; Vanguri, 1995). Indeed, we have demonstrated that IL-6 is a major cytokine mediating brain MT-I+II induction by traumatic brain injury (Penkowa et al., 1999c) and by endotoxin-mediated inflammatory response (Carrasco et al., 1998). These studies, however, also indicated that other factors should be involved in brain MT regulation since the response was not completely blunted. Part of the brain MT response to traumatic brain injury could be related to the associated stress response that the animals will likely develop (Hidalgo et al., 1994b, 1997b). Cytokines such as IL-6 and TNF-α are released not only after inflammatory stimuli but also after stressful stimuli (Arck et al., 1995; Lemay et al., 1990; Weinstock et al., 1997; Yamashu et al., 1992; Zhou et al., 1993). However, cytokines seem unlikely candidates for mediating brain MT response to stress (Carrasco et al., 1998; Hernández et al., 1997), while glucocorticoids appear to be involved only in some brain areas (Belloso et al., 1996; Hidalgo et al., 1997a). Since zinc is a major regulator of brain MT-I+II (Ebadi et al., 1995; Hidalgo et al., 1997b) which acts through transcriptional regulatory mechanisms (Bittel et al., 1998; Heuchel et al., 1994; Palmiter, 1994), we have evaluated the putative role of this essential trace metal on MT-I+II and also MT-III responses to both stress and inflammation by feeding mice a zinc deficient diet.

2. Materials and methods

2.1. Experimental procedures

Male Swiss mice were used, which were 3 weeks old at the beginning of the experiment. The animals were maintained under standard laboratory conditions (light cycle from 07:30 to 19:30, temperature 22°C, food and water provided ad libitum) for 1 week before starting the experiments.

The mice were divided randomly into two groups, which were given a semisynthetic diet (Panlab SL, Barcelona, Spain) for up to 38 days consisting of either a normal diet containing 43.3 mg Zn/kg Cu/kg (n = 16), or a zinc-deficient diet containing 1.9 mg Zn/kg (n = 16). These mice were allowed to eat ad libitum. The amount of food of each diet ingested by all mice was controlled every day.

At the end of the feeding period, the two groups were subdivided into (a) those mice which were killed without any further disturbances, (b) those mice which were killed after 5 h of restraint stress, and (c) those mice which were killed 5 h after a i.p. injection of lipopolysaccharide (1 mg/kg). All the mice were killed by cervical dislocation. The brains and livers were immediately frozen in liquid nitrogen and stored at −80°C.

2.2. In situ hybridization

Immediately after the animals were killed, their brains were removed, frozen in liquid nitrogen and stored at −80°C. Serial sagittal sections (20 μm in thickness) were obtained from the frozen brains with a cryostat and mounted on slides coated with poly-L-lysine, which were then maintained at −80°C until the day of analysis. We have analyzed the MT-I and MT-III isoforms. Since MT-I and MT-II are coordinately regulated (Yagle and Palmiter, 1985), we assume that the results described for MT-I are representative of the MT-I+II isoforms.
For MT-I mRNA studies, we used the mouse cDNA generously provided by Dr R. D. Palmiter (University of Washington, Seattle, WA). For MT-III mRNA studies, and in order to avoid cross-hybridization with MT-I and MT-II mRNAs, we have used a specific DNA fragment of 153 bp that contains the coding region for the terminal 15 amino acids and the 3’ untranslated region until the poly G stretch of MT-III mRNA (generously provided by Dr G. K. Andrews, Dept. Biochemistry, Kansas City, Kansas, USA). The procedure has been described previously (Carrasco et al., 1998; Hernández et al., 1997). Autoradiography was performed exposing the film (hyperfilm-MP, Amersham) to the slides for several days. All sections to be compared were prepared simultaneously and exposed to the same autoradiographic film. MT-I or MT-III mRNA levels were semiquantitatively determined in several sections per brain area and animal, by measuring the optical densities and the number of pixels in defined areas with a Leica Q 500 MC system. The MT-I and MT-III mRNA values shown are expressed in arbitrary units (number of pixels × optic density).

2.3. Statistical assays

Results were analyzed with two-way ANOVA, with time and type of diet (body weight gain) and type of diet and treatment (stress or LPS) as main factors.

3. Results

Fig. 1 shows the body weight gain and food intake of the two groups of mice. Body weight gain and food intake were progressively impaired by the zinc deficient diet ($p < 0.001$).

Fig. 2 shows representative in situ hybridization results of all experimental groups for both MT-I and MT-III mRNA. The sense probes produced a very weak signal compared with the antisense MT-I and MT-III probes (not shown). The pattern of hybridization of the two MT probes differed significantly. Thus, in control mice we observed an intense hybridization of the MT-III probe to pyramidal neurons of the hippocampus in the CA1–CA3 regions as well as granule cells of the dentate gyrus (DG), whereas MT-I expression was basically absent in all but the CA3 neurons and was prominent in the lacunomolecular. In the remaining brain areas, the MT-I and MT-III antisense probes produced comparable signals in unstressed mice, with clear hybridization throughout the neocortex, thalamus, hypothalamus, brainstem and cerebellar cortex (especially the Purkinje cell layer). A prominent MT-I and MT-III signal was also observed in the ependymal cells.

