Universitat Autònoma de Barcelona

Regulació de les metal.lotioneïnes hepàtiques i cerebrals

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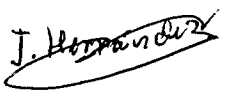

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IL-6 AND TNF RECEPTOR 1 DEFICIENT MICE REVEAL ESSENTIAL PHYSIOLOGICAL ROLES OF IL-6 AND TNF-α IN LIVER METALLOTHIONEIN REGULATION[1]

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ABSTRACT

Metallothioneins are a family of low molecular weight proteins, which in rodents is comprised of at least four isoforms (MT-I to -IV). MT-I and MT-II are widely expressed isoforms which have been shown to function in zinc and copper metabolism and against oxidative stress, and in the liver they are strongly up-regulated by stress through unknown mechanisms. In this report we have studied the putative role of IL-6 and TNF-α in the hepatic MT response to a basically psychogenic stress model-immobilization stress, by using mice carrying a null mutation in either the IL-6 gene (IL-$6^{-/-}$) or the TNF-α type 1 receptor (TNFR$1^{-/-}$) or both (IL-$6^{-/-}$xTNFR$1^{-/-}$). For comparison, the response to bacterial lypopolysaccharide (endotoxin) and turpentine was also studied. Liver MT response to stress of IL-$6^{-/-}$ mice was significantly lower than that of the two parental mouse strains used to generate the IL-$6^{-/-}$ mice, namely C57BL/6 and 129/Sv mice, and that of the F_1 C57BL/6x129/Sv offspring mice. Liver MT-I+II protein levels were in accordance with MT-I mRNA changes, suggesting a transcriptional level of regulation for both stress and IL-6. The functional TNF-α deficiency, in contrast, did not affect liver MT response to stress. The effect of IL-6 deficiency on hepatic MT response to endotoxin and turpentine was more dramatic that for stress, and in this case TNF-α appeared to contribute albeit modestly. Thus, the present results demonstrate that IL-6 and, to a lower extent, TNF-α, are major regulators of the liver MT isoforms in physiological conditions. Furthermore, the results suggest that IL-6 is an important component not only of the normal response to an immunological insult but also of the physiological response of the organism to stress.

INTRODUCTION

MTs are a family of small, cysteine-rich, metal-binding proteins (1, 2). There are several isoforms in mammals; 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 localized in the brain and stratified squamous epithelia, respectively (3-5). The biochemical and molecular properties of the MT-I and MT-II isoforms are well documented, but their physiological roles and regulation in physiological conditions are poorly characterized.

Stress is a major physiological insult to which animals respond with a plethora of mechanisms. It is well known that stress increases MT-I+II production in several tissues but especially in the liver (6-10), suggesting that these isoforms are involved in the physiological adaptation of the organism to stress. The factors implicated in the control of MT-I+II during stress are not clear. Since the activity of the hypothalamic-pituitary-adrenal (HPA) axis is increased by stress, it has generally been assumed that glucocorticoids could mediate liver MT response to stress. However, the experimental results in rats do not support such a mediating role (7). Furthermore, the role of the sympathomedulloadrenal system is also unclear (9) as it is that of endogenous opioids (10). Therefore, factors other than the prototypical stress hormones should participate in the control of liver MT during stress. A putative set of factors that could be involved is that of cytokines, a group of essential mediators of the immune system but also of other physiological systems (11-18). MT induction by the administration of cytokines such as IL-1α/β, IL-6, TNF-α and IFN-γ has repeatedly been demonstrated (19-26). However, the only evidence that these and/or other cytokines have a role in MT regulation in physiological conditions is suggested by the fact that the liver MT induction by endotoxin is attenuated in the

C3H/HeJ strain (20, 21). Indeed, these proteins have tentatively been considered acute-phase proteins (26), but this has not been thoroughly demonstrated.

It has been reported that circulating TNF-α and especially IL-6 levels are strongly increased by stress (27-31). Since these cytokines have been regarded as major regulators of the MT genes specifically in the liver (20), it seems plausible that the hepatic MT response to stress could be mediated by IL-6 and/or TNF-α. The present report demonstrates by employing knock-out mice carrying a null mutation in the IL-6 or the TNF-α type 1 receptor genes (32, 33) that IL-6 but not TNF-α is involved in the control of liver MT synthesis during immobilization stress, which suggests that this cytokine is not only important during an immunological insult but also during the adaptation to stress. The present results also demonstrate that IL-6 is the major cytokine responsible for liver MT induction by the inflammatory response and suggest that liver MTs could be considered acute-phase proteins.

MATERIALS AND METHODS

Production of IL-6 and TNF-R1 deficient mice. Generation and development of the IL-6 (IL-6$^{-/-}$) and TNF-α type 1 receptor (TNF-R1$^{-/-}$) deficient mice was as described previously (32, 33). We used as controls the wild-type strains, C57BL/6 (Jackson, Germany) and 129/Sv, and the F_1 mice C57BL/6x129/Sv (provided by Biological Research Lab. Ltd., Basel, Switzerland).

Maintenance of the animals. The animals were housed individually and maintained under standard laboratory conditions (light cycle from 07:30 to 19:30, temperature 22°C, food and water provided *ad libitum*) for at least one week before starting the experiments.

Experiment 1. In this first experiment we studied the effect of IL-6 deficiency on liver MT induction by immobilization stress using only C57BL/6 mice as control animals. To this end, the mice subjected to stress were immobilized in the morning by wrapping them in a metallic net, whereas the basal mice remained undisturbed in the animal room. Basal and stressed mice were killed together 4 hours after the onset of stress. Only MT-I mRNA levels were measured in these animals. Since the regulation of the MT-I and MT-II genes is coordinated (34), we assume that the results for MT-I are representative of both isoforms, and indeed when the MT-I+II proteins were measured (see below) that assumption was confirmed.

Experiment 2. In order to rule out effects related to the genetic background of the knock-out mice, in this experiment we used C57BL/6, 129/Sv and C57BL/6x129/Sv mice as controls. Furthermore, to verify that the MT-I mRNA changes observed have physiological significance, the MT-I+II protein levels were

also measured. Thus, animals subjected to stress were immobilized by wrapping them in a metallic net, and were killed after 4 or 18 hours of stress along basal, unstressed mice. MT-I mRNA levels were measured in the basal and 4-hour stressed groups, whereas the MT-I+II protein levels were measured in the basal and 18-hour stressed groups to allow induced protein synthesis to occur.

Experiment 3. Since IL-6 and TNF-α are related cytokines which have some overlapping functions (35), and both cytokines have been shown to induce liver MT synthesis, it might be hypothesized that liver MT response to stress was not completely eliminated in IL-6 deficient mice because of a compensatory effect of TNF-α. To test this hypothesis, an attempt to completely blunt liver MT response to stress was made with IL-6$^{-/-}$xTNF-R1$^{-/-}$ mice. IL-6$^{-/-}$ and TNF-R1$^{-/-}$ mice were also included as controls. Since the previous results demonstrated that MT-I+II protein levels reflected MT-I mRNA changes and that the three types of control animals responded similarly, in this experiment only MT-I mRNA levels were measured and C57BL/6x129/Sv mice used as controls. All animals were killed 4-5 hours after the onset of stress.

Experiment 4. From the current literature, a role of IL-6 and/or TNF-α on liver MT induction by the inflammatory response might be predicted. Thus, in parallel to Exp. 3, we analyzed liver MT induction by endotoxin (0.1 mg/kg, s.c.) in the IL-6$^{-/-}$, TNF-R1$^{-/-}$ and IL-6$^{-/-}$xTNF-R1$^{-/-}$ mice in comparison to that observed in control C57BL/6x129/Sv mice. In addition, we also studied liver MT induction by turpentine (50 µl, s.c.) in IL-6$^{-/-}$ and C57BL/6 mice. All animals were killed 4-5 hours after the injection.

Experiment 5. The low cytokine producer mice strain C3H/HeJ (36), which presents a mutation in the *Lps* gene, has been shown to have an attenuated liver MT response to endotoxin (20, 21), presumably because of a reduced endotoxin-induced cytokine release. In this experiment we evaluated the putative attenuation of the liver MT response to immobilization stress. The strain CD-1 was used as control animals in accordance with previous MT studies (20). Mice from the two strains were subjected to 0, 3 and 8 hours of stress, and their liver MT-I mRNA and total MT-I+II protein levels were measured.

Liver MT-I mRNA analysis. Livers were removed and immediately snap-frozen in liquid nitrogen. MT-I mRNA levels were quantified by dot-blot analysis as previously described (37) using as probe the mouse cDNA generously provided by Dr. R. D. Palmiter (University of Washington, Seattle, WA). A γ-actin probe (cDNA generously provided by Dr. A. Boronat, University of Barcelona, through Dr. X. Avilés, Autonomous University of Barcelona) was used to normalize the MT-I mRNA.

Total RNA was extracted by the method of Chomczynski and Sacchi (38). For Northern blot studies, RNA samples (10 μg RNA) were denatured for 5 min at 65°C in a solution of MOPS buffer (0.2 M MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0), 50% formamide and 2.2 M formaldehyde, and electrophoresed in a 1% agarose gel containing 1x MOPS buffer and 2.2 M formaldehyde. RNAs were then transferred to nylon filters in 20x SSC (3 M NaCl and 0.3 M sodium citrate, pH 7.0) by capillary elution. For dot blot, RNA samples (10 μg RNA) were denatured in 6x SSC (1.2 M NaCl, 0.12 M sodium citrate, pH 7.0) containing 2.5 M formaldehyde, and usually 100 μl were applied onto duplicate nylon filters, one for MT-I mRNA and another for γ-actin mRNA. The mouse MT-I and γ-actin cDNAs were used as templates for the synthesis of ^{32}P DNA probes.

The membranes were prehybridized at 46°C for 2 h in a mixture containing 6x SSC (3 M NaCl, 0.3 M sodium citrate), 5x Denhart's solution (50x Denhart's: 50 mg each of polyvinylpyrrolidone, Ficoll and bovine serum albumin per ml), 0.5% SDS, 50% formamide and 100 μg/ml of denatured herring sperm DNA. Hybridization incubation was done at 46°C for 18 h containing the labelled probe in a solution of 6x SSC, 0.5% SDS, 50% formamide, 10% dextran sulphate and 100 μg/ml of denatured herring sperm DNA. Following hybridization, filters were washed for 30 min in 1x SSC containing 0.1% SDS at 30°C and 30 min in 1x SSC containing 0.1% SDS at 46°C. For the Northerns, autoradiographs were developed by exposing the X-ray film with a high-plus intensifying screen at -70°C. For the dots, the radioactivity was quantitatively determined by scintillation counting of each dot after their excision in a β counter.

MT-I+II protein assay. MT-I+II levels were measured by radioimmunoassay as described previously (39) using a polyclonal antibody which cross-reacts with rat and mouse MT-I and MT-II.

Statistical assays. Results were analyzed with MANOVA, one- or two-way ANOVA. Logarithmic transformation of the data was applied when necessary.

RESULTS

Experiment 1. Table 1 shows the ratio of hepatic MT-I mRNA vs γ-actin mRNA of control (C57BL/6) and IL-$6^{-/-}$ mice. Two-way MANOVA revealed that stress significantly increased the MT-I mRNA levels in both control and IL-$6^{-/-}$ mice, but that the increase was smaller in IL-6 deficient mice ($p<0.05$).

Experiment 2. Northern blot studies with pooled samples demonstrated that stress increased MT-I mRNA levels (not shown) and that IL-$6^{-/-}$ mice responded less to stress than all control mice (C57BL/6, 129/Sv and C57BL/6x129/Sv) (Figure 1), suggesting that the lower MT-I response of IL-$6^{-/-}$ mice is not related to the genetic background of the knock-out mice but to IL-6 deficiency. Individual mRNA measurements by dot-blot (Figure 2 top), which allowed statistical evaluation, confirmed these Northern results. Thus, liver MT-I mRNA levels were significantly ($p<0.05$) increased by immobilization stress in all mice studied, and this increase was significantly ($p<0.05$) lower in the IL-$6^{-/-}$ strain compared with the three control strains. MT-I+II protein levels (Figure 2 bottom) correlated with the MT-I mRNA changes, clearly suggesting a transcriptional level of regulation for both stress and IL-6. Furthermore, basal MT regulation appears to be influenced by IL-6 too, since liver MT-I+II levels of IL-$6^{-/-}$ mice were significantly ($p<0.05$) lower than those of the three control strains. This decrease could not be observed in the MT-I mRNA levels, presumably because of the superior sensitivity of the radioimmunoassay.

Experiment 3. The results with TNF-$R1^{-/-}$ and IL-$6^{-/-}$xTNF-$R1^{-/-}$ mice (Figure 3) demonstrated that liver MT response to stress ($p<0.001$ in all mice) was decreased by IL-6 deficiency ($p<0.05$) but not by TNF-α functional deficiency.

Experiment 4. Figure 4 shows that liver MT response to the inflammatory response elicited by endotoxin was greatly decreased in IL-6 deficient mice ($p<0.001$). In contrast to MT induction by stress, MT induction by endotoxin was also decreased significantly by TNF-α functional deficiency ($p<0.01$), although to a lower extent than by IL-6 deficiency. Consequently, in the double mutant mice the response was virtually eliminated.

Figure 5 shows that shows that liver MT induction by the inflammatory response elicited by turpentine was also severely decreased in IL-$6^{-/-}$ mice compared to C57BL/6 mice ($p<0.001$).

Experiment 5. Figure 6 shows that liver MT response to stress is also decreased in the low cytokine producer mice strain C3H/HeJ. Thus, both MT-I mRNA (Fig. 6 top) and MT-I+II protein (Fig. 6 bottom) were significantly decreased ($p<0.05$) in C3H/HeJ mice compared to controls.

DISCUSSION

In this report we have examined the hypothesis that cytokines such as IL-6 and TNF-α could be involved in liver MT induction by a non-immunological challenge, acute immobilization stress. To this end, we have used mice carrying a null mutation for the IL-6 gene or the TNF-α type I receptor or both. The results demonstrate that IL-6 deficiency decreases consistently liver MT induction by stress, suggesting that this cytokine is involved in the hepatic MT regulation during stress, and, consequently, in the adaptation of the organism to stress. Furthermore, this is also the case in basal conditions. These roles of IL-6 are somewhat unexpected but consistent with previous in vivo and in vitro studies which demonstrated that MT synthesis is up-regulated by this cytokine (20, 21, 25), and with the well-known fact that exposure to psychological and physical stressors increases plasma IL-6 levels (27, 28). This is the first time, however, that IL-6 is directly linked to MT regulation in vivo in physiological conditions, i.e., not after the exogenous administration of the cytokine.

In contrast to IL-6, TNF-α functional deficiency did not affect liver MT response to stress. This is somewhat surprising since it is well-known that the exogenous administration of TNF-α up-regulates liver MT synthesis (21-23) and that at least some types of stress increase plasma TNF-α levels (29-31). Thus, analogously to IL-6 deficiency, TNF-α functional deficiency was expected to decrease liver MT induction by stress. This lack of effect of the functional deficiency of TNF-α could be attributable to a less relevant biological role of this cytokine in liver MT regulation than IL-6, or, alternatively, to a lack of secretion of TNF-α to the blood during immobilization stress. Unfortunately, we could not measure blood TNF-α levels in these animals. However, the results with endotoxin (see below) support the first

possibility. Regardless of whether or not immobilization stress increases circulating TNF-α levels, the present results demonstrate that a role of this cytokine in liver MT regulation during stress is unlikely. Moreover, they also rule out a compensatory effect of TNF-α in IL-6 deficient mice, a plausible possibility in principle since it is well-known that IL-6 and TNF-α share some physiological functions to some extent (35). However, this does not necessarily mean that TNF-α is not biologically relevant in terms of MT regulation. Indeed, a physiological role of TNF-α in liver MT regulation is evidenced in a different context, i.e. in the inflammatory response elicited by endotoxin (see below).

Thus, the above results clearly indicated that IL-6 but not TNF-α was a mediating factor in liver MT response to a non-immunological stimulus, immobilization stress. For comparison, we also studied the role of these cytokines in a context where they presumably have a greatest importance, namely, during the inflammatory response. To this end, mice were injected with endotoxin or turpentine, two well-known inflammatory agents. It has long been recognized that both factors induce liver MT synthesis (1, 2), as well as the release of many cytokines including IL-6 and TNF-α (35). That liver MT induction occurs because of cytokine release was originally suggested by results with the C3H/HeJ strain of mice, which have a mutation in the *Lps* gene that results in a defective response to endotoxin (36), since these mice showed an attenuated hepatic MT induction by endotoxin (20, 21). However, these results were only suggestive of the involvement of cytokines, and if they were actually involved and which of them was responsible for the observed MT changes has never been demonstrated. Another source of concern is the stress associated with the administration of endotoxin. It is well-known that endotoxin injections activate the hypothalamic-pitutary-adrenal axis (14-16), which is suggestive of a stress response. Since stress is a major hepatic MT inducer, these

findings cast doubts of whether the C3H/HeJ mice responded less to endotoxin because of a diminished cytokine production or because they responded differently to the stress associated with endotoxin. To gain some insight in this regard, we studied the liver MT response of C3H/HeJ mice to immobilization stress. The results clearly indicated that these mice responded less to stress as far as liver MT is concerned, supporting therefore the second possibility and highlighting the need of further studies to demonstrate the involvement of cytokines in liver MT induction by endotoxin.

The present results with knock-out mice for IL-6 and TNF-α type 1 receptor definitively demonstrate that these cytokines do mediate liver MT induction by endotoxin. The effect of IL-6 deficiency was especially dramatic, and blunted most of the hepatic MT induction by endotoxin and also turpentine. In contrast to during stress, the functional TNF-α deficiency also contributed significantly to liver MT induction by endotoxin. Since TNF-α induces IL-6, TNFR1$^{-/-}$ mice show a decreased IL-6 secretion in response to endotoxin (33). Thus, it could be argued that the observed effects of TNF-α functional deficiency on liver MT induction by endotoxin were simply the consequence of reduced IL-6 production. However, since an effect of TNF-α functional deficiency was still observed in IL-6 deficient mice, we conclude that TNF-α has a specific role, independent of IL-6 production, on liver MT regulation. It must be acknowledged, nevertheless, that this is less relevant biologically than that of IL-6. A residual MT induction by endotoxin was still observed in the double mutant mice, which might be mediated by other cytokines.

Thus, taken together the results clearly demonstrate that IL-6 is a major hepatic MT regulator in physiological conditions, contributing substantially to liver MT induction during stress and essentially during the inflammatory response.

Furthermore, the results suggest that IL-6 is involved in the adaptation of the organism to stress in addition to the physiological response to inflammation. Since liver MT appears to be good antioxidant proteins (40), the teleological basis for this MT control by IL-6 could be to promote cell protection against oxidative stress. Finally, given the prominent role of IL-6 in the liver acute-phase response, the long held view of MT as an acute-phase protein (26) receives now strong support. Work is in progress to establish whether the pattern of acute-phase proteins induced in the liver by stress is comparable to that induced by endotoxin.

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Table 1. Effect of IL-6 deficiency on hepatic MT-I mRNA levels of basal and stressed mice.

	Ratio MT-I vs Actin	
	Basal	**4h Stress**
C57BL/6	0.719 ± 0.096	42.125 ± 3.75*
IL-6$^{-/-}$	0.387 ± 0.102▲	14.146 ± 2.07*▲

Results are mean ± SE (n=3-8). Two-way MANOVA indicates a significant effect of the stress (*) and the strain (▲) (p at least <0.05).

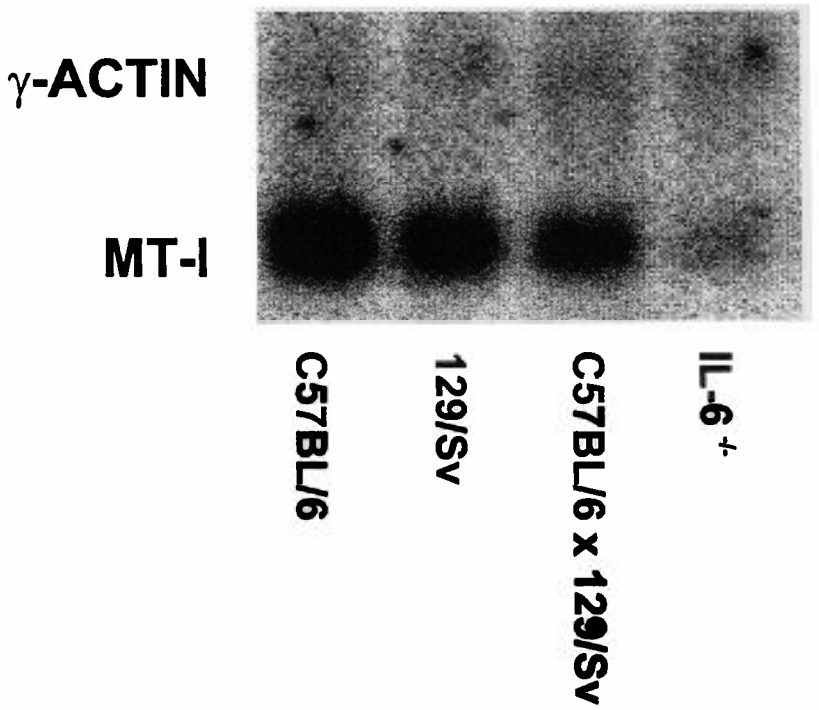

FIGURE 1. Northern blot analysis of the effect of IL-6 deficiency on liver MT-I mRNA response to 4 hours of immobilization stress. Pooled samples from stressed animals were electrophoresed, blotted and hybridized with the MT-I and γ-actin probes as described in Materials and Methods. It is clear that MT-I mRNA levels are lower in the IL-6-/- mice than in the three types of control mice (C57BL/6, 129/Sv, B57BL/6x129/Sv). Basal animals are not shown because of their low signal compared with the stressed ones.

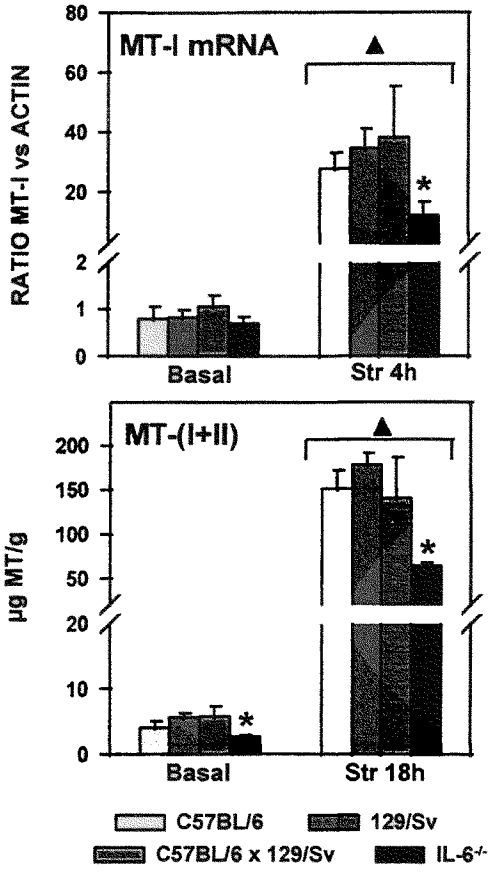

FIGURE 2. Effect of IL-6 deficiency on liver MT response to immobilization stress. Liver MT-I mRNA (ratio vs actin mRNA, top) and total MT-I+II protein levels (µg MT/g tissue, bottom) are shown. Results are mean ± SE (n=5-8). Data were evaluated with two-way MANOVA with strain and stress as main factors. A significant inducing effect of stress on mRNA and protein levels was observed in all cases, and IL-6$^{-/-}$ mice showed a significantly decreased MT-I mRNA and total protein MT-I+II response to stress compared to control mice. Furthermore, they had decreased basal (unstressed) MT-I+II protein levels ($p<0.05$). ▲ $p<0.001$ vs unstressed, basal mice. * $p<0.05$ vs control (C57BL/6, 129/Sv, B57BL/6 *x* 129/Sv) mice.

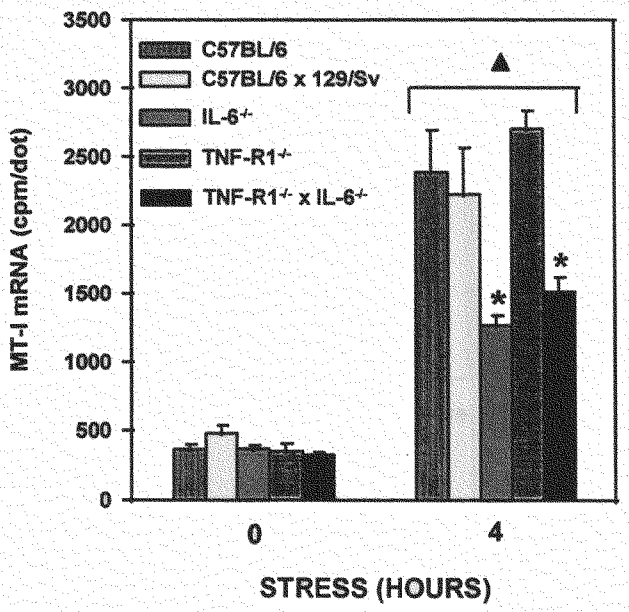

FIGURE 3. Effect of IL-6 and TNF-α functional deficiency on liver MT response to immobilization stress. Results are mean ± SE (n=5-12). A significant inducing effect of stress on mRNA and protein levels was observed in all cases, and IL-6 but not TNF-α functional deficiency significantly decreased liver MT response to stress. ▲ $p<0.001$ vs the respective unstressed, basal mice. * $p<0.05$ vs IL-$6^{+/+}$ mice.

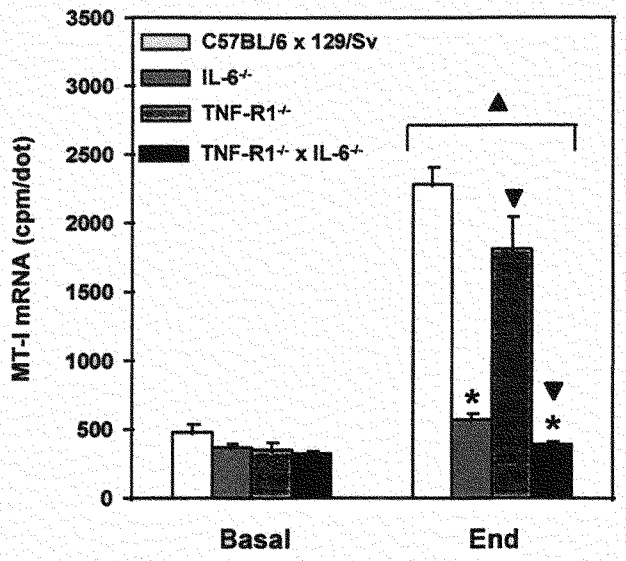

FIGURE 4. Effect of IL-6 and TNF-α functional deficiency on liver MT response to endotoxin. Results are mean ± SE (n=4-8). A significant inducing effect of endotoxin was observed in all cases, and IL-6 and to a lower extent TNF-α functional deficiency significantly decreased liver MT response to endotoxin. ▲ $p<0.001$ vs the respective control (basal) mice. * $p<0.05$ vs IL-$6^{+/+}$ mice. ▼ $p<0.01$ vs the respective TNFR$1^{+/+}$ mice.

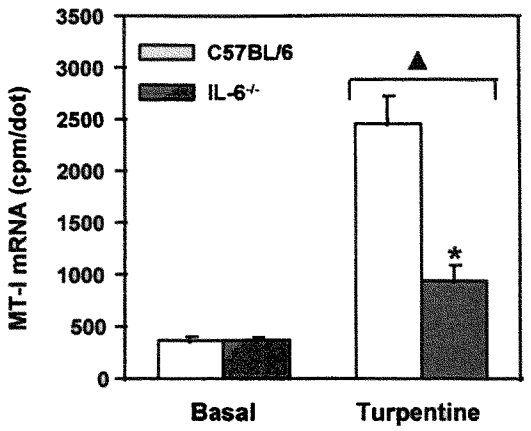

FIGURE 5. Effect of IL-6 deficiency on liver MT response to turpentine. Results are mean ± SE (n=5-7). A significant inducing effect of turpentine was evident, and in line with the previous experiment with endotoxin, IL-6 deficiency decreased liver MT response. ▲ $p<0.001$ vs the respective control (basal) mice. * $p<0.001$ vs IL-$6^{+/+}$ mice.

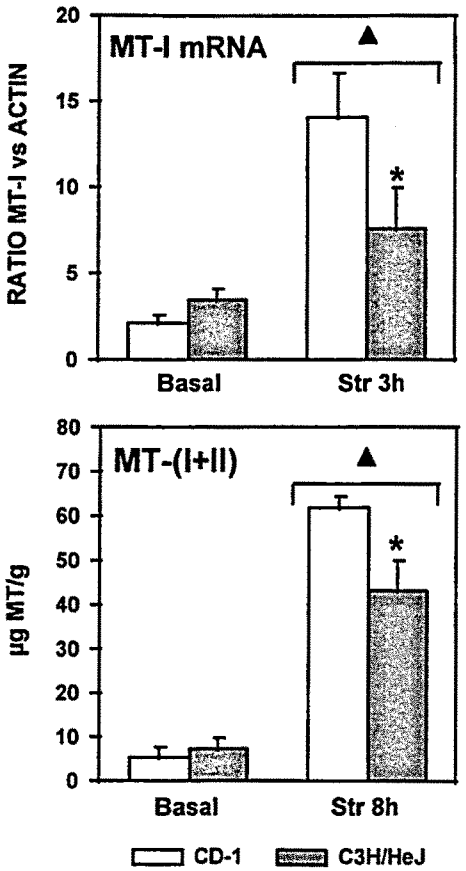

FIGURE 6. Liver MT response to immobilization stress in the C3H/HeJ strain. Results are mean ± SE (n=6-7). Stress increased both MT-I mRNA (top) and MT-I+II protein (bottom) levels. MT induction was significantly decreased in the C3H/HeJ mice compared to control mice. ▲ $p<0.001$ vs unstressed, basal mice. * $p<0.05$ vs control mice.

Treball 6

Hernández, J., Bluethmann, H. and Hidalgo, J.
Physiological role of IL-6 in brain metallothionein regulation in stress situations using a IL-6 deficient mice.
Manuscript.

PHYSIOLOGICAL ROLE OF IL-6 IN BRAIN METALLOTHIONEIN REGULATION IN BASAL AND STRESS SITUATIONS USING A IL-6 DEFICIENT MICE.

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ABSTRACT

Interleukin-6 (IL-6) is a major cytokine with, among other physiological functions, a prominent role in the control of the liver acute-phase response. The present mini-report studing the role of IL-6 on the brain metallothionein (MT) response in a basically psychogenic stress model, i.e. immobilization stress.

Stress has a general increasing effect in the brain MT-(I+II) protein, but a mice carrying a null mutation in the IL-6 gene (IL-$6^{-/-}$) did not respond differently to control mice in the selected brain areas (cerebellum, hypothalamus, hippocampus, medulla+pons and remaining brain), indicating that it is unlikely that IL-6 mediates brain MT-(I+II) response to stress. Only in the hypothalamus of IL-$6^{-/-}$ mice the MT-(I+II) protein levels were lower than in the control strains.

INTRODUCTION

Interleukin 6 (IL-6) is a major mediator of the immune and inflammatory responses in the organism. Originally, IL-6 was named "hepatocyte-stimulating factor" because of its induction of the acute-phase response in the liver (Gauldie *et al.*, 1987; Andus *et al.*, 1991). IL-6 is also involved in the B-cell differentiation, the activation of T-cells and the promotion of hematopoiesis (reviewed in ref. (Van Snick, 1990)). Furthermore, IL-6 has specific effects in the nervous system, stimulating the activity of the hypothalamus-adrenocortical axis, inducing fever or causing neuronal differentiation (Pousset, 1994; Mandrup-Poulsen *et al.*, 1995; Rothwell and Hopkins, 1995). It has also been demonstrated that transgenic mice expressing IL-6 under the control of the glial fibrillary acidic protein gene promoter develop a chronic progressive neurodegenerative disease characterized by neurodegeneration, astrocytosis, microgliosis, angiogenesis, and induction of acute-phase protein synthesis (Campbell *et al.*, 1993). It is therefore clear that IL-6 has major functions in both the liver and the brain.

It is known that the exogenous administration of a number of cytokines including IL-6 increases metallothionein (MT) expression in different tissues (Bell *et al.*, 1987; Cousins and Leinart, 1988; De *et al.*, 1990; Sato *et al.*, 1992) and cell lines (Friedman and Stark, 1985; Karin *et al.*, 1985; Schroeder and Cousins, 1990; Bauer *et al.*, 1993; Vanguri, 1995). MTs are a family of small, cysteine-rich, metal-binding proteins (Kägi and Kojima, 1987; Suzuki *et al.*, 1993). There are several isoforms in mammals; 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 localized in the brain and stratified squamous epithelia, respectively (Uchida *et al.*, 1991; Palmiter *et al.*, 1992; Quaife *et al.*, 1994). The biochemical and molecular properties of the MT-I and MT-II isoforms are well documented (Kägi and Kojima,

1987; Suzuki *et al.*, 1993), but their physiological role and regulation in physiological conditions are poorly characterized.