As expected, both restraint stress and LPS caused a significant MT-I induction throughout the brain. In contrast, minor effects were observed for the MT-III isoform. The zinc deficient diet blunted the MT-I response to stress but not to LPS. Furthermore, a decreasing effect was also evident in control mice. MT-III mRNA levels also appeared to be decreased by the zinc deficient diet. For a more quantitative approach and statistical evaluation of the results, we carried out MT-I and MT-III mRNA quantitations in defined working fields in representative brain areas (Figs. 3 and 4). Results were analyzed by two-way ANOVA with type of diet and treatment (either stress of LPS) as main factors. The results demonstrate that stress increased significantly ($p < 0.05$) MT-I mRNA levels in all areas analyzed except the CA3 (pyramidal neuron layer) hippocampal area, and that the zinc deficiency blunted that induction significantly ($p < 0.05$) in the CA1–CA3 (pyramidal neuron layer) and lacunomolecular hippocampal areas, and in the neocortex. The same tendency was observed in the other brain areas. In contrast, MT-I induction by endotoxin, which was significant ($p$ at least $< 0.05$) in all areas,
was not decreased by the zinc deficient diet in any of the brain areas analyzed.

The results for MT-III mRNA demonstrated that this isoform behaves differently from MT-I. As stated above, a very prominent signal was observed in the pyramidal neurons of the CA1–CA3 areas and the granule cells of the DG, and, as expected, stress tended to increase mildly the MT-III levels of these areas. In contrast, the administration of LPS did not affect significantly MT-III mRNA levels in all the brain areas analyzed, except the Purkinje cell layer of the hippocampus. Mice fed the zinc deficient diet tended to have decreased MT-III mRNA levels in most cases (see Fig. 4).

4. Discussion

Mice were fed a zinc deficient diet until unequivocal symptoms of zinc deficiency (decreased body weight gain and food intake) were obvious (Bremner et al., 1987; Gasull et al., 1994). This took a considerable length compared to rats fed the same diets (unpublished observations). Given the importance of zinc and MTs for the CNS (see section 1), it seems important to characterize the regulation of these proteins in normal and zinc-deficient conditions in two essential physiological scenarios, stress and inflammation.

It is well known that the MT-I+II isoforms are strongly induced by a number of stressful and inflammatory conditions (Hidalgo et al., 1997b; Sato and Bremner, 1993) and as expected we observed a very significant upregulation of the MT-I expression during both stress and LPS-induced inflammatory response. However, the factors mediating these responses are still a matter of investigation. We have shown previously that glucocorticoids appear to mediate brain MT-I induction in some but not all brain areas (see Hidalgo et al., 1997b for review), indicating that other factors must be involved. By a number of approaches we have observed that the cytokines IL-6, TNF-\(\alpha\) and IFN-\(\gamma\) are unlikely mediators of the response of brain MT-I+I to stress (Carrasco et al., 1998; Hernández et al., 1997). In this report, we evaluated that response in
mice fed a zinc deficient diet, and found that zinc deficiency blunted MT-I upregulation throughout the brain. This suggests that zinc mediates the response of this MT isoform (and presumably that of MT-II, since both are regulated coordinately (Yagle and Palmiter, 1985)) to stress. It is well known that zinc is a primary inducer of the MT-I+II genes (Bittel et al., 1998; Heuchel et al., 1994; Palmiter, 1994; Yagle and Palmiter, 1985), that this upregulation may occur in neurons, astrocytes and microglia (Agullo et al., 1998; Hidalgo et al., 1994a; Kramer et al., 1996a,b), and that zinc is released by brain cells not only during normal brain activity but also in response to restraint stress (Assaf and Chung, 1984; Itho et al., 1993). Furthermore, intracerebral administration of zinc clearly increases MT-I+II synthesis, a process apparently driven by increased cellular zinc uptake (Gasull et al., 1994; Paliwall et al., 1990). Taken together, the results indicate that zinc is an important MT-I+II inducer in the brain in basal and stress conditions.

In contrast to during stress, during the inflammatory response elicited by LPS no decreasing effect of the zinc deficiency was observed in any of the brain areas analyzed. Moreover, a tendency to potentiate MT-I induction was observed in the CA1 neuronal layer of the hippocampus, although that was not statistically significant. Therefore, the results indicate that it is unlikely that zinc is an essential factor in brain MT-I+II induction by LPS. A number of studies have suggested that cytokines are the likely candidates for mediating MT-I+II regulation in peripheral tissues (De et al., 1990; Liu et al., 1991), and indeed previous results in IL-6 and TNF-R1 deficient mice demonstrated that these cytokines are actually important in that process in the brain (Carrasco et al., 1998).