Stress is a major physiological insult to which animals respond with a plethora of mechanisms. It is well known that stress increases MT production in the brain and especially the liver (Oh *et al.*, 1978; Hidalgo *et al.*, 1988; Hidalgo *et al.*, 1990; Hidalgo *et al.*, 1991a; Gasull *et al.*, 1994b), suggesting that MT is involved in the physiological adaptation of the organism to stress. The factors implicated in the control of MT during stress are not clear. In the rat, it appears that liver MT response to stress is not mediated by glucocorticoids, catecholamines or endogenous opioids (Hidalgo *et al.*, 1988; Hidalgo *et al.*, 1991b; Gasull *et al.*, 1994a), which implicates that other factors must be involved in the control of this response. IL-6 appeared to us to be a major candidate, since as stated above this cytokine induces readily liver MT expression (De *et al.*, 1990; Liu *et al.*, 1991) and circulating IL-6 levels are strongly increased by stress (Lemay *et al.*, 1990; Zhou *et al.*, 1993).

EXPERIMENTAL PROCEDURES

Production of IL-6 deficient mice. Generation and development of the IL-6 deficient mice (IL-6$^{-/-}$) was as described previously (Kopf *et al.*, 1994). The IL-6$^{-/-}$ mice were created by insertion of a *neo*^{r} cassette in the second exon of the IL-6 gene. Heterozygous mice were interbred to obtain mice homozygous for the disrupted IL-6 allele. The mice were used for the experiments at the age of 8-9 weeks. We used as controls the wild-type strains, C57BL/6 (Jackson, Germany), 129/Sv and the IL-6$^{+/+}$ F_1 mice C57BL/6 x 129/Sv (provided by Biological Research Lab. Ltd., Basel, Switzerland).

Maintenance of the animals. The animals were housed individually and maintained under standard laboratory conditions (light cycle from 07:30 to 19:30, temperature 22°C, food and water provided *ad libitum*) for at least one week before starting the experiments.

Experimental procedures. We studied the effect of immobilization stress on mouse total MT-(I+II) protein levels in the following brain areas: hypothalamus, cerebellum, medulla+pons, hippocampus, and remaining brain. Mice were immobilized by wrapping them in a metallic net. The animals were immobilized for either 4 or 18 hours and then killed using unstressed (0 hours) animals as controls. MT-I mRNA levels were measured in the 0- and 18-hour groups.

MT protein assay. MT levels were measured by radioimmunoassay (RIA) as described previously (Gasull *et al.*, 1993) using a polyclonal antibody which cross-reacts with rat MT-I and MT-II but not with MT-III. We have also observed cross-reactivity of the antibody with mouse MT-I and MT-II.

Statistical assays. Results were analyzed with MANOVA, one- or two-way ANOVA and Mann-Whitney U-test. Logarithmic transformation of the data was applied when necessary.

RESULTS

Brain MT-(I+II) induction by stress in C57BL/6, 129/Sv, C57BL/6 x 129/Sv and IL-6$^{-/-}$ mice. The MT-(I+II) protein induction by stress and the putative effect of IL-6 deficiency was studied in hypothalamus, cerebellum, medulla+pons, hippocampus and remaining brain (Fig. 1). As expected (Hidalgo *et al.*, 1990; Hidalgo *et al.*, 1991a; Gasull *et al.*, 1994a; Gasull *et al.*, 1994b), we observed a significant effect of stress in all strains studied in cerebellum, medulla+pons and hippocampus, but no effect of IL-6 deficiency was observed in any brain area studied. In basal conditions, only in the hypothalamus of IL-6$^{-/-}$ mice the MT-(I+II) protein levels were lower than in the control strains. These results indicate a minor role of IL-6 regulating MT-(I+II) in both basal and stress conditions in the brain, suggesting a different MT regulating pathway compared with the liver.

DISCUSSION

Stress is a major physiological inducer of liver MT synthesis (Oh *et al.*, 1978; Hidalgo *et al.*, 1988; Hidalgo *et al.*, 1990; Hidalgo *et al.*, 1991a; Hidalgo *et al.*, 1991b; Gasull *et al.*, 1994a; Gasull *et al.*, 1994b). Because the activity of the hypothalamic-pituitary-adrenal (HPA) axis is increased by stress, it has generally been assumed that glucocorticoids could mediate liver MT response to stress. However, in rats the experimental results do not support such a mediating role of these hormones (Hidalgo *et al.*, 1988). Furthermore, the role of the sympathomedulloadrenal system is also unclear (Gasull *et al.*, 1994a) as it is that of endogenous opioids (Hidalgo *et al.*, 1991b). Therefore, factors other than the usual hormones responsive to stress should participate in the control of liver MT during stress. A putative set of factors that could be involved is that of cytokines, a group of essential mediators of the immune system but also of other physiological systems (Gauldie *et al.*, 1987; Van Snick, 1990; Andus *et al.*, 1991; Pousset, 1994; Mandrup-Poulsen *et al.*, 1995; Rothwell and Hopkins, 1995), e.g. liver MT induction by bacterial lypopolysaccharide (endotoxin) (De *et al.*, 1990; Liu *et al.*, 1991). Indeed, because endotoxin and endotoxin-induced factors such as IL-1 and IL-6 are major inducers of hepatic MT (Karin *et al.*, 1985; Cousins and Leinart, 1988; De *et al.*, 1990), this protein has been considered temptatively as an acute-phase protein by some authors (Karin *et al.*, 1985).

It is unclear whether a psychological stressor such as immobilization causes an acute-phase liver response comparable to that induced by endotoxin, especially because stress has been reported to exert in general immunosuppresive effects (reviewed in (Khansari *et al.*, 1990)). However, recent reports have demonstrated that exposure to a psychological or physical stressor increases plasma IL-6 levels (Lemay *et al.*, 1990; Zhou *et al.*, 1993), which has been related to the control of the

HPA axis activity and likely to the maintenance of homeostasis by acting as a hormone (Zhou *et al.*, 1993).

We have evaluated the effect of IL-6 deficiency on brain MT regulation. The studies about brain MT have raised great expectations since the discovery of a brain-specific MT isoform, MT-III, and its apparent neurotrophic role (Uchida *et al.*, 1991; Palmiter *et al.*, 1992; Erickson *et al.*, 1994). However, our knowledge of brain MT regulation is still scarce. As for the liver, studying brain MT regulation in mice carrying a null mutation in the IL-6 gene is an unique approach for characterizing the putative role of this cytokine. To this end, we measured MT-(I+II) protein levels in selected brain areas, namely, hypothalamus, cerebellum, medulla + pons, hippocampus, and remaining brain. From these, IL-6 deficiency only affected MT-(I+II) protein accumulation in the hypothalamus of unstressed mice, which was slightly decreased compared to controls, indicating a specific rather than a general effect of IL-6 deficiency in the brain. This is in accordance with previous reports which suggest that the hypothalamus is a major brain area regarding central IL-6 action (Pousset, 1994; Mandrup-Poulsen *et al.*, 1995; Rothwell and Hopkins, 1995). Nevertheless, this general lack of effect of IL-6 deficiency on brain MT-(I+II) levels is in principle puzzling, since it is well-known that peripheral administration of endotoxin, which presumably will increase IL-6 production (Laye *et al.*, 1994), increases brain MT mRNA levels (De *et al.*, 1990). Furthermore, the i.c.v. injection of IL-6 in rats and the expression of IL-6 under the control of the GFAP promoter in mice increases brain MT-(I+II) levels (Hernández *et al.*, 1997; Hernández and Hidalgo, 1998). It is possible, of course, that basal IL-6 production is small or even negligible in brain areas other than hypothalamus of normal, not challenged animals, which would explain, at least in part, the lack of effect of IL-6 deficiency. It is also possible that IL-6 needs to act in concert with other factors to exert a significant effect on brain MT-(I+II) levels which might not be available in basal animals.

In contrast to the liver, brain MT-(I+II) reponse to stress was not affected by IL-6 deficiency. In agreement with previous reports (Hidalgo *et al.*, 1990; Hidalgo *et al.*, 1991a; Gasull *et al.*, 1994a; Gasull *et al.*, 1994b), stress had a general increasing effect in the brain areas studied, but IL-6$^{-/-}$ mice did not respond differently to control mice, indicating that it is unlikely that IL-6 mediates brain MT-(I+II) response to stress. This could be due to the fact that IL-6 might not be increased during stress in the brain. It is also possible that other cytokines such as TNFα could compensate for IL-6 deficiency, since it is well-known that IL-6 and TNFα share some physiological effects to some extent (Bluethmann *et al.*, 1994), and therefore it would be important to study brain MT regulation in mice with a double null mutation for both cytokines. In summary, the present results demonstrate that *in vivo* IL-6 has a unlikely role in the regulation of brain MT response to stress. IL-6 deficiency only affected MT-(I+II) in the hypothalamus of unstressed mice, which was slightly decreased. These results suggest that other factors are also involved in the control of this protein during stress. In mice, in contrast to rats, one of them could be glucocorticoids, and other could be TNF.

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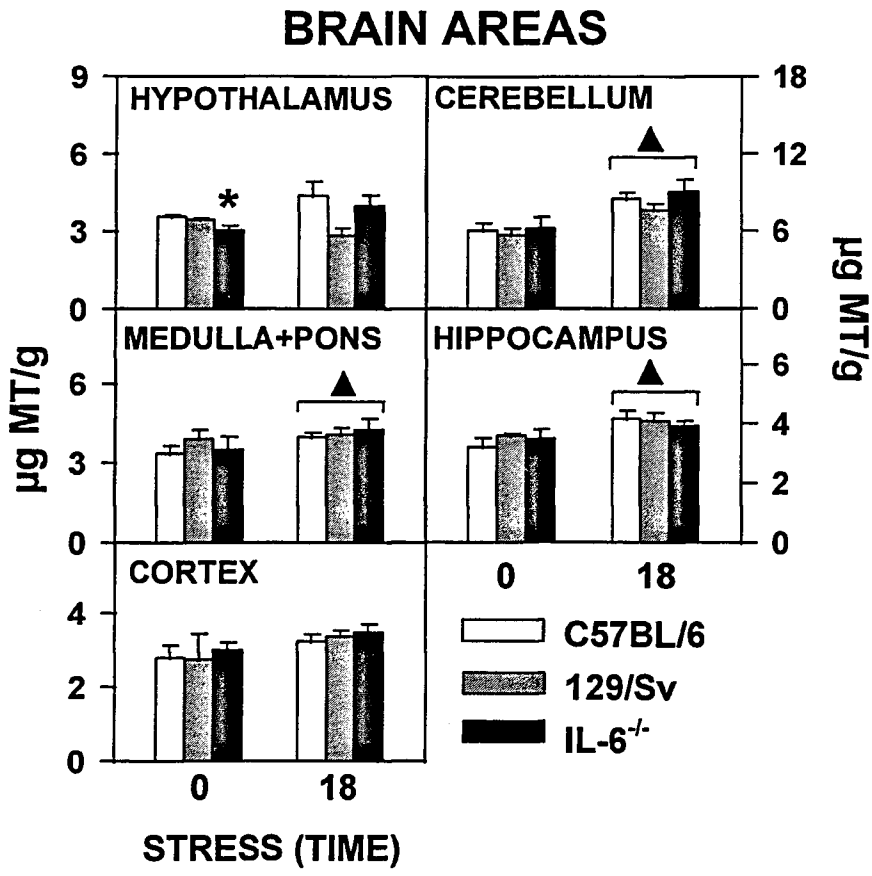

FIGURE 1. Effect of immobilization stress on MT-I+II levels in 5 brain areas. The brains were removed immediately after sacrifice and frozen at -80 °C. The areas were dissected and processed the same day of the MT assay. Results are mean ± SE (n=3-8). Groups of mice were compared statistically with two-way MANOVA, except for the unstressed group of 129/Sv mice, consisting of n=2, which precludes appropriate comparisons. Stress significantly increased MT-(I+II) levels in cerebellum, medulla+pons and hippocampus (p at least <0.05), but IL-6 deficiency did not affect the response to stress. ▲ significantly different compared to unstressed mice. * significantly different compared to control (C57BL/6) mice.

Treball 7

Hernández, J., Molinero, A., Campbell, I. L. and Hidalgo, J. (1997).

Transgenic expression of interleukin 6 in the central nervous system regulates brain metallothionein-I and -III expression in mice.

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MOLECULAR BRAIN RESEARCH

Research report

Transgenic expression of interleukin 6 in the central nervous system regulates brain metallothionein-I and -III expression in mice

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Abstract

The metallothionein (MT) gene family consists of several members (MT-I–IV) that are tightly regulated during development. MT-I and MT-II are expressed in many tissues, including the brain, whereas MT-III is expressed mainly in the central nervous system. However, the physiological roles of these isoforms in the brain and their regulation are poorly characterized. In this report, we have studied the putative role of IL-6 in the regulation of brain MT. The present results demonstrated that transgenic mice expressing IL-6 under the regulatory control of the glial fibrillary acidic protein gene promoter (GFAP-IL6 mice), and which develop chronic progressive neurodegenerative disease, show significantly increased MT-I + II protein levels in specific brain areas. Thus, the MT-I + II levels of 1- and 3-month-old GFAP-IL6 mice (G16 and/or G36 lines) were not altered in hippocampus but they were elevated in the cerebellum (highest induction), medulla plus pons, hypothalamus and remaining brain (lowest induction). The effect of the transgenic expression of IL-6 was more dramatic for MT-I + II protein than for MT-I mRNA levels, with the latter only marginally elevated in the G16 line at 3 months but not at 6 months of age where there was a tendency to decreased levels. Brain MT-I mRNA levels also tended to decrease in the higher expressor G36 line in 3-month-old mice despite the strongly elevated MT-I + II protein levels at this age. Therefore, in addition to increasing MT gene transcription, these results suggest a post-transcriptional effect of IL-6 or of a IL-6-dependent factor, in this chronic situation. The up-regulated brain MT-I + II protein levels in the GFAP-IL6 mice was comparable to the expression of the acute-phase response gene EB22/5, suggesting that these MT isoforms could be considered acute-phase response proteins in the brain. Brain MT-III mRNA levels followed a somewhat similar pattern that those of MT-I mRNA but the decreasing effect of IL-6 transgene production with age was more dramatic for the former, suggesting differential regulation of these MT isoforms by IL-6. The results indicate that these transgenic mice might be a valuable tool for further examining the role of the MT isoforms in brain physiology and pathobiology.

Keywords: Metallothionein-I; Metallothionein-II; Metallothionein-III; Growth inhibitory factor; IL-6; Endotoxin; Brain; Liver

1. Introduction

Metallothioneins (MTs) are a class of small, cysteine-rich, heavy metal-binding proteins. To date, several isoforms have been characterized in mammals, with MT-I and MT-II being expressed in virtually all tissues [25,43] and MT-III (also called Growth Inhibitory Factor) and MT-IV being expressed specifically in the brain [36,45] and keratinizing epithelia [38], respectively. The role of this family of proteins in brain function is potentially important since they could serve in the control of Zn and Cu metabolism [14,20,31,35], in the adaptation to stress [13,17,19,21], as antioxidant agents [18,28,40,44] or in the regeneration of damaged brain areas [2,22,37,47,48].

In order to understand the physiological functions of these proteins in the brain, it is essential to characterize the factors that control their expression. It is now well-established that the essential heavy metals Zn and Cu [14,20,35], glucocorticoids [14,20,36] and catecholamines [13] are involved in the control of brain MT-I + II levels. Regarding MT-III, the data suggest that this isoform is under a quite different control compared to the other two isoforms since it is mostly unresponsive to the inducers of MT-I + II [10,27,36,49] and substantial differences exist in their promoter regions [23,34]. A set of factors that could be involved in the control of brain MT levels is that com-

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prised by the cytokines, a group of essential mediators of cellular communication not only of the immune system but also of the nervous system [1,32]. Indeed, previous studies have demonstrated that cytokines, such as IL-1 and especially IL-6, are important inducers of liver MT synthesis [9,11,41].

In the present report, MT regulation was studied in transgenic mice (termed GFAP-IL6) in which IL-6 was expressed under the regulatory control of the glial fibrillary acidic protein (GFAP) gene promoter which targeted expression of IL-6 to astrocytes [6,7]. The results demonstrate that IL-6 has a significant role in the control of brain MT isoforms.

2. Materials and methods

2.1. Production of GFAP-IL6 transgenic mice

Construction and characterization of the GFAP-IL6 transgenic mice was described previously [6]. Briefly, an expression vector derived from the murine glial fibrillary acidic protein (GFAP) gene was used to target expression of IL-6 to astrocytes. Heterozygous offspring of the low expressor G16 (3 and 6 months of age) and the high expressor G36 (1 and 3 months of age) lines were studied in parallel with age- and sex-matched non-transgenic littermates.

2.2. Effect of endotoxin in vivo

The effect of endotoxin (i.p.) was studied in adult mice. Endotoxin (lipopolysaccharide from *Escherichia coli* 0127:B8, Sigma, St. Louis, MO) was administered (3 mg/kg) i.p. and the animals were killed 6 h (for MT mRNA assay) or 24 h (for MT protein assay) later. Control mice received saline ($n = 6$ in all cases).

2.3. Primary cultures

Astrocyte-enriched and neuronal primary cultures were prepared from the cerebral hemispheres of newborn mice (within 24 h-post-natal) or from 16-day-old embryos as described previously for rats [20] according to the method of Löffner et al. [30].

2.4. RNA isolation and analysis

Organs were removed and immediately snap-frozen in liquid nitrogen. In the case of GFAP-IL6 mice and their controls, poly(A)$^+$-enriched RNA was isolated by a rapid procedure [3] performed as described previously [7]. In the case of endotoxin-treated mice, total RNA was extracted as described [8]. mRNA levels were measured by Northern blot hybridization as follows: poly(A)$^+$-enriched RNA samples (3 (μg RNA) or total RNA (10 (μg RNA) were denaturated for 10 min at 65°C in a solution of MOPS buffer (0.2 M MOPS, 5 mM sodium acetate, 1 mM EDTA pH 7.0), 50% formamide and 2.2 M formaldehyde. Denatured RNAs were electrophoresed in a 1% agarose gel containing 1 × MOPS buffer and 2.2 M formaldehyde. RNAs were then transferred to nylon filters in 20 × SSC (3 M NaCl and 0.3 M sodium citrate pH 7.0) by capillary elution as described by Sambrook et al. [39]. RNAs were fixed by exposing the nylon membrane (Hybond-N, Amersham, Buckinghamshire, UK) to UV irradiation. The membranes were kept dry until hybridization.

For MT-I and MT-III mRNA analyses, mouse cDNAs were used which were kindly provided by Dr. R.D. Palmiter, University Of Washington, WA, USA. For γ-actin mRNA, a cDNA was used, kindly provided by Dr. Albert Boronat, University of Barcelona, through Dr. X. Avilés, Autonomous University of Barcelona. The cDNA for the murine acute-phase response gene EB22/5 [24] was kindly provided by Dr. John Inglis, Medical Research Council Human Genetics Unit, Edinburgh, UK. The cDNAs were used as templates for the synthesis of DNA probes using ^{32}P from Amersham and an Oligolabelling Kit from Pharmacia Biotech (Uppsala, Sweden). The specific activity of each probe was $1–2 \times 10^9$ dpm/μg. The labeled probes were purified by chromatography (Quick Spin Columns Sephadex G-50, Boehringer Mannheim, Germany). The filters were pre-hybridized at 46°C for 2 h in a mixture containing 6 × SSC, 5 × Denhardt's solution (50 × Denhardt's: 50 mg each of polyvinylpyrrolidone, Ficoll and bovine serum albumin per ml), 0.5% SDS, 50% formamide and 100 μg/ml of denatured herring sperm DNA. Hybridization incubation was done at 46°C for 18 h containing the labeled probe in a mixture solution of 6 × SSC, 0.5% SDS, 50% formamide, 10% dextran sulphate and 100 μg/ml of denatured herring sperm DNA. Following hybridization, filters were washed for 30 min twice in 1 × SSC containing 0.1% SDS. Brain and liver MT-I and γ-actin mRNA levels were analyzed simultaneously; after autoradiography the probes were stripped by heating the filter at 65°C for 2 h in 50 ml of 5 mM Tris–HCl pH 7.5, 2 mM EDTA and 0.1 mM Denhardt's solution. The filters were then subsequently re-probed for MT-III mRNA after it had been verified that the previous two probes were removed. The MT-III probe was then stripped as above and the blot was re-hybridized with the EB22/5 probe. Autoradiographs were developed by exposing X-ray film (XAR-05, Kodak, Rochester, NY) with a high-plus intensifying screen (Wolf X-Ray, New York, NY) at −70°C. The bands were quantitated with the Molecular Analyst software (Bio-Rad). In some cases, MT and γ-actin mRNAs were measured by dot-blot hybridization and scintillation counting of the dots.

2.5. MT protein assay

MT protein levels were measured by RIA [15] using a polyclonal antibody which cross-reacts with MT-I and

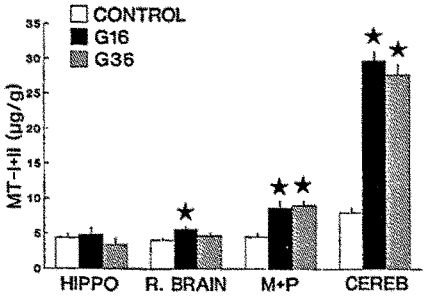

Fig. 1. Effect of transgenic IL-6 expression on brain MT-I+II levels in specific brain areas. Mice of the G16 and G36 lines were killed at 3 months of age along appropriate controls and the brains immediately frozen in liquid nitrogen. For MT assay, brains were thawed, dissected into the specified brain areas and immediately processed for MT RIA. Hippo, hippocampus; M+P, medulla plus pons; Cereb, cerebellum; R. Brain, remaining brain. Results are mean±S.E. $n=3$. $^{*}P<0.05$ vs. control mice (Duncan procedure).

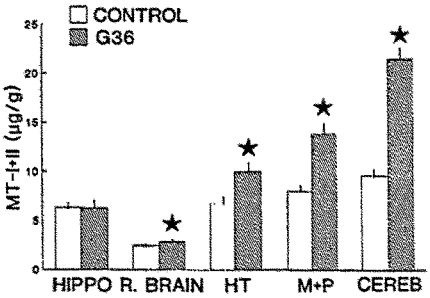

Fig. 2. Effect of transgenic IL-6 expression on brain MT-I+II levels in specific brain areas. Mice of the G36 line were killed at 1 month of age along appropriate controls and processed as in Fig. 1. Hippo, hippocampus; HT, hypothalamus; M+P, medulla plus pons; Cereb, cerebellum; R. Brain, remaining brain. Results are mean±S.E. $n=4$. $^{*}P<0.05$ vs. control mice (Student's t-test).

MT-II but not with human MT-III [14]. We have also observed no cross-reaction with recombinant rat MT-III (unpublished data).

2.6. Statistical assays

Results were analyzed with Student's t-test or one-way ANOVA, after logarithmic transformation of the data when necessary. Multiple comparisons of the means were carried out with the Duncan procedure.

3. Results

Initially, we examined MT-I + II protein levels by RIA in several brain areas of the G16 and G36 lines at 3 months of age (Fig. 1). MT-I + II protein levels were strongly increased in the cerebellum and medulla plus pons and slightly in the remaining brain except for the hippocampus where no effect of the transgenic expression of IL-6 was observed. These results were confirmed and extended in a further set of animals of the G36 line at 1 month of age (Fig. 2) which again showed significant MT-I + II increases in cerebellum, medulla plus pons, hypothalamus and remaining brain except hippocampus where no effect of IL-6 was noticed.

Fig. 3 shows the expression of γ-actin, MT-I, MT-III and EB22/5 mRNAs in whole brain from control and GFAP-IL6 mice as well as liver γ-actin and MT-I mRNAs. Table 1 shows the ratios of the different mRNAs vs. γ-actin mRNA level. As expected, the brain acute-phase reactant EB22/5 mRNA levels were strongly increased in the G16 and G36 mice at the ages studied (3 and 6 months) ($P<0.05$). The brain MT-I mRNA levels in the G16 line were increased at 3 months of age ($P<0.05$) but tended to be decreased at 6 months while no effect was observed in the G36 line in this set of animals. In contrast, brain MT-III mRNA levels remained unaltered at 3 months of age and decreased at 6 months ($P<0.05$) in the G16 line and also tended to decrease in the G36 line at 3 months of age.

Since it was shown previously that transgene encoded IL-6 expression was higher in the cerebellum [6], the MT expression was analyzed separately in the cerebrum and cerebellum in an additional set of mice (Table 2). In the cerebrum, MT-I mRNA levels again were slightly elevated in the G16 line at 3 months of age but decreased at 6 months of age whereas in the G36 line a slight decrease was observed in MT-I RNA levels at 3 months of age. MT-III mRNA levels were significantly ($P<0.05$) decreased in the G16 line at 6 months of age but not at 3 months of age whereas the G36 line had decreased MT-III levels at 3 months of age. The results for the cerebellum were similar to those for the cerebrum, with a tendency to

Table 1
Relative levels of the MT-I, MT-III and EB22/5 mRNAs of brain and liver

	Brain MT-I mRNA	Brain MT-III mRNA	Brain EB22/5 mRNA	Liver MT-I mRNA
Control 3 months	0.29 ± 0.06	1.80 ± 0.87	0.06 ± 0.04	2.29 ± 1.51
Control 6 months	0.19 ± 0.04	1.06 ± 0.06	0.03 ± 0.01	7.36 ± 3.90
G16 3 months	0.84 ± 0.05 [a]	2.06 ± 0.76	1.32 ± 0.10 [a]	13.8 ± 4.38
G16 6 months	0.21 ± 0.03	0.48 ± 0.12 [a]	0.34 ± 0.10 [a]	8.74 ± 1.94
G36 3 months	0.31 ± 0.05	0.92 ± 0.22	1.33 ± 0.77 [a]	0.24 ± 0.14

MT and EB22/5 mRNAs were normalized with γ-actin mRNA levels. Results are mean ± S.E. of the Northerns shown in Fig. 3.
[a] $P<0.05$ vs. respective control mice.

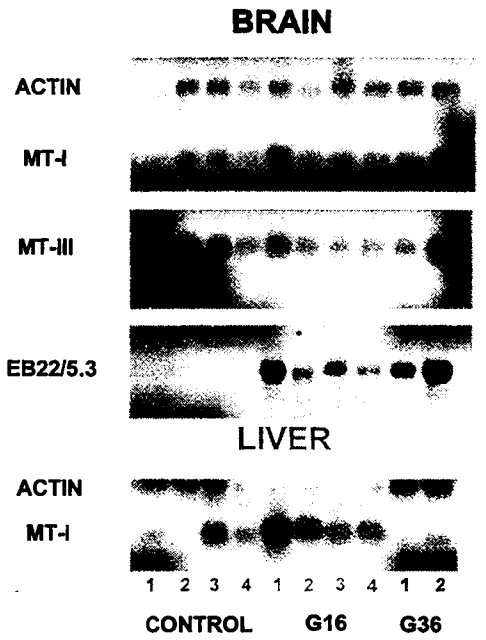

Fig. 3. Northern blot analysis of poly(A)$^+$ RNA (3 μg) isolated from brains and livers of 4 non-transgenic mice (control) and 6 GFAP-IL6 mice, 4 from the low expression G16 line and 2 from the high expression G36 line. Animals 1 and 2 are of 3 months of age. Animals 3 and 4 are of 6 months of age. Blots were hybridized with the actin and MT-I probes together first and, subsequently, with those of MT-III and EB22/5 (in the case of brains) as outlined in Materials and methods.

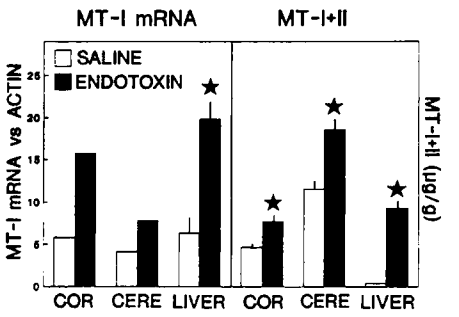

Fig. 4. Effect of endotoxin on mouse brain cortex (Cor) and cerebellum (Cere) and liver MT-I mRNA and MT-I + II protein levels. Mice were injected i.p. with 3 mg/kg endotoxin and were killed 6 h later. Brain MT-I mRNA levels were measured in pooled samples by Northern blotting and densitometric quantification of the autoradiographs whereas liver MT-I mRNA levels were measured individually by dot-blot and scintillation counting of the dots. Results are mean ± S.E. (except brain MT-I mRNA). $n = 6$. $^*P < 0.05$ vs. control mice (Student's t-test).

up- and down-regulation of MT-I and MT-III mRNAs, respectively, in a time-dependent manner. Taken together, the results clearly indicate that the effect of the transgenic expression of IL-6 was more dramatic on brain MT-I + II protein levels than on MT-I mRNA levels.

In contrast to the brain, liver MT-I mRNA and MT protein levels were not affected significantly (Fig. 3); previously, it has been noted that IL-6 does not leak significantly from the brain in the GFAP-IL6 mice (I.L. Campbell, unpublished observation).

To determine if MT gene expression was regulated acutely in the brain in a similar pattern to the GFAP-IL6 mice we next investigated the effects of endotoxin administration. Fig. 4 shows the effect of endotoxin on mouse brain and liver MT-I mRNA and total MT-I + II protein levels. As expected from previously published reports studying MT mRNA levels [11,49], endotoxin administration significantly increased brain and liver MT-I mRNA levels which is accompanied by a concomitant increase in total MT-I + II protein levels. These results demonstrate that in an acute inflammatory state, the MT-I + II protein levels appear to reflect closely the MT-I mRNA levels. This is in contrast to the poor correlation between MT protein and mRNA levels in the brain exposed to chronic production of IL-6 as seen in the GFAP-IL6 mice (see above). Brain MT-III mRNA levels were also measured in pooled samples, and in contrast to MT-I, were found to be decreased by endotoxin (59.7 vs. 19 for control and endotoxin mice, respectively, arbitrary units).

In order to examine the effect of IL-6 on the MT-I + II levels on the two main cell types of the brain, neurons and astrocytes, we carried out several experiments with primary cultures enriched in these cells (data not shown). The results indicated a marginal effect, if any, of IL-6 on MT-I + II protein levels of both cell types, suggesting that in vivo IL-6 could interact with other factors to induce MT synthesis in the brain.

Table 2
Relative levels of the MT-I and MT-III mRNAs of cerebrum, cerebellum and liver

	Brain MT-I mRNA		Brain MT-III mRNA		Liver MT-I mRNA	Liver MT-I + II protein (μg/g)
	Cerebrum	Cerebellum	Cerebrum	Cerebellum		
Control 3 months	1.35 ± 0.32	1.36 ± 0.40	3.04 ± 0.78	14.8 ± 4.68	1.12 ± 0.43	11.7 ± 2.79
Control 6 months	1.13 ± 0.06	1.70 ± 0.37	3.67 ± 0.66	10.5 ± 1.77	6.01 ± 1.78	
G16 3 months	1.91 ± 1.11	1.32 ± 0.35	4.70 ± 2.05	8.49 ± 0.31	0.46 ± 0.12	15.1 ± 2.01
G16 6 months	0.93 ± 0.14	2.71 ± 0.33	2.34 ± 0.19 [a]	6.14 ± 0.42	3.70 ± 2.29	
G36 3 months	0.81 ± 0.08 [a]	1.99 ± 0.41	1.96 ± 0.09 [a]	4.96 ± 0.72 [a]	3.61 ± 1.66	15.8 ± 1.30

MT and EB22/5 mRNAs were normalized with γ-actin mRNA levels. Results are mean ± S.E. of the Northerns carried out similarly to those of Fig. 3. $n = 2$–3.

[a] $P < 0.05$ vs. respective control mice.

4. Discussion

In this study, MT regulation was examined in transgenic mice in which chronic expression of IL-6 was targeted to astrocytes under the regulatory control of the GFAP promoter [6,7]. These mice show profound effects of IL-6 in the brain, with clear symptoms of neurodegeneration, astrocytosis, microgliosis, angiogenesis and up-regulation of several inflammatory and other host-response genes, including IL-1 α/β, TNFα, GFAP, ICAM-1, the acute-phase protein EB22/5 and complement C3 protein. The present results indicate that brain MT regulation is also profoundly affected by the transgene production of IL-6. Endotoxin treatment also significantly increased brain MT-I mRNA and total MT-I + II protein levels. Since it is well-known that peripheral administration of endotoxin increases the release of a number of cytokines, including IL-6 in the brain [33], it seems likely that endotoxin induced brain MT-I + II synthesis through cytokine production. Indeed, IL-6 is a potent MT-I + II inducer in other tissues [9,11,41].