The research on brain MT gained great importance since the discovery of the brain specific isoform, MT-III (or Growth Inhibitory Factor), and its putative relationship to Alzheimer disease (Uchida et al., 1991). However, as stated above such relationship has been challenged, but nevertheless recent studies have demonstrated an important role of MT-III as neuroprotective during kainic acid brain damage (Erickson et al., 1997). In agreement with previous studies (Carrasco et al., 1998; Choudhuri et al., 1995; Dalton et al., 1995; Palmiter et al., 1992; Zheng et al., 1995), the
results demonstrate that MT-III remains mostly unaffected in comparison with MT-I. Nevertheless, stress slightly but consistently increased MT-III mRNA levels in the CA1–CA3 neuronal fields of the hippocampus and in the Purkinje cell layer of the cerebellum, which is consistent with a previous study (Carrasco et al., 1998). Also in agreement with this study, LPS tended to increase MT-III mRNA levels only in the Purkinje cell layer of the cerebellum. The effect of feeding a zinc deficient diet on brain MT-III mRNA levels has not been evaluated previously. A general tendency to decrease MT-III mRNA levels was observed, especially in the neurons of the CA1–CA3 and the DG, the richest region regarding MT-III expression. This decreasing tendency was obvious in control mice, but it was more important during stress and the LPS-induced inflammation, suggesting complex interactions between zinc levels and the factors elicited by those scenarios. The mechanisms underlying these changes are unknown. Studies carried out in vitro with neurons and astrocytes indicated that zinc decreased slightly (in neurons) and had no effect (in astrocytes) on MT-III mRNA levels (Kramer et al., 1996a,b), which again suggests interactions of the zinc deficiency with other factors rather than direct effects on MT-III expression.

In summary, the present results demonstrate that in vivo zinc deficiency affects substantially the response of MT-I+II to stress but not to inflammation, while that of MT-III is affected in both cases. This suggests that attention must be paid to the zinc status of the animals in CNS studies given the neurobiological importance of brain MTs.

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References


Trabajo 7

Identification of a signal transducer and activation of transcription (STAT) binding site in the mouse metallothionein-I promoter involved in interleukin-6-induced gene expression

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Identification of a signal transducer and activator of transcription (STAT) binding site in the mouse metallothionein-I promoter involved in interleukin-6-induced gene expression

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Mechanisms of regulation of mouse metallothionein (MT)-I gene expression in response to bacterial endotoxin-lipopolysaccharide (LPS) were examined. Northern blot analysis of hepatic MT-I mRNA in interleukin (IL)-6 or tumour necrosis factor (TNF)-receptor type I knock-out mice demonstrated that IL-6, not TNF-α, is of central importance in mediating hepatic MT-I gene expression in vivo after LPS injection. In vivo genomic footprinting of the MT-I promoter demonstrated a rapid increase, after LPS injection, in the protection of several guanine residues in the −250 to −300 bp region of the MT-I promoter. The protected bases were within sequences which resemble binding sites for the signal transducers and activators of transcription (STAT) transcription factor family. Electrophoretic mobility-shift assays using oligonucleotides from footprinted MT-I promoter regions showed that injection of LPS resulted in a rapid increase in the expression of several high-affinity, in vitro binding of STAT1 and STAT3 to a binding site at −297 bp (TTTCCGTAA).

INTRODUCTION

The acute-phase response is elicited in the host in response to infection, tissue injury or inflammation [1]. One of the major components of this response is the coordinate stimulation of hepatic synthesis of the acute-phase plasma proteins (APPs) [2]. Interleukin-6 (IL-6) is considered to be the principal regulator of most APP genes, but other cytokines including IL-1 and tumour necrosis factor-α (TNF-α) also contribute to stimulation of type-I APP genes. Glucocorticoids also participate, in a poorly defined way, in the induction of several APPs [1].

The Jak/STAT and the mitogen-activated protein kinase signal transduction pathways play an important role in mediating transcriptional activation by extracellular signals from cytokines and growth factors [3]. The binding of IL-6 to its receptor complex leads to phosphorylation of Jak kinases and subsequent dimerization and nuclear translocation of STAT proteins. In particular, it is thought that STAT3 plays a central role in the response to IL-6 [4–6]. STAT3 binding to specific sequences in the promoter regions of cytokine-responsive genes leads to activation of transcription [7,8]. The interaction of STAT3 with cis-acting promoter elements has been shown to participate in the activation of several APP genes, such as α₂-macroglobulin (α₂MG); [9–11], γ-fibrinogen [12] and C-reactive protein [13].