MT-I + II protein levels were significantly increased by transgenic IL-6 expression in specific brain areas, viz. cerebellum, medulla plus pons, hypothalamus and remaining brain but not in the hippocampus. The induction of MT-I + II levels correlated with the associated inflammatory response observed in these brain areas [6,7]. The effect of transgenic IL-6 expression on brain MT-I + II protein levels is in accordance with previous results which had shown that these proteins are increased by factors, such as stress [13,17,19,21], experimental brain damage [37] and in neurodegenerative disorders, such as AD or amyotrophic lateral sclerosis [12,42]. In addition, IL-6 is a major regulator of liver MTs [16]; given the essential role of this cytokine in the expression of the liver acute-phase protein response [1,16], it has generally been assumed that liver MTs could be members of the acute-phase response. The results with GFAP-IL6 mice suggest that MT-I and MT-II could also be involved in such a response in the brain, presumably under the control of IL-6 and/or a downstream factor activated by IL-6. The results with endotoxin also support this assumption. Our findings, therefore, add MT-I + II to the other acute-phase response genes EB22/5 and complement C3 that are up-regulated in the brain of the GFAP-IL6 mice.

Surprisingly, whereas MT-I + II levels were strongly increased in the brains of both transgenic lines at 3 months of age, MT-I mRNA levels were only marginally increased in the G16 line and tended to decrease in the G36 line. MT-I mRNA levels also tended to be decreased at 6 months of age in the G16 line. In view of the fact that the brain is chronically exposed to IL-6 in the GPAP-IL6 mice, this finding may reflect a compensatory down-regulation of the MT-I + II expression. Somewhat comparable results have been recently reported for the cerebral expression of the complement C3 gene in GFAP-IL6 mice [4]. This lack of concordance between MT mRNA and protein levels is not likely to be attributable to a lack of sensitivity or specificity of the analysis of the mRNA levels performed since, using the same hybridization and washing conditions, we could observe a clear increase in brain MT-I mRNA which was paralleled by increased protein levels in mice injected with endotoxin. This suggests that in the GFAP-IL6 mice, a chronic model of IL-6 stimulation, factors other than MT mRNA levels are important for the control of brain MT levels. Indeed, in line with this notion, the evidence for the post-transcriptional control of MT gene expression is mounting [29,46].

Several studies suggest that in the brain astrocytes are the main site of MT-I + II production [20,37]. We have analyzed the effect of IL-6 on MT-I + II protein levels in primary cultures of mouse astrocytes and neurons. Similar to previous reports [20,27], we found that IL-6 did not affect mouse astrocyte MT levels. In addition, we were unable to find an effect of IL-6 on MT levels in mouse neurons. Therefore, these results suggest that IL-6 probably interacts with other factors in vivo for inducing brain MT. The reason for the high inducibility of MTs in established astrocytic and neuronal cell lines [5,26], in contrast to primary cultures, is unknown.

Regarding MT-III, previous reports have demonstrated a substantial differential control of this isoform compared to the widely expressed MT-I + II isoforms [10,23,34,36,49]. In most cases, inducers, including endotoxin, produce up-regulation of the latter with no change or down-regulation of the former and, accordingly, we observed increased and decreased MT-I and MT-III mRNA levels, respectively, in endotoxin-injected mice. The results with GFAP-IL6 mice were somewhat similar to those for MT-I mRNA since there was a tendency to be increased at 3 months of age in the G16 line whereas a down-regulation was observed at 6 months. While cerebral MT-III mRNA levels were significantly decreased at 3 months in the G36 line. The age- and IL-6-dependent down-regulation of MT-III mRNA levels appeared to be more dramatic and consistent than for MT-I mRNA, suggesting a differential regulation of these two MT isoforms by IL-6. Since GFAP-IL6 mice develop a progressive neurodegenerative process, the observed down-regulation of MT-III in these animals is reminiscent of that observed in patients with the well-known brain neurodegenerative disease, Alzheimer disease (AD) [45]. Interestingly, the down-regulation of MT-III mRNA levels was higher in cerebellum than in cerebrum which correlates with the higher neurodegeneration of this brain area.

In summary, the present results demonstrate that the widely expressed MT isoforms, MT-I + II, are strongly induced in the brain of transgenic mice which have the cytokine IL-6 under the control of the GFAP promoter. The induction is specific for some brain areas, especially the cerebellum, medulla plus pons and hypothalamus, whereas other areas, such as the hippocampus, are insensi-

tive to increased IL-6 levels. Furthermore, the i.c.v. administration of IL-6 also induces brain MT-I + II production in specific brain areas in the rat (unpublished data). Thus, the results suggest that IL-6 is a major regulator of brain MT-I + II and that these proteins could function as acute-phase proteins in the brain as appears to be the case in the liver.

Acknowledgements

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Endotoxin and intracerebroventricular injection of IL-1 and IL-6 induce rat brain metallothionein-I and -II

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Abstract

Metallothioneins (MTs) are a family of proteins which in mammals is comprised of four isoforms (MT-I–IV). MT-I and MT-II are expressed in many tissues, whereas MT-III is expressed exclusively in the central nervous system (CNS). In contrast to the liver, the knowledge of the regulation of the different MT isoforms in the brain is scarce. A number of cytokines have been shown to be important regulators of MT synthesis *in vivo* and *in vitro*. In accordance with this concept, the i.p. administration of endotoxin, which elicits the release of cytokines not only in peripheral tissues but also in the brain, caused an overall increase of MT-I + II levels in the rat brain which was very significant in medulla + pons and cerebellum. Among the putative cytokines involved in endotoxin-elicited brain MT-I + II induction, interleukin-1 (IL-1) and interleukin-6 (IL-6) are likely candidates. These cytokines have a variety of effects in the brain, and they are major regulators of MT-I + II synthesis in tissues such as the liver. Here we show the administration of IL-1 and IL-6 into the third ventricle increased MT-I + II protein levels in specific brain areas in the rat. IL-1 tended to increase MT-I + II levels in all brain areas studied, but significantly in the striatum, hypothalamus, medulla + pons and cerebellum. The effect of IL-6 was more restricted, but a significant increase of MT-I + II levels was still observed in frontal cortex, hypothalamus and cerebellum. The results suggest that IL-1 and IL-6 are important regulators of brain MT-I + II and that these cytokines could mediate MT-I + II induction after an immunological insult. © 1997 Elsevier Science Ltd

Key words: ???

Introduction

Cytokines are produced in the brain by resident cells, particularly macrophages and microglia, and play a key role during infectious and inflammatory diseases of the central nervous system (CNS). Most cytokines are synthesized in response to different stimuli including bacterial lipopolysaccharides, viral antigens or other cytokines (Pousset, 1994; Hopkins and Rothwell, 1995; Dinarello, 1996). Cytokines such interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) have been proposed as principal mediators of central effects including fever, behaviour alterations induced by sickness or activation of the hypothalamic-pituitary-adrenal (HPA) axis (Besedovsky *et al.*, 1986; Dinarello, 1988; Laye, 1994; Chai *et al.*, 1996; Dinarello, 1996; Fiore *et al.*, 1996; Zhou *et al.*, 1996).

It is known that the exogenous administration of IL-1 and IL-6 increases metallothionein (MT) expression in different tissues, specially the liver (Bell *et al.*, 1987; Cousins and Leinart, 1988; De *et al.*, 1990; Liu *et al.*, 1991; Sato *et al.*, 1992) and some cell lines (Friedman and Stark, 1985; Karin *et al.*, 1985; Schroeder and Cousins, 1990; Bauer *et al.*, 1993; Vanguri, 1995). It has also been demonstrated that transgenic mice expressing IL-6 under the control of the glial fibrillary acidic protein gene (GFAP) promoter induces MT (Hernández *et al.*, 1997).

MTs are a family of low molecular-weight, cysteine-rich, heavy metal-binding proteins (Kägi and Kojima, 1987). There are several isoforms in mammals; 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 localized in the brain and stratified squamous epithelia, respectively (Uchida *et al.*, 1991; Palmiter *et al.*, 1992; Quaife *et al.*, 1994). The biochemical and molecular properties of the MT-I and MT-II isoforms are well documented (Kägi and Kojima, 1987; Suzuki *et al.*, 1993). To date, our knowledge of the physiological functions of MT-I and MT-II and the factors that control their expression in the brain

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is scarce in comparison with the liver. It is known that brain MT-I+II are regulated *in vivo* by the essential metals Zn and Cu (Ebadi, 1986; Paliwal *et al.*, 1990; Gasull *et al.*, 1994b; Hidalgo *et al.*, 1994), glucocorticoids (Gasull *et al.*, 1994b), stress stimuli (Gasull *et al.*, 1994a) and endotoxin (Choudhuri *et al.*, 1995; De *et al.*, 1990). MT-I+II isoforms have also been reported to be induced when brain injury or inflammation occurs in the central nervous system (CNS), such as after kainic-induced seizures (Dalton *et al.*, 1995), freeze lesions (Penkowa and Moos, 1995) or in a transgenic mice expressing IL-6 under the regulatory control of the GFAP promoter (Hernández *et al.*, 1997), which develop a chronic progressive neurodegenerative disease (Campbell *et al.*, 1993). *In vitro* studies have shown the induction of brain MT-I+II by dexamethasone, heavy metals and IL-1 (Kikuchi *et al.*, 1993; Hidalgo *et al.*, 1994; Kramer *et al.*, 1996a,b).

Despite the previous reports, increases in brain MT-I+II protein levels *in vivo* after either endotoxin or cytokines have not been demonstrated. In the present report, brain MT-I+II induction was studied after peripheral administration of endotoxin and central injection of IL-1 and IL-6. The results demonstrate that IL-1 and IL-6 are inducers of brain MT-I+II, suggesting that these cytokines could be involved in the control of the MT-I+II genes in the brain during injury and inflammation.

Experimental Procedures

Maintenance of the animals

Adult male Sprague–Dawley weighing 250–300 g were housed individually because of the experimental procedures carried out in the i.c.v. injected rats, and maintained under standard laboratory conditions (light cycle from 07:30–19:30 h, temperature 22°C, food and water provided *ad libitum*) for at least one week before starting the experiments.

Peripheral administration of endotoxin

Endotoxin (lipopolysaccharide from *Escherichia coli* 0127:B8) was purchased from Sigma (St. Louis, MO). Rats were injected with 1 mg/kg intraperitoneally (i.p.) and the animals were killed 24 h later to allow MT synthesis to proceed. Control rats were injected with saline. MT-(I+II) protein levels were measured in the following areas: frontal cortex, cerebellum, medulla+pons and remaining brain.

Intracerebroventricular (i.c.v.) administration of recombinant human IL-1 and IL-6

Rats were injected with 1000 U of recombinant human IL-1α (kind gift from Hoffman-La Roche, Nutley, NJ, U.S.A.) in the third ventricle in a volume of 10 µl of sterile saline. Control rats received saline. Under ether anesthesia, the animals were placed in a sterotaxic instrument (Stoelting, IL, U.S.A.) and were injected in the third ventricle (4.2 mm posterior from bregma, 0.0 mm lateral and 4.5 below the skull surface at the point of entry) according to the atlas of Paxinos and Watson (1986). After surgery, the animals were returned to the cages and killed 24 h later to allow MT synthesis to proceed. MT-(I+II) protein levels were measured in the following brain areas: hippocampus, striatum, frontal cortex, cerebellum, hypothalamus, medulla+pons and remaining brain.

Another group of rats were injected into the third ventricle with 1000 U of recombinant human and mouse IL-6 (Boehringer Mannhein, Mannhein, Germany) in a volume of 10 µl of sterile saline. Control rats received saline. MT-(I+II) protein levels were measured in the same areas above described for IL-1.

MT protein assay

MT levels were measured by radioimmunoassay (RIA) as described previously (Gasull *et al.*, 1993) using a polyclonal antibody which cross-reacts with rat MT-I and MT-II but not with MT-III. Unfortunately, MT-III protein levels could not be measured because antibodies are not commercially available.

Statistical assays

Results were analyzed with Student's *t*-test. Logarithmic transformation of the data was applied when necessary.

Results

Brain MT-(I+II) induction by peripheral administration of endotoxin

Figure 1 shows the effect of a i.p. injection of endotoxin on MT(I+II) levels of cerebellum, frontal cortex, medulla+pons and remaining brain. As expected from previous reports (De *et al.*, 1990; Zheng *et al.*, 1995), endotoxin administration significantly increased brain MT-I+II protein levels.

Brain MT-(I+II) induction by central administration of IL-1 and IL-6

Figure 2 shows the effect of IL-1 administered in the third ventricle on MT-I+II levels of specific brain areas. There was a clear trend in all areas, but a statistically significant ($P < 0.05$) increasing effect of IL-1 was observed only in striatum, hypothalamus, medulla+pons and cerebellum. IL-6, in contrast, had a more restricted effect, since no

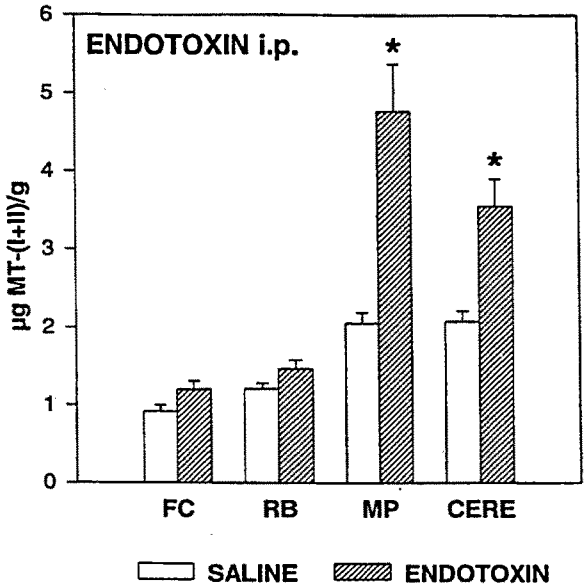

Fig. 1. Effect of endotoxin on MT-(I+II) protein levels (µg MT/g tissue). Results are mean ± SE ($n = 6$). The rats were injected i.p. with 1 mg/kg and killed 24 h later. *P at least < 0.05 vs saline rats). FC: Frontal cortex, RB: Remaining brain, MP: Medulla+pons, CERE: Cerebellum.

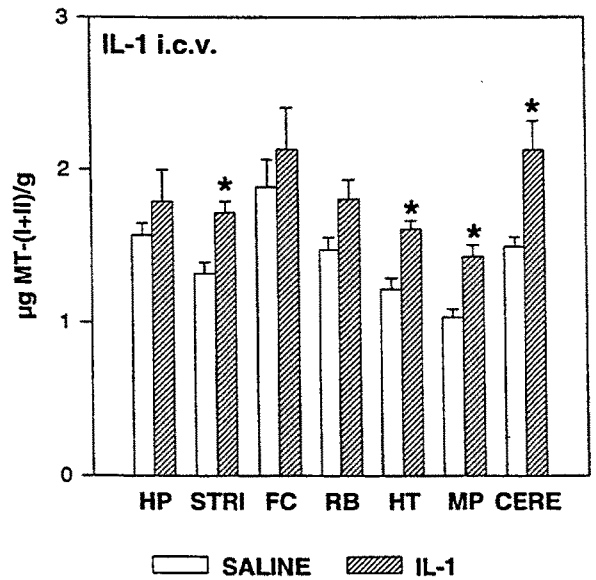

Fig. 2. Effect of central administration of IL-1 (third ventricle) on MT-(I+II) levels in specific brain areas. The brains were removed immediately after sacrifice, dissected and frozen at −80°C. All areas were processed the same day of the MT assay. Results are mean ± SE ($n = 3$–4). *P at least < 0.05 vs saline rats). HP: Hippocampus, STRI: Striatum, FC: Frontal cortex, RB: Remaining brain, HT: Hypothalamus, MP: Medulla+pons, CERE: Cerebellum.

inducing trends were observed in brain areas such as hippocampus, medulla+pons and remaining brain (mainly cortex) (Fig. 3). It did, however, significantly ($P < 0.05$) increase MT-I+II levels in frontal cortex, hipothalamus and cerebellum. The results, however, clearly suggest a role of both interleukins in brain MT regulation.

Discussion

Endotoxin administration (i.p.) increased brain MT-I+II levels significantly in a brain area-specific manner, as measured 24 h after its administration. This is in accordance with previous studies where MT mRNA levels were measured in the brain (De *et al.*, 1990; Itano *et al.*, 1991; Palmiter *et al.*, 1992; Zheng *et al.*, 1995). In tissues such as the liver, it appears that endotoxin induces MT-I+II levels through cytokines (Liu *et al.*, 1991). This is also likely in the brain, since several reports have demonstrated that central but also peripheral administration of endotoxin increase the expression of a number of cytokine transcripts in the brain, including those for IL-1β, IL-6 and TNF-α (Muramami *et al.*, 1993; Laye *et al.*, 1994). Furthermore, increased bioactive levels of IL-1 in the brain after endotoxin have also been observed (Quan *et al.*, 1994).