In addition to the activation of expression of hepatic APPs, inflammation also leads to increased expression of several predominantly intracellular proteins, including haem oxygenase I (HO-I) [14], manganese superoxide dismutase [15] and the metallothioneins (MTs). MTs are cysteine-rich, heavy-metal-binding proteins that have been highly conserved during evolution [16]. Of the four mouse MT genes, the MT-I and MT-II genes are the most widely expressed. These genes are constitutively expressed in the liver and are highly induced in response to a variety of stresses including inflammation [17]. Bacterial endotoxin [lipopolysaccharide (LPS)] is a potent inducer of hepatic MT gene expression [18,19]. Studies using LPS-resistant C3H/HeJ mice suggest that LPS indirectly affects MT gene expression in the liver by activation of inflammatory cytokines [18]. Injection of a variety of cytokines, such as IL-1, IL-6, TNF-α and interferon (IFN), can induce hepatic MT gene expression in vivo [18,20–22]. IL-6 has been suggested to be a direct mediator of hepatic MT gene expression in mice [18,22–24]. Studies of rat hepatocytes cultured in vitro support the concept that IL-6, in combination with zinc and glucocorticoids, directly mediates MT gene expression during inflammation [25,26]. However, a direct role for IL-6 in mediating MT-I gene expression in vivo has not been directly demonstrated.

Studies of the mouse MT-I promoter in transgenic mice indicate that the LPS-responsive region is located between −185 bp and −350 bp of the proximal promoter [19], but the transcription factors involved have yet to be identified. The present study used IL-6 and TNF-α receptor type 1 knockout mice to demonstrate that IL-6 plays a key role in LPS induction

Abbreviations used: APPs, acute-phase plasma proteins; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; /Gal, β-galactosidase; HO-I, haem oxygenase I; IFN, interferon; IL, interleukin; LM-PCR, ligation-mediated PCR; LPS, lipopolysaccharide; α₂MG, α₂-macroglobulin; MRE, metal regulatory elements; MT, metallothionein; NP40, Nonidet P40; SAA, serum amyloid A; TNF, tumour necrosis factor; USF/ARE, composite upstream stimulating factor/antioxidant response element.

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of hepatic MT-I gene expression. Analyses of protein interactions in vivo and in vitro with the MT-I promoter suggest that LPS induces activation of STAT1 and STAT3 binding to a site at –297 bp, and transfection studies demonstrated that this site is IL-6 responsive. Therefore, regulation of the mouse MT-I gene during inflammation is likely to be mediated, in part, by the same signal transduction cascades that regulate expression of genes encoding APPs.

**EXPERIMENTAL**

**Materials and methods**

Tissue collection, RNA isolation and Northern blot analysis

All experiments involving animals were conducted in accordance with National Institutes of Health guidelines for the care and use of experimental animals. Two- to three-month old C57BL/6, 129/Sv and F1 (C57BL/6 x 129/Sv) wild-type (non-transgenic) and homozgyous IL-6 (IL-6−/−) knockout mice [27] and homozgyous IL-6 (IL-6−/−) knockout mice [28] were injected with LPS (0.5 mg/ml, *Escherichia coli* serotype 0127:B8; Sigma Chemical Co, St. Louis, MO, U.S.A.) intraperitoneally at a dosage of 100 µg/mouse. Livers were collected at 0, 1, 3.5 and 8 h after treatment and frozen immediately in liquid nitrogen. RNA was extracted from frozen liver as described previously [29], separated by 2.2 M formaldehyde (%), agarose gel electrophoresis, transferred to Nytran membranes (Schleicher and Schuell, Keene, NJ, U.S.A.) and cross-linked by UV irradiation as described [29,30]. Blots were prehybridized, hybridized and washed as described [18,30]. Blots were hybridized successively with 32P-labelled cRNA probes for mouse MT-I [18], HO-I [29] and serum amyloid A (SAA) [31]. Membranes were stripped of probe before each rehybridization, as described [18]. Hybrids were detected by autoradiography at –70 °C with intensifying screens. The blot was further exposed to a phosphor screen, scanned with a PhosphorImager SI, and quantified using the ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA, U.S.A.). In all experiments, duplicate gels were stained with Acridine Orange to verify integrity and equal loading of RNA.

**In vivo footprinting**

Nuclei were prepared from freshly collected pieces of liver by homogenization, with a glass–Teflon homogenizer, in 50 vols. (v/w) of homogenization buffer [10 mM Hepes, pH 7.9/10 mM KCl/1.5 mM MgCl2/0.1 % NP40/1 mM DTT/20 mM NaF/1 mM EDTA/1 mM EGTA/1 mM NaVO4/0.5 mM PMSF/1 µg/ml pepstatin A/1 µg/ml leupeptin/1 µg/ml aprotinin]. Nuclei were collected by centrifugation at 800 g (Sorvall, HB-4) for 5 min at 4 °C, and washed once with homogenization buffer without NP40. The nuclei were suspended in 3 vols. of nuclear extraction buffer [20 mM Hepes, pH 7.9/420 mM KCl/1.5 mM MgCl2/25 % (w/v) glycerol/1 mM DTT/20 mM NaF/1 mM EDTA/1 mM EGTA/1 mM NaVO4/0.5 mM PMSF/1 µg/ml pepstatin A/1 µg/ml leupeptin/1 µg/ml aprotinin]. The nuclear suspension was stirred for 30 min on ice, and then centrifuged at 100000 g (Beckman, TL-100) for 30 min at 4 °C. The supernatant was collected and concentrated in a Microcon3 microconcentrator (Amicon Inc., Beverly, MA, U.S.A.) by centrifugation at 25000 g (Sorvall, SS-34) for 3 h at 4 °C. The concentrated extract was diluted with an equal volume of dilution buffer (extraction buffer without KCl) and frozen in aliquots at –80 °C. The concentration of nuclear proteins was determined using the Bradford method with BSA as a standard (Bio-Rad, Hercules, CA, U.S.A.).

Electrophoretic mobility-shift assay (EMSA)

STAT binding activity was detected as follows. Hepatic nuclear proteins (5 µg) were incubated in binding reaction buffer containing 12 mM Hepes (pH 7.9), 60 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 12 % (w/v) glycerol, 5 mM MgCl2, 2 µg of poly(dI)-poly(dC), 0.5 mM PMSF and 2–3 fmol of end-labelled double-stranded oligonucleotide (5000 c.p.m./fmol) in a total volume of 20 µl for 20 min at room temperature. MT-I promoter oligonucleotides and the rat zMG promoter functional STAT-binding site [10] oligonucleotide sequences were as follows:

**MT-I**/–297  5’GATCGAGTTCTCGTAAACTC3’

**rat zMG**  5’GATCCTTCTGGAATTCCTA3’

**MT-I**/–277  5’GATCGCGATAGGCCGTAATATCGGGGAAAGCCTA3’

In binding-site competition experiments, a 100-fold molar excess of unlabelled double-stranded oligonucleotide was included in the EMSA binding reaction. In antibody supershift assays, the indicated antiserum (1 µl) was added to the binding reaction followed by addition of the labelled oligonucleotide. The entire mixture was incubated for 30 min at room temperature. The antibodies against Sp1 (sc-059x), STAT1 (sc-346x), STAT3 (sc-482x), STAT5b (sc-835x) and glucocorticoid receptor (sc-1002x) were purchased from Santa Cruz Biochemicals (Santa Cruz, CA, U.S.A.). The EMSA gel was polymerized and then pre-run at 4 °C in buffer (pH 8.5) consisting of 25 mM Tris, 0.19 M glycine and 0.5 mM EDTA. Protein–DNA complexes were separated electrophoretically in a 4 % polyacrylamide gel at 15 V/cm at 4 °C. After electrophoresis, the gel was dried and labelled complexes were detected by autoradiography.

**Western blotting**

Equal amounts (5 µg) of nuclear extract were subjected to SDS/10 % PAGE with the discontinuous buffer formulation of Laemmli [36] and transferred to nitrocellulose membranes (Schleicher and Schuell), using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Membranes were incubated in a blocking solution of 5 % non-fat dried milk in TBST buffer (10 mM Tris/HCl, pH 7.4/150 mM NaCl/0.5 % Tween-20) overnight at 4 °C. The membrane was incubated with antiserum in the same blocking solution for 1 h at room temperature and then washed in TBST buffer. The membrane was then incubated in TBST containing goat anti-rabbit immunoglobulin conjugated
with horseradish peroxidase for 1 h at room temperature and washed with TBST buffer. Specific protein complexes were visualized with the enhanced chemiluminescence (ECL) system (Amersham Life Sciences, Arlington Heights, IL, U.S.A.).

**Luciferase reporter plasmids**

Two tandem copies of the MT-I/−297 oligonucleotide, two tandem copies of the rat zMG oligonucleotide or a single copy of an oligonucleotide spanning the −300 bp to −251 bp region of the MT-I promoter were subcloned in front of the MT-I minimal promoter (−42 to +62) [29] in the luciferase reporter (Luc) vector, pGL-2 basic (Promega Biotech, Madison, WI, U.S.A.). The TATA box and transcription start point were provided by the MT promoter in these Luc fusion genes. Oligonucleotides were synthesized by the Biotechnology Support Center (University of Kansas Medical Center, Kansas City, KS, U.S.A.).

**Transient transfection assay**

Human HepG2 cells were obtained from the American Type Culture Collection (Rockville Pike, MD, U.S.A.). HepG2 cells were cultured in complete medium (Dulbecco’s modified Eagle’s medium)-rich glucose supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine and 100 units/ml penicillin/100 µg/ml streptomycin. Cells were seeded at a density of 70000 per well in 24-well plates and incubated for 36 h at 37 °C in a humid atmosphere of 5% CO2 in air. Cells were transfected using LipofectAMINE reagent according to the manufacturer’s suggestions (Life Technologies, GIBCO BRL, Gaithersburg, MD, U.S.A.). Each well was incubated for 5 h at 37 °C in 0.25 ml of Dulbecco’s modified Eagle’s medium containing 2 µl of LipofectAMINE (2 mg/ml), 200 ng of SV-βGal (Promega Biotech) transfection control plasmid and 300 ng of Luc reporter plasmid. Dulbecco’s modified Eagle’s medium containing 20% fetal bovine serum (0.25 ml) was added to each well and the incubation was continued for 18 h. This medium was replaced with 0.5 ml of complete medium and, after 8 h, human recombinant IL-6 (Preprotech Inc., Rockhill, NJ, U.S.A.) was added (2 ng/ml or 20 ng/ml). The cells were treated with IL-6 for 18 h and then lysed and assayed for Luc and βGal activities using the luminescence assays, as described [37]. βGal activity was used to correct for transfection efficiency. Each experimental condition tested was replicated six times within each experiment, and the entire experiment was repeated at least twice. Statistical significance was determined using analysis of variance and Student’s t-test. Differences were considered significant when the P value was < 0.001. Values are given as means ± S.E.M.