IL-1 and IL-6 are major regulators of MT-I+II synthesis in peripheral tissues (Karin *et al.*, 1985; Cousins and Leinart, 1988; De *et al.*, 1990; Schroeder and Cousins, 1990; Liu *et al.*, 1991) and are therefore putative

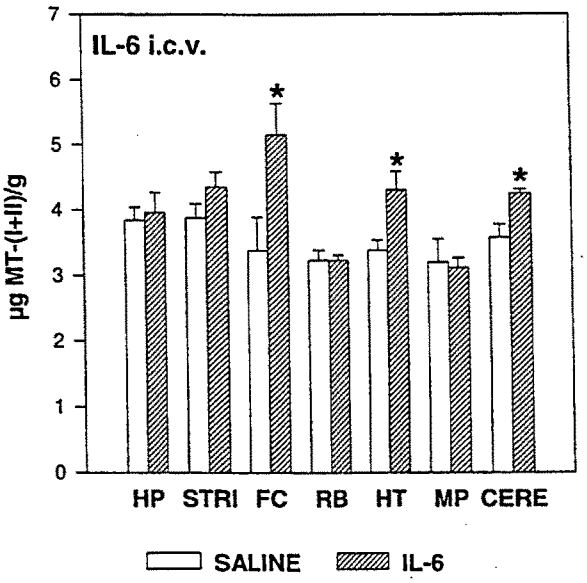

Fig. 3. Effect of i.c.v administration of IL-6 (third ventricle) on MT-(I+II) levels in specific brain areas. The brains were processed as in Fig. 2. Results are mean ± SE ($n = 4$–5). *P at least < 0.05 vs saline rats). HP: Hippocampus, STRI: Striatum, FC: Frontal cortex, RB: Remaining brain, HT: Hypothalamus, MP: Medulla+pons, CERE: Cerebellum.

candidates for mediating brain MT-I+II response to endotoxin. CNS sources of IL-1 and IL-6 includes microglia, astrocytes, neurons and endothelial cells (Arvin *et al.*, 1996), and specific binding sites for IL-1 and IL-6 have been localized in the brain, especially in the hippocampus, hypothalamus and anterior pituitary (Bandtlow *et al.*, 1990; Schöbitz *et al.*, 1992; Takao *et al.*, 1993). Thus, to give some insight into the putative role of these cytokines on brain MT-I+II regulation, we carried out experiments where IL-1 and IL-6 were injected intracerebroventricularly. The results clearly demonstrate that both cytokines increase brain MT-I+II levels in an area-specific manner. IL-1, however, appeared to have a more general effect than IL-6, which in principle is consistent with the fact that IL-1 is capable of inducing the release of other cytokines, such as TNF-α and IL-1β, and also IL-6 (Laye *et al.*, 1994), which are known inducers of MT-I+II synthesis (De *et al.*, 1990; Sato *et al.*, 1992). The results are also consistent with what is known of the role of these cytokines in other tissues regarding MT-I+II regulation (see Introduction), but also point out that both IL-1 and IL-6 will likely act in concert with other factors. This is substantiated by the fact, for instance, that IL-6 did not affect MT-I+II levels in the hippocampus despite the presence of IL-6 receptors in this area (see above). It is also known that these cytokines do not induce MT synthesis in primary cultures of either neurons or astrocytes (Hidalgo *et al.*, 1994; Kramer *et al.*, 1996a; Hernández *et al.*, unpublished results). Finally, MT-I+II synthesis in transgenic mice expressing IL-6 under the regulatory control of the GFAP promoter correlates more with the inflammatory response of these mice than with the IL-6 transgene expression (Hernández *et al.*, 1987). Nevertheless, the results do demonstrate that if IL-1 or IL-6 are released *in vivo* they are likely to induce MT-I+II synthesis. The teleological basis for this MT response could reasonably be cytoprotection against heavy metals or free radical-induced damage that one could expect after brain injury or inflammation (Aschner, 1996). Hence, MT-I+II might be considered acute-phase proteins in the CNS as they appear to be in the liver.

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Anex 1

Role of zinc, dexamethasone and IL-6 regulating metallothionein -I and -II in primary cultured astrocytes and neurons of mouse.

ROLE OF ZINC, DEXAMETHASONE AND IL-6 REGULATING METALLOTHIONEIN -I AND -II IN PRIMARY CULTURED ASTROCYTES AND NEURONS OF MOUSE.

EXPERIMENTAL PROCEDURES

Primary cultures and experiments. Astrocyte-enriched and neuronal primary cultures were prepared from the brain hemispheres of newborn (within the first 24h) and 16-day-old-embryos of Swiss mice, respectively, following the method of Löffner et al. (Löffner *et al.*, 1986) as previously described for the rat (Hidalgo *et al.*, 1994). For astrocyte cultures tissue was dissociated into cells by successive passages through nylon cloths of 211- and 135- μm mesh. Brain cells were suspended in 90% Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 20 units/ml penicillin and 20 μg/ml streptomycin (0.6×10^6 viable cells/ml) and then seeded onto 35-mm diameter plastic Petri dishes (2 ml) and incubated at 37°C in a humidified atmosphere of 90% air/10% CO_2. Medium was changed once a week, and confluent monolayers were used at 13-14 days in culture because at this time cells are confluent and the number of contaminating cells is low.

For neuronal cultures, tissue was passed through nylon cloths of 135- and 22- μm mesh and cells were suspended in 90% DMEM, 10% horse serum, 20 units/ml penicillin, and 20 μg/ml streptomycin. Two milliliters of the cell suspension (0.6×10^6 viable cells/ml) were seeded onto 35-mm diameter plastic Petri dishes previously coated with poly-L-α-ornithine (0.01% wt/vol). After 1 day in culture the initial culture medium was replaced by serum-free-glia-conditioned medium containing insulin (5 μg/ml), transferrin (100 μg/ml), putrescine (100 μM), progesterone (20 nM), and Na_2SeO_3 (30 nM). Two days later cytosine arabinoside (5 μM final concentration) was added and after 24h the medium was replaced by hormone-supplemented conditioned medium. Neuronal cultures were incubated in the same way as the astrocytes and were used at 11-12 days in culture.

At the day of the experiment the medium was replaced by DMEM medium containing 2 mg/ml of BSA and 10 μM of $ZnSO_4$. Then, the MT-I+II inducers were added to the medium in small aliquots to get the following final concentrations: Zn^{2+} (50 μM), the synthetic glucocorticoid dexamethasone (Dex) (10^{-6} M), and/or IL-6 (100 Units/ml) (Boehringer Mannheim, Mannheim, Germany). The cells were incubated for

24h, and the medium was then removed and the cells gently washed two times with 1 ml of 10 mM Tris-HCl (pH 8.2), scraped with a spatula and sonicated for 20 s at 4°C. After centrifugation (3000 x *g*, 15 min, 4°C), the supernatant was stored at -20°C until required for MT assay.

MT protein assay. MT levels were measured by radioimmunoassay (RIA) as described previously (Gasull *et al.*, 1993) using a polyclonal antibody which cross-reacts with rat MT-I and MT-II but not with MT-III. We have also observed cross-reactivity of the antibody with mouse MT-I and MT-II.

Indirect immunofluorescence staining of MT-(I+II) in astrocytes and neurons primary cultures. The cultures were developed as described above. Immunostaining using rabbit antisera against MT-(I+II) was performed as described by Weber et al. (Weber *et al.*, 1975) modified by Löffner et al. (Löffner *et al.*, 1986). At the day of the experiment the cells were incubated for 24h with the inducers Zn^{2+} (50 μM) and dexamethasone (Dex) (10^{-6} M), as described above. Then, the medium was removed and the plates (35-mm diameter plastic Petri dishes) were fixed with 3.5% paraformaldehyde in phosphate-buffered saline, pH 7.2 (PBS) for 45-60 min at 4°C. The cells were washed twice with PBS for 10 min and once with PBS containing 0.3% Triton X-100 for 10 min. Later, the cells were incubated with PBS containing 10% fetal bovine serum (FBS) at room temperature for 30 min. MT antisera (developed in our laboratory, see (Gasull *et al.*, 1993)) were diluted in PBS with 0.1% Triton X-100 containing 50% FBS (1:200). The plates were incubated with 700 μl of MT antisera dilution for 2 h at 4°C. The cells were washed twice with PBS containing 0.1% Triton X-100 for 10 min at room temperature with a slow shaking. FITC anti-rabbit IgG (Sigma, USA) (diluted 1:30 in PBS with 0.1% Triton X-100 containing 50% FBS) were added to the plates (700 μl each plate) and incubated for 2h at room teperature in a dark and humidified chamber. After washing twice for 10 min with PBS containing 0.1% Triton X-100 in a slow shaking, coverslips were added to the plates previously embedded in PBS-glycerol (1:1, v/v). The plates were viewed in a Zeiss fluorescence microscope equipped with a FITC-fluorescence. Photomicrographs were taken with Ektachrome Elite II color slide film (200 ASA).

Indirect immunofluorescence staining of glial fibrillary acidic protein (GFAP) and neuron-specific enolase (NSE) in astrocytes and neurons primary cultures. Immunostaining using rabbit antisera against GFAP and NSE (Dako, Denmark) was

performed as described by Weber et al. (Weber *et al.*, 1975) modified by Löffner et al. (Löffner *et al.*, 1986). At the day of the experiment the cells were incubated 24h with DMEM medium containing 2 mg/ml of BSA and 10 μM of $ZnSO_4$. The staining was performed as described above. GFAP and NSE antisera were diluted 1:300. Anti-rabbit IgG FITC conjugate (1:30) (Sigma, St. Louis, USA)was used as secondary antiserum in NSE treated plates. Anti-rabbit IgG-Rhodamine (1:10) (Boehringer Mannheim, Mannheim, Germany) was added to GFAP treated plates.

Statistical analysis. Results were analyzed with two-way or one-way ANOVA. Logarithmic transformation of the data was applied when necessary. Multiple comparisons of the means were carried out with the Duncan procedure.

RESULTS

Immunocytochemical characterization of the cultures. The results of the immunostaining for GFAP and NSE in the primary cultures of astrocytes show a typical immunopositive for GFAP marker and a astrocyte-rich culture where the cells are distributed homogenously (figure 1). Furthermore, the cultures show a reduced NSE imnunostaining. In the primary culture of neurons, the immunostaining for GFAP and NSE reveal a immunopositive distribution of the NSE marker in the culture and a clear negative immunostaining for GFAP (figure 2).

Effect of Zn^{2+}, Dex and IL-6 on MT-(I+II) levels in astrocytes and neurons primary cultures. Figure 3 shows the effect of IL-6 alone or together with the MT-I+II inducers Zn^{2+} and dexamethasone. In agreement with previous studies (Hidalgo *et al.*, 1994; Kramer *et al.*, 1996a; Kramer *et al.*, 1996b), Zn^{2+} and dexamethasone significantly increased MT-(I+II) levels in astrocytes, whereas IL-6 did not affect MT levels either when added alone or in combination with the other inducers. Surprisingly, in cultured mouse neurons there was no induction of MT-(I+II) by Zn^{2+}, Dex or IL-6, but the combination of these factors did slightly increase MT levels.

Immunocytochemical localization of MT-(I+II) in primary cultures of astrocytes from newborn mouse. Figure 4 shows the induction and the localization of MT-(I+II) in primary cultures of astrocytes. The cells were stimulated with 50 μM Zn^{2+} and Dex (10^{-6} M), and incubated for 24h with the inducers. The fluorescence intensity is increased in the treated cells as expected with the MT-(I+II) levels obtained in the figure 3 and previous reports (Hidalgo *et al.*, 1994; Kramer *et al.*, 1996a; Kramer *et al.*, 1996b).

Immunocytochemical localization of MT-(I+II) in primary cultures of neurons from embryonic (E16) mouse brain cells. The induction and the localization of MT-(I+II) in primary cultures of astrocyte is showed in the figure 5. The cells were stimulated with 50 μM Zn^{2+} and Dex (10^{-6} M), and incubated for 24h with the inducers. The induction is less than in astrocytes but MT-(I+II) is increased clearly in the axons of the treated cells. It's known from previous results (Hidalgo *et al.*, 1994) that the levels of MT-(I+II) in the neurons are 10-fold less than the levels in the astrocytes.

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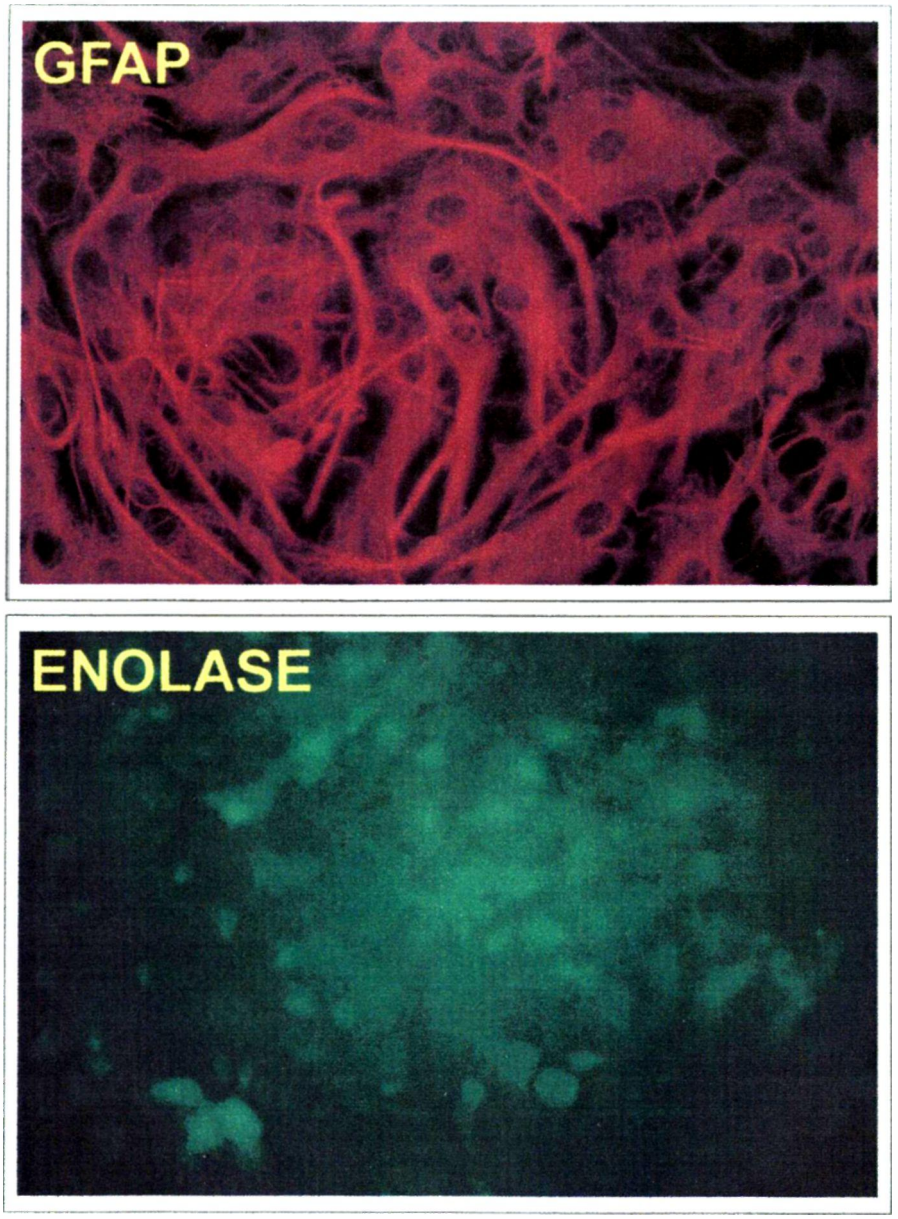

FIGURE 1. Immunofluorescence of GFAP and NSE in astrocytes primary cultures of mouse. As expected, an astrocyte-enriched cells were observed with the GFAP staining.