**RESULTS**

**LPS-induced hepatic MT-I gene expression is severely attenuated in IL-6−/− mice, but not in TNFR1−/− mice**

The effects of LPS injection on hepatic MT-I mRNA levels was examined in non-transgenic mice and in IL-6−/− and TNFR1−/− mice. In non-transgenic mice, a dramatic (> 60-fold) induction of MT-I mRNA was noted at 8 h after injection. Similar results were obtained using three strains of mice as wild-type (non-transgenic) controls: C57BL/6, 129/Sv and F1. C57BL/6 × 129Sv F1 mice served as non-transgenic controls in the experiments shown (Figure 1).

In the IL-6−/− mice, LPS induction of MT-I mRNA was attenuated to only 14% of that found in the non-transgenic mice at 8 h (Figure 1A). In sharp contrast, the time-course and extent of induction of MT-I mRNA in TNFR1−/− mice was indistinguishable from that in the non-transgenic controls (Figure 1A). Changes in SAA mRNA were monitored as a positive control for the acute-phase response [38]. In non-transgenic mice, a dramatic induction (> 80-fold) of hepatic SAA mRNA was noted by 8 h after LPS injection. LPS induction of this mRNA was severely attenuated in both the IL-6−/− and the TNFR1−/− mice and the effect of IL-6 deficiency (24% of non-
Liver was collected from CD-1 mice at the indicated time (0–4 h) after an injection of LPS (100 μg/mouse). Nuclei were isolated and treated with 0.1% dimethyl sulphate to methylate guanine residues, as described in the Materials and methods section. Methylated genomic DNA was extracted, cleaved with piperidine and the proximal region of the MT-I promoter was specific amplified using LM-PCR. The G-ladder (G) was generated by LM-PCR of naked genomic DNA methylated in vitro. LM-PCR products were separated on a 6% sequencing gel and detected by autoradiography. Guanine residues are numbered relative to the transcription start point. The locations of known regulatory elements in the MT-I promoter are as indicated: MRE, metal regulatory elements (a–e); Sp1, Sp1 binding site; USF/ARE, composite upstream stimulating factor/antioxidant response element (USF/ARE), Sp1 and E-box sites (Figures 2A and 2B). Similar results have been reported previously in cultured mouse cell lines [34,40]. In vivo footprinting was not noted over the metal regulatory elements (MRE) core sequences in control liver, nor in the putative LPS-responsive region upstream of the E-box (–225 bp) (Figure 2A). However, by 1 h after LPS injection several new footprints were noted. Guanine –292 was protected. Similarly, guanines in a four guanine cluster at –256 to –259, and guanine –267 were protected. These guanines remained protected for at least 4 h after LPS injection. In contrast, a weak, transient footprint was noted over MRE-d. Guanine –146, in the MRE core sequence, was protected up to 1 h after LPS, but not thereafter (Figure 2B). No footprints were detected over other MRE sequences (Figures 2B and 2C).

Within the more proximal MT-I promoter, after LPS injection, guanine –32 became strikingly hypersensitive (Figure 2C) and remained so for up to 4 h. This base is immediately upstream of the TATA box (Figure 2A). In addition, a change in the constitutive footprint over the USF/ARE was noted by an apparent increase in the hypersensitivity of guanine –101 and by increased protection of guanine –94. Metals and oxidative stresses also cause changes in the in vivo footprint around the USF/ARE [34].

Identification of a STAT-binding site at –297 bp in the MT-I promoter and of LPS-dependent in vitro binding of STAT1 and STAT3

Analysis of the MT-I promoter region from –250 bp to –300 bp, around those bases in vivo footprinted after LPS injection (Figure 2), revealed three STAT-like binding sites. The consensus STAT sequence (TTN,AA) is present beginning at –297 bp and this sequence (TTTCGATA) is similar to the IL-6-responsive STAT-binding site (TTCTGGGAA) in the rat aMG promoter [10]. Two tandem STAT-like binding sites were located at –272 bp and –262 bp. Although neither conforms to the STAT-binding consensus sequence, the last five bases of the –272 bp site are identical with those of the –297 bp site (CGTAA), and seven bases of the –262 bp site (ATCGGGGA, indicated in bold) are identical with those in the aMG site.

EMSA was used to detect protein interactions with oligonucleotides containing the –297 bp site (MT-I/-297) or an oligonucleotide spanning the –272 bp and –262 bp sites (MT-I/-277) (see below). Hepatic nuclear protein was extracted from liver taken after LPS injection. Two MT-I/-297 binding complexes (I and II; Figure 3A) were detected by 1 h and remained until 4 h after LPS injection. The amount of complex I increased between 1 h and 4 h, whereas the amount of complex II was apparently maximal by 1 h and declined by 4 h. Formation of both binding complexes was effectively inhibited by a 100-fold molar excess of unlabelled MT-I/-297 oligonucleotide, as well as by a 100-fold molar excess of unlabelled rat aMG oligonucleotide (Figure 3B). Supershift analysis using antisera specific for STAT1 and STAT3 showed that complex I contained STAT1 and complex II contained STAT3 (Figure 3B). Neither complex was supershifted using antisera against STAT5, Sp1 or glucocorticoid receptor.

protein–DNA interactions were visualized as either less intense (protected) or more intense (hypersensitive) relative to DNA from control mice and relative to surrounding bases within the same region of the gel. Genomic footprinting was repeated at least three times using DNA prepared from separate experiments and the results were reproducible (Figures 2B and 2C).

In control mouse liver, constitutive footprints were evident over the composite upstream stimulating factor/antioxidant response element (USF/ARE), Sp1 and E-box sites (Figures 2A and 2B).
Interleukin-6 induces STAT activation of metallothionein

EMSA was performed using hepatic nuclear extracts prepared at the indicated times after LPS injection (100 μg/mouse). Nuclear protein (5 μg) was incubated with the 32P-labelled double-stranded MT-I/−297 oligonucleotide (A and B) or the labelled rat α2MG oligonucleotide (C) for 20 min at room temperature, as described in the Materials and methods section. Protein–DNA complexes were analysed by 4% polyacrylamide-Tris/glycine gel electrophoresis and detected by autoradiography. P, free probe. The arrows indicate specific binding complexes (I and II). (B) Nuclear extract was prepared 2 h after LPS injection. Co refers to EMSA binding reactions which contained the indicated unlabelled double-stranded oligonucleotide as a competitor. Ab refers to EMSA supershift reactions which contained the indicated STAT antisera. No STAT supershift was detected and competition with excess unlabelled oligonucleotide was incomplete.

LPS-induced in vitro binding activity for the −277 bp region of MT-I promoter does not involve STAT proteins

The two tandem STAT-like binding sites, located at −272 bp and −262 bp, were footprinted in vivo after LPS treatment, although neither site conforms to the STAT-binding consensus sequence. EMSA revealed two relatively faint complexes with the MT-I/−277 oligonucleotide using nuclear extracts from liver (Figure 4A). One of these complexes was transiently induced after LPS injection, and reached a peak at 1 h, after which it decreased. Competition experiments demonstrated that formation of this binding complex was completely inhibited by a 100-fold molar excess of the indicated unlabelled double-stranded oligonucleotide as a competitor. Ab refers to EMSA supershift reactions which contained the indicated STAT antisera. No STAT supershift was detected and competition with excess unlabelled oligonucleotide was incomplete.

The STAT binding site at −297-bp in the MT-I promoter directs the response to IL-6 in transiently transfected HepG2 cells

Transient transfection assays were used to determine whether the −297 bp STAT-binding site (MT-I/−297) or the −300 bp to −251 bp region of the MT-I promoter represent functional IL-6-responsive promoter elements. Human HepG2 cells were transfected in these experiments. This cell line has been used extensively to study IL-6 regulation of acute-phase protein gene expression [2,10,13]. Two tandem copies of the MT-I/−297 oligonucleotide or the rat α2MG oligonucleotide were cloned in front of the minimal MT-I (−42 bp) promoter in a Luciferase expression vector. These constructs were compared in transfection assays, since it is known the rat α2MG oligonucleotide is IL-6 responsive under these conditions [2]. A single copy of the −300 bp to −251 bp oligonucleotide was also cloned into this vector and
200 ng of SV-in this reporter construct. HepG2 cells were transfected using LipofectAMINE reagent containing for Luc and plasmid. Transfected cells were treated for 18 h with human recombinant IL-6 at 2 ng/ml or addition, a single copy of the MT-I promoter region from 0.001 relative to untreated. **

![Diagram](image)

**Figure 5** The STAT binding site at −297 bp in the mouse MT-I promoter directs the response to IL-6 in transiently transfected HepG2 cells

The MT-I −297 and the rat zMG were each cloned as two tandem copies in the forward orientation in front of a minimal (−42 bp) MT-I promoter [29] driving Luc expression. In addition, a single copy of the MT-I promoter region from −300 to −251 bp was cloned in this reporter construct. HepG2 cells were transfected using LipofectAMINE reagent containing 200 ng of SV-βGal transfection control plasmid and 300 ng of the indicated Luc reporter plasmid. Transfected cells were treated for 18 h with human recombinant IL-6 at 2 ng/ml or 20 ng/ml, as indicated. The cells were treated with IL-6 for 18 h and then lysed and assayed for Luc and βGal activities. (A) Luc activity was normalized to βGal activity to correct for transfection efficiency and the values shown are means ± S.E.M. of six determinations. *P value was < 0.001 relative to untreated. **P value was 0.0013 relative to untreated. (B) Data from (A) are expressed as fold induction in the IL-6-treated samples relative to the untreated control for each of the Luc reporter constructs.