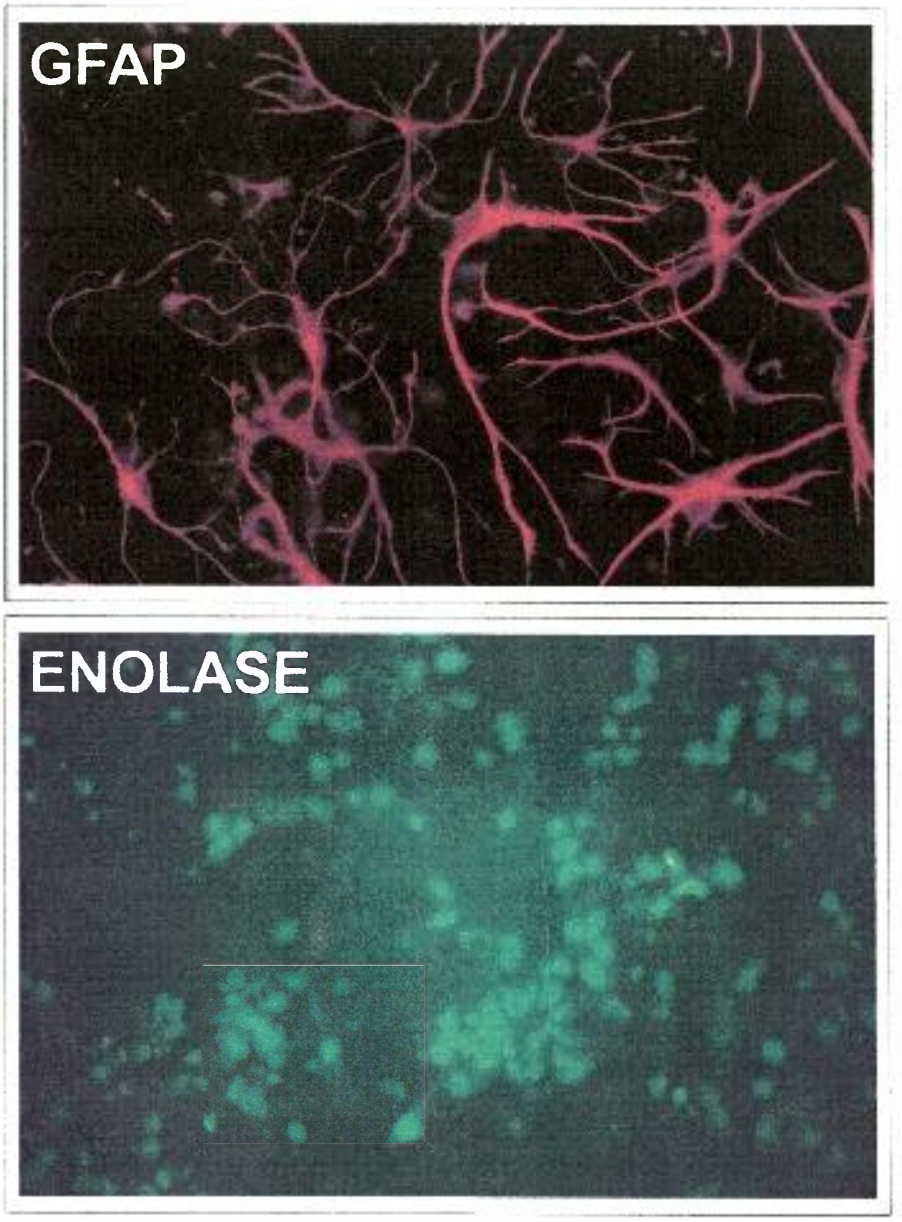

FIGURE 2. Immunofluorescence of GFAP and NSE in neurons primary cultures of mouse. As expected, a neuron-enriched cells were observed with the NSE staining. Some cells were observed with GFAP staining and with a different morphology vs figure 1.

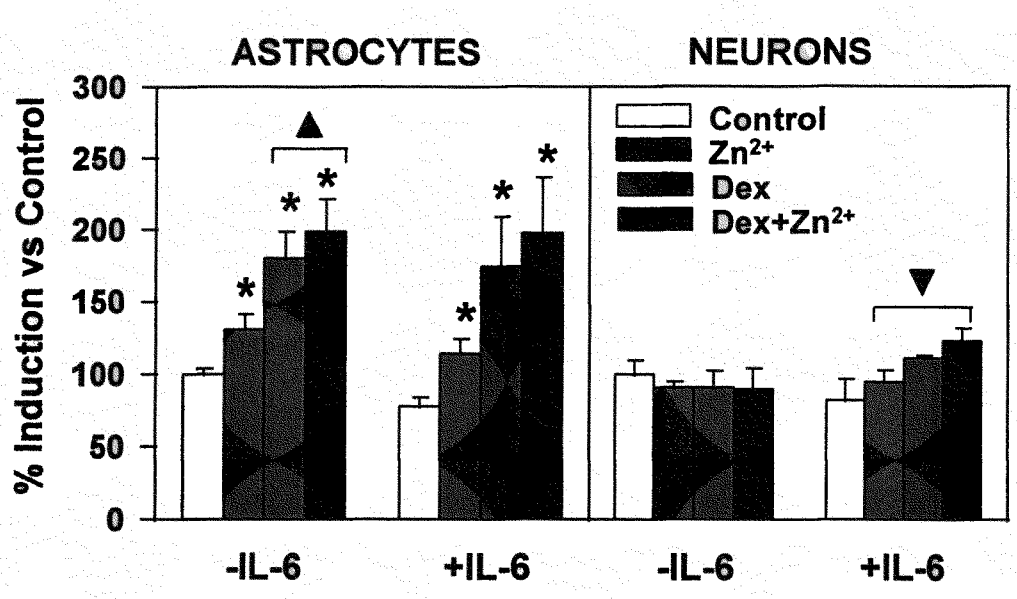

FIGURE 3. Effect of Zn^{2+}, Dex and IL-6 on MT-(I+II) protein levels of cultured astrocytes and neurons of mouse hemispheres. In astrocyte cultures, the MT levels were significantly increased by Zn^{2+}, Dex and Zn^{2+}+Dex treatments without IL-6 ($p<0.05$). The simultaneous presence of IL-6 did not affect MT-(I+II) levels. In neurons cultures, Zn^{2+}, Dex and Zn^{2+}+Dex treatments at the concentrations studied did not affect MT-(I+II) levels, but in the presence of IL-6 a significant induction of MT-(I+II) was observed ($p<0.05$). ✳ indicates significantly different from control cells; ▲ significantly different from Zn^{2+}-treated cells. ▼ significantly different from -IL-6 cells.

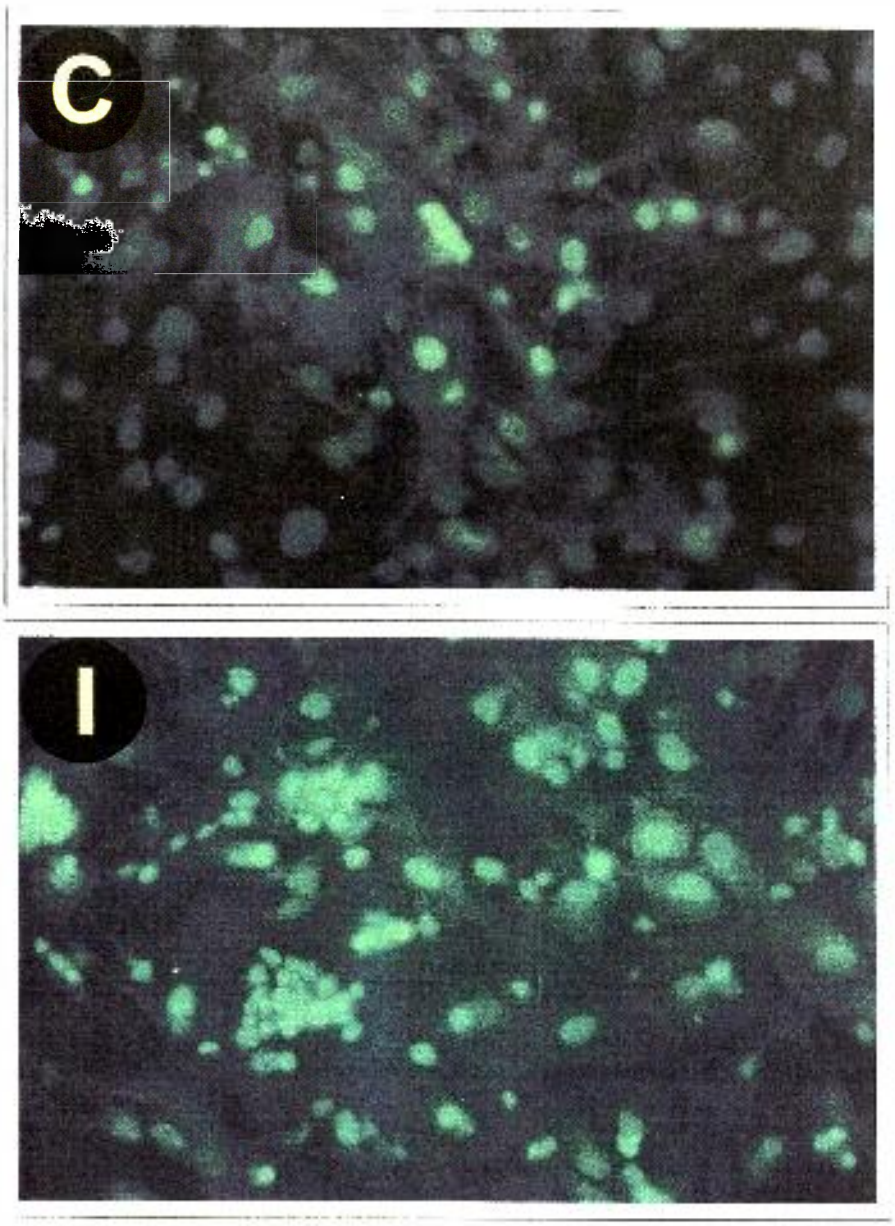

FIGURE 4. Immunofluorescence of MT-(I+II) in astrocytes primary cultures of mouse. **C** indicates untreated cells. **I** indicates astrocytes treated with 50 μM Zn^{2+} + 10 μM Dex.

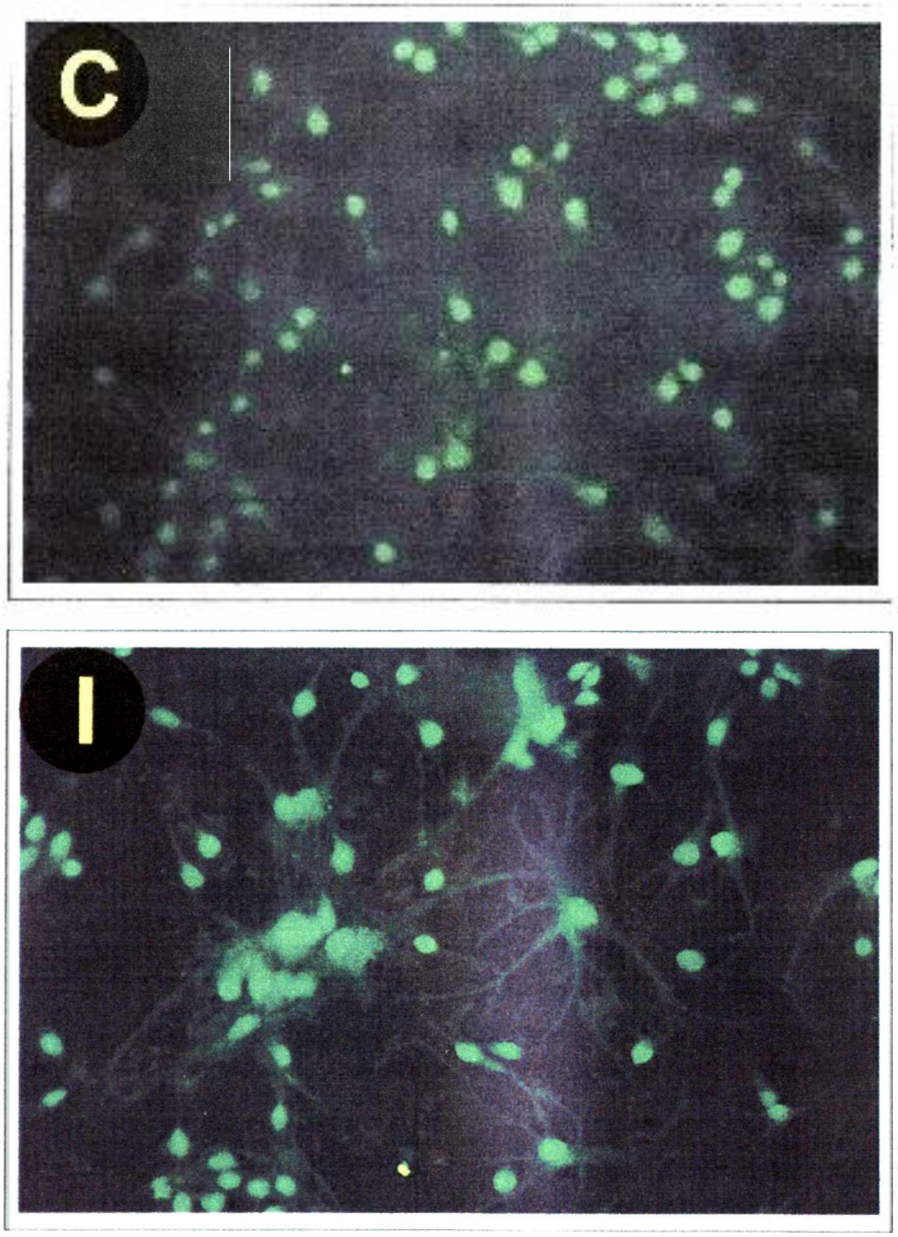

FIGURE 5. Neurons primary cultures of mouse showing immunofluorescence of MT-(I+II) antibody. **C** indicates untreated cells. **I** indicates neurons treated with 50 μM Zn^{2+} + 10 μM Dex.

Discussió

DISCUSSIÓ

Els estudis presentats en aquesta Tesi han estat realitzats amb la finalitat d'aprofondir més en la caracterització de la regulació de les metal.lotioneïnes. D'aquesta forma, els estudis s'han centrat en dos òrgans, el fetge i el cervell, el primer com un dels principals llocs d'expressió i amb més coneixement sobre les MTs, i el segon com un dels òrgans més estudiats des del descobriment de la MT-III. Per una altra banda ens hem centrat en dues situacions fisiològiques, la inflamació i l'estrès, com marc de referència als nostres estudis sobre la regulació de les MTs.

Fetge

La MT s'indueix en resposta a una amplia sèrie d'agents estressants (Oh *et al.*, 1978). Com era d'esperar, l'estrès d'immobilització va induir la síntesi de MT hepàtica en tots els experiments realitzats (Hidalgo *et al.*, 1988c). Si bé el control de la expresssió gènica d'aquestes proteínes està ben caracteritzat, es desconeixen quins factors intervenen en la inducció de les MTs per l'estrès. En aquest treball hem evaluat el possible paper dels glucocorticoides i de les citocines com la IL-6, l'IFN-γ i el TNF-α.

Abans d'entrar amb aquests factors es interessant assenyalar els resultats obtinguts en relació al paper del Zn en la resposta de la MT hepàtica a l'estrès. El paper del Zn com inductor de la MT-(I+II) en el fetge està bastant ben caracteritzat. A més de ser un inductor primari (Bremner and Davies, 1975), la privació de Zn disminueix la síntesi de MT, i en situacions d'estrès el Zn sèric disminueix i s'eleva el Zn hepàtic, la qual cosa sembla ser conseqüència de la inducció de la MT hepàtica i la posterior unió del Zn a la proteïna (Cousins, 1986; Bremner and Beattie, 1990). Això podria suggerir que la mobilització del Zn des dels teixits perifèrics no seria important. Malgrat això, s'han observat alguns efectes sinèrgics del Zn i altres inductors de la MT, entre ells l'estrès (Hidalgo *et al.*, 1988a). En aquest treball es va tornar a examinar aquest sinergisme i es va comprovar que existeix en els cas de l'estrès + Zn, però no en el cas de l'estrès + endotoxina. Les dades suggereixen que la mobilització del Zn durant l'estrès des dels teixits perifèrics pot contribuir a la síntesi hepàtica de la MT. Per una altra banda també s'han observat sinergismes *in vitro*. Així, la IL-6 no té efecte inductor de la MT *per se*, mentre que la presència de

glucocorticoides incrementa els nivells de MT (Schroeder and Cousins, 1990; Coyle *et al.*, 1993). Per tal d'estudiar les interaccions entre diferents inductors hem analitzat el paper combinat de diversos factors com el Zn, glucocorticoides i les citocines TNF-α i l'IFN-γ en cultiu primari d'hepatòcits (Hernández *et al.*, 1996a). Altres estudis (Coyle *et al.*, 1993) indiquen que contrariament a estudis *in vivo*, el TNF i l'IFN tenen un paper modest en la regulació de la MT, el qual no es optimitzat pel Zn ni els glucocorticoides, en contrast amb els resultats observats amb la IL-6 (Schroeder and Cousins, 1990; Coyle *et al.*, 1993).

Els glucocorticoides serien els candidats més lògics per intervenir en la inducció hepàtica de la MT durant l'estrès, ja que s'ha observat la presència de GREs en el gen de la MT (Kelly *et al.*, 1997). Malgrat això, resultats del nostre laboratori i d'altres no abonen aquesta possibilitat en rata (Brady and Burger, 1979; Hidalgo *et al.*, 1988c; Hidalgo *et al.*, 1992). En aquest treball hem tornat a examinar el paper dels glucocorticoides, però en ratolí, encara que s'han realitzat experiments en rata de forma comparativa.

Com s'esperava, l'adrenalectomia (ADX) en rata no disminueix els nivells de MT, si no que causa un increment dels nivells proteics com conseqüència de l'increment del mRNA (Hidalgo *et al.*, 1992; Gasull *et al.*, 1994b). Resulta sorprenent aquest efecte dels glucocorticoides sobre la MT atès que l'administració exògena incrementa els nivells hepàtics de la MT (Etzel *et al.*, 1979; Cousins and Coppen, 1987), així com la Dex també indueix clarament els nivells proteics de la MT-(I+II) *in vitro* (Karin and Herschman, 1979; Karin *et al.*, 1984a; Coyle *et al.*, 1993; Hernández *et al.*, 1996a). La presència de GREs al gen de la MT explica aquests increments tant *in vivo* com *in vitro* (Karin *et al.*, 1984b; Yu and Lin, 1995; Kelly *et al.*, 1997). Malgrat això, aquests resultats i els de treballs anteriors (Brady and Burger, 1979; DiSilvestro and Cousins, 1984; Hidalgo *et al.*, 1988c) mostren que els glucocorticoides estarien inhibint la síntesi constitutiva de MT en la rata. Aquest efecte és revertit per la corticosterona però no per l'aldosterona (Hidalgo *et al.*, 1988c). Durant l'estrès els nivells hepàtics de mRNA de la MT en rates ADX es troba incrementat com a les rates SHAM i tendeix a un cert decreixement a les 8 h d'estrès. En anterior treballs s'ha vist també un cert efecte decreixent però no així de la proteïna, probablement perquè els glucocorticoides podrien permitir el fluxe de MT del fetge a la circulació (Hidalgo *et al.*, 1988c; Hidalgo *et al.*, 1992).

En ratolí, l'ADX i sobretot l'administració de RU 486 ocasionen la disminució dels nivells de mRNA de la MT-I dels nivells proteics de la MT-(I+II) durant l'estrès.

La Dex, però no la progesterona, és capaç de revertir l'efecte del RU 486, la qual cosa suggereix que aquest bloquejant actua a través del receptor dels glucocorticoides. En definitiva, els glucocorticoides intervenen en la resposta a l'estrès de la MT en ratolí, i aquests resultats estan d'acord amb l'efecte de l'estrès sobre aquestes hormones i els recentment descoberts GREs en aquesta espècie (Kelly *et al.*, 1997).

Els resultats obtinguts amb l'ADX i el RU 486 no mostren una reversió completa de la inducció de la MT durant l'estrès, i això ens va fer pensar que potser algun altre factor podria contribuir a la regulació de la MT, ja que aquestes proteïnes són multiregulades. Així, ens vam plantejar la possibilitat que les citocines tinguessin un paper rellevant, molt més quan s'ha demostrat que els nivells circulants d'algunes citocines com la IL-1 , la IL-6 i el TNF poden incrementar-se en situacions d'estrès físic i psicològic (Lemay *et al.*, 1990; Dobbin *et al.*, 1991; Yamasu *et al.*, 1992; Zhou *et al.*, 1993) i, a més, aquestes citocines són inductores de la MT (De *et al.*, 1990).

Primerament es va estudiar la soca C3H/HeJ, ja que té la particularitat de produir nivells molt baixos de citocines en resposta a la endotoxina (Vogel, 1992) i perquè previament s'hi havia observat que produïa menys MT (Maitani *et al.*, 1986; De *et al.*, 1990). Els resultats obtinguts van ser prometedors ja que els nivells de mRNA de la MT-I i els nivells proteics de la MT-(I+II) van ser significativament menors a la soca C3H/HeJ durant l'estrès, abonant la idea que les citocines tenen un paper en la regulació de la MT hepàtica durant l'estrès.

Aquestes dades eren prou interessants com per continuar en aquesta línia. En aquest sentit, una aproximació molt fina per demostrar el paper d'una citocina en el control de l'expressió d'un gen com el de la MT és la utilització d'animals *knock-outs*, ja sigui del gen o del seu receptor. D'aquesta forma, i gràcies a la cessió de varies línies de ratolins *knock-outs* per la IL-6, el TNF-αR1 o l'IFN-γR1, hem estudiat la resposta a l'estrès de la MT.

En situació basal, les úniques diferències les veiem en el ratolí IL-$6^{-/-}$, que mostra uns nivells de mRNA de la MT-I disminuïts respecte a les soques controls. A nivell proteic també es veu aquesta disminució en el ratolí IL-$6^{-/-}$. Això suggereix un paper en la regulació basal de la MT. Els resultats durant l'estrès en el ratolí IL-$6^{-/-}$ ens indiquen clarament que tant el mRNA de la MT-I com els nivells proteics de la MT-(I+II) hepàtics es troben disminuïts durant l'estrès, d'acord amb el fet que la IL-6 es troba incrementada durant l'estrès (Zhou *et al.*, 1993) i que és inductora de la MT (De *et al.*, 1990). És la primera vegada que es demostra l'implicació d'una citocina en la regulació hepàtica de la MT durant l'estrès i també a nivell basal. Podem

especular sobre els possibles mecanismes a través dels quals pot actuar la IL-6 sobre el gen de la MT, ja que s'ha vist en el promotor de la MT-I_X humana la presència de l'element AABS (Stennard *et al.*, 1994), el qual està associat a gens involucrats en la resposta de fase aguda del fetge mitjançant la IL-6. Aquest podria ser un dels mecanismes.

El ratolí TNFR1$^{-/-}$ no ha mostrat cap diferència respecte als controls en els seus nivells de MT durant l'estrès, si bé el TNF sembla estar incrementat durant l'estrès en sang (Yamasu *et al.*, 1992). El ratolí IFN-γR1 tampoc sembla mostrar cap efecte regulatori sobre la MT durant l'estrès, i podria estar d'acord amb el fet que els nivells circulants d'IFN-γ durant les situacions d'estrès es veuen disminuïts (Dobbin *et al.*, 1991). Les citocines formen una xarxa d'accions molt complexe i en molts casos encara no estan estarblerts molts dels mecanismes d'acció. A més, molts d'ells poden alterar-se en diferents situacions fisològiques com ara l'estrès.

Globalment podem dir que durant l'estrès la MT hepàtica resulta ser una proteïna multiregulada, al menys per metalls, glucocorticoides i la IL-6 en ratolí.

L'inflamació és l'altra situació fisiològica que hem estudiat. Moltes situacions inflamatòries i agents que causen inflamació com la endotoxina o la turpentina indueixen la MT (Sobocinski *et al.*, 1981; Durnam *et al.*, 1984; De *et al.*, 1990). De la mateixa forma aquests dos agents ens van induir la MT en tots els experiments realitzats. Abans però d'entrar en detall en la regulació de la MT hepàtica durant inflamacions comentarem breuement els resultats obtinguts sobre el paper del Zn en la resposta de la MT a l'inflamació.

Els nivells de mRNA de MT-I són incrementants després de l'administració amb endotoxina (Hernández *et al.*, 1996b), com era d'esperar. Si la MT és preinduïda per Zn, els nivells són incrementats de forma sinèrgica com passava amb l'estrès. Si la preinducció és per estrès, els nivells de MT són incrementats posteriorment per endotoxina de forma additiva i no sinèrgica. El mateix succeeix amb els nivells de proteics de MT-(I+II). Això ens indica que de la mateixa manera que succeïa amb l'estrès, la mobilització de Zn té un important paper en aquest tipus de situacions.

La inducció de la MT per la endotoxina es dóna en part per l'acció directa en el gen a causa de l'existència de la seqüència cis per endotoxina en ratolí (Durnam *et al.*, 1984), però en situacions d'inflamació s'activen a més una sèrie de mecanismes del sistema immunitari en què destaca l'alliberació de citocines. Si bé l'administració de citocines ha demostrat induir la MT (De *et al.*, 1990), en realitat no està clar quines d'aquestes citocines i en quin grau participen en el control de l'expressió de

la MT durant els procesos inflamatoris. Alguns treballs han realitzat una primera aproximació gràcies a la soca C3H/HeJ, que com ja hem explicat, no respon a la endotoxina (Vogel, 1992), i s'observa que produeix menys MT en situacions inflamatories (Maitani *et al.*, 1986; De *et al.*, 1990). En aquest sentit, i seguint la línia d'estudis que hem realitzat amb l'estrès, hem estudiat la resposta de la MT hepàtica a l'endotoxina o la turpentina en els ratolins IL-6$^{-/-}$, TNF-R1$^{-/-}$ i IFN-γR$^{-/-}$ per tal de trobar quines són les citocines implicades.

En aquests experiments hem demostrat que els nivells de mRNA de la MT-I són clarament disminuïts en el ratolí IL-6$^{-/-}$ després d'administrar l'endotoxina. Aquesta disminució va ser observada també després d'administrar turpentina. Podem afirmar que la IL-6 és la principal citocina que regula l'expressió de la MT durant l'inflamació i és la primera demostració de la implicació directa de la IL-6 en la regulació de la MT per inflamació. Abans hem suggerit que el possible mecanisme d'acció de la IL-6 podria estar motivat per la presència de certs elements com els AABS en el gen de les MTs. En tot cas aquests resultats suggereixen un clar suport a la consideració de la MT com una proteïna de la resposta de fase aguda.

En el cas del ratolí TNFR1$^{-/-}$ hem vist una certa disminució dels nivells de mRNA de la MT-I després d'administrar l'endotoxina, la qual cosa indica un cert paper en la participació de la inducció hepàtica de la MT en inflamacions. Els resultats amb el knock-out IL-6$^{-/-}$ x TNFR1$^{-/-}$ van ratificar els resultats anteriors. Aquest paper podria ser més important si s'estudiés el bloqueig dels dos receptors del TNF. Cal apuntar a més que les possibles vies d'inducció del TNF durant l'inflamació poden ser les mateixes que s'han suggerit per la IL-6, ja que per una banda el TNF indueix la IL-6 i per una altra banda, també intervé en la regulació de les proteïnes *acute phase* o de fase aguda.

Per últim, els nivells proteics de la MT-(I+II) han estat estudiats després de tractar el ratolí IFN-γR$^{-/-}$ a la endotoxina. En aquest cas observem que el ratolí IFN-γR$^{-/-}$ mostra una inducció més alta de MT-(I+II) que els controls. D'aquesta manera es suggereix que l'IFN-γ està exercint un efecte inhibitori durant la vía d'activació que genera la endotoxina en la regulació de la MT-(I+II). Si bé el IFN-γ indueix a la MT (De *et al.*, 1990), aquests resultats són oposats als mostrats amb el ratolí IL-6$^{-/-}$ i el TNFR1$^{-/-}$. Les citocines són peces clau dintre del sistema immunitari i exerceixen els seus efectes a través de múltiples vies. Si bé en alguns casos les vies implicades semblen estar ben estarblertes, en altres, com en aquest cas, no estan definides les rutes d'actuació.

Aquestes dues situacions fisiològiques, l'estrès i la inflamació, provoquen un increment considerable dels nivells de MT hepàtics. La regulació que hi té lloc en ambdós casos és multifactorial.

Cervell

Al cervell s'expressa la MT(I+II), a l'igual que al fetge, però a més també s'expressa una altra isoforma, la MT-III, isoforma específica d'aquest teixit descoberta recentment (Uchida *et al.*, 1991; Palmiter *et al.*, 1992).

Estudis previs del nostre laboratori han mostrat que els nivells de MT(I+II) s'eleven amb l'estrès d'immobilització en rata. En aquesta tesi hem observat que en el ratolí l'estrès també augmenta els nivells de la MT(I+II), possiblement per un increment de la transcripció gènica ja que el mRNA de la MT-I també s'hi eleva.

El plantejament va ser analitzar el paper dels glucocorticoides en la resposta a l'estrès de les MT-(I+II) d'àrees específiques, per la qual cosa l'utilització de la rata ens va permetre obtenir quantitats suficients de RNA. Estudis previs indiquen que aquestes hormones participen en la regulació dels nivells basals de MT-(I+II) en cervell de rata (Gasull *et al.*, 1994b; Hidalgo *et al.*, 1994a) i aquí s'ha tornat a confirmar, observant-se com els nivells de mRNA de MT-I decreixen en l'hipotàlam de rates ADX però no a l'hipocamp. Durant l'estrès decreixen els nivells de mRNA a l'hipocamp i a l'hipotàlam, desapareixent l'efecte a l'hipotàlam a les 8 h. Els resultats del còrtex s'han d'agafar entre cometes atès que els nivells d'actina no es van poder mesurar en aquesta àrea. Al contrari del que s'observa al fetge, els glucocorticoides podrien ser importants en el control del mRNA de la MT-I de rata durant l'estrès a l'hipocamp mentre que l'oposat passaria a l'hipotàlam. En conjunt, aquests resultats del mRNA de la MT-I suggereixen diferents patrons de regulació de la MT-I cerebral segons l'àrea i la situació fisiològica.

Respecte la MT-III hem observat que, al contrari que succeeix amb els nivells de MT-(I+II), el mRNA de la MT-III tendeix a disminuir durant l'estrès en el cervell sencer de ratolí. En rata, l'estrès tendeix a fer decréixer els nivells de MT-III a l'hipocamp mentre que a la resta d'àrees tendeix a incrementar-se. Si comparem el patró seguit pel mRNA de la MT-III en les diferents àrees de cervell de rata i en cervell sencer de ratolí veiem que aquest és diferent, i els resultats suggereixen una regulació diferencial durant l'estrès de la MT-III respecte la MT-(I+II). En definitiva, els resultats mostrats d'aquesta isoforma en estrès necesiten més estudis.

També s'ha estudiat el paper dels glucocorticoides durant l'estrès sobre la MT-III- El mRNA de la MT-III tendeix a quedar inalterat o a decréixer en totes les àrees de les rates ADX, i té un efecte significatiu en el còrtex. Aquesta tendència decreixent no seria esperada ja que no s'han vist de moment elements reguladors dels glucocorticoides en el gen de la MT-III de ratolí ni d'humà (Naruse *et al.*, 1994). Per últim, hem observat com en cèl.lules glials de rata sembla estar induït el mRNA de la MT-III per Dex, d'acord amb dades obtingudes en ratolí (Kramer *et al.*, 1996a). En cultius neuronals els nivells de mRNA de la MT-III semblen estar disminuits tant en rata com en ratolí (Kramer *et al.*, 1996b). Es suggereix doncs una regulació diferenciada segons el tipus cel.lular de la MT-III per glucocorticoides, al menys *in vitro*, regulació diferent de la mostrada per la MT-(I+II), la qual en el mateix sistema veu incrementada la seva síntesi en ambdós tipus cel.lulars, astròcits i neurones, amb la presència de Dex (Hidalgo *et al.*, 1994a).

Seguint els mateixos criteris que hem explicat anteriorment, hem realitzat dues aproximacions al possible paper de les citocines durant l'estrès en la regulació de les MTs cerebrals. Per una banda es coneix que les citocines s'expressen en el cervell (Plata-Salaman, 1991) i per una altra banda hem observat com les injeccions i.c.v. de IL-1 i IL-6 incrementen la MT-(I+II) cerebral en varies àrees de rata, sent aquesta la primera demostració *in vivo* d'inducció directa de les MTs cerebrals per la IL-1 i la IL-6 (Hernández and Hidalgo, 1998). Aquests resultats són concordants amb el paper d'aquestes citocines en la regulació de la MT en diferents teixits i en especial al fetge (Cousins and Leinart, 1988; De *et al.*, 1990; Liu *et al.*, 1991), però indiquen que les dues citocines poden estar actuant conjuntament amb altres factors, ja que varies dades així ho indiquen; per una banda, la IL-6 no afecta els nivells de MT-(I+II) en hipocamp tot i que és una àrea on hi ha receptors de IL-6 (Schöbitz *et al.*, 1992), i per un altra banda, els nostres resultats confirmen que la IL-6 necessita d'altres factors en combinació com el Zn i la Dex per induir la síntesi de MT en cultiu primari d'astròcits i neurones, d'acord amb altres treballs (Hidalgo *et al.*, 1994a; Kramer *et al.*, 1996a; Kramer *et al.*, 1996b). Davant d'aquests resultats pot esperar-se que les citocines poden particpiar en la regulació de les MTs