examined in these assays. HepG2 cells were transfected and then treated overnight with human recombinant IL-6 (Figure 5). Under these conditions, none of these promoter constructs had an increased basal level of expression relative to the −42 bp promoter (Figure 5A). However, each of the oligonucleotides examined conferred IL-6 responsiveness on the −42 bp promoter. The response was dose dependent, was detectable at an IL-6 concentration of 2 ng/ml and was near maximal at 20 ng/ml (higher concentrations are not shown). Luciferase expression driven by the MT-I/−297 and the rat zMG oligonucleotides was increased 3.9- and 2.4-fold respectively. In repeated experiments, the −300 to −251 promoter element was responsive to IL-6, whereas the −42 bp promoter was not. These results suggest that IL-6 responsiveness of the MT-I promoter involves the −300 bp to −251 bp region which contains a STAT binding site.

**DISCUSSION**

Expression of mouse MT genes is upregulated in response to a variety of stresses including inflammation [17,41–43]. The observation that hepatic expression of MT is dramatically elevated in response to bacterial infection, an effect mediated by endotoxin (LPS) [41], has led to the classification of MT as an acute-phase protein. This effect of LPS on hepatic MT gene expression has been conserved during evolution [44], which suggests a fundamental physiological role of MT in the acute-phase response. Results presented herein suggest that IL-6 is a direct mediator of hepatic MT gene expression in mice exposed to endotoxin, as it is for the expression of many APPs [1]. Induction of SAA, haptoglobin, z1-acid glycoprotein, z2MG and serum amyloid P is dramatically decreased in IL-6−/− mice during acute-phase responses [27,45,46]. Therefore, the effects of other cytokines on hepatic MT gene expression, reported previously [18,21,22,47,48], may be attributable to IL-6. Consistent with this concept, IL-6 has little effect on IL-1 or TNF-z gene expression in the mouse liver. In contrast, IL-1 or TNF-z can rapidly induce IL-6 mRNA in the liver [18]. Recent studies demonstrate that antisera against IL-6 can block the ability of serum from LPS-treated mice to induce expression of MT in a hepatoma cell line [24], and IL-6 has been shown to induce MT gene expression in rat hepatocyte cultures [25,26]. Remarkably, IL-6 has also been suggested to play a key role in hepatic MT gene expression during inflammation caused by organic solvents [23] and by immobilization stress (J. Carrasco, J. Hernandez, H. Bluethmann, G. K. Andrews and J. Hidalgo, unpublished work).

Multiple promoter elements may be involved in LPS-induced MT-I gene expression. In the mouse liver and in mouse Hepa cells, MT-I is expressed constitutively and it appears that constitutive interactions of Sp1-, USF- (at two sites) and ARE-binding proteins occur within the proximal 250 bp of the MT-I promoter [34,49]. In addition to those protein–DNA interactions, LPS apparently induces the binding of STAT proteins (1 and 3) to a binding site at −297 bp. Furthermore, changes in protein–DNA interactions occur downstream of the STAT-binding site, near the TATA box and at the USF/ARE. Thus, efficient induction of MT-I gene transcription during inflammation is likely to involve the cooperation of multiple promoter elements.

The functionality of the MT-I promoter STAT site was demonstrated in transiently transfected cells. This site maps within a region of the MT-I promoter that is important for LPS responsiveness in vivo [19], and LPS induces STAT1 and STAT3 binding to this site, as suggested by increased in vitro binding and increased in vivo protein interactions at this site. Furthermore, STAT1 and STAT3 binding in vitro to the MT-I STAT-binding site was qualitatively similar to that with the rat zMG IL-6-responsive element (STAT-binding site) [10,11]. These results suggest that the Jak/STAT signal-transduction cascade potentiates transcription of the MT-I gene, as it does the APP genes [27]. The individual roles that activated STAT1 and/or STAT3 may play in regulating MT-I gene expression remain to be determined. STAT1 and STAT3 are activated by a variety of growth factors and cytokines, including IL-6 [7,8,50]. However, studies of STAT1 knockout mice demonstrate that STAT1 functions to regulate genes that provide innate immunity [51]. Thus, loss of STAT1 activity attenuates the response to IFN, but not to several other cytokines [8,51]. Whether STAT1 is important for IL-6 activation of MT-I gene expression has not been demonstrated. However, LPS induction of the mouse MT-I gene is not attenuated in IFN-γ receptor knockout mice [52]. STAT3 is expressed in many cell-types [53], and is activated by the IL-6 family of cytokines [4,7,10,54]. STAT3 is also activated in the liver in response to LPS [50]. Targeted disruption of the mouse STAT3 gene is lethal to the embryo, but over-expression of dominant negative mutants of STAT3 block IL-6 activation of murine erythroblastic cells [5,55]. Therefore, STAT3 plays an important role in transcriptional activation of IL-6-responsive genes.
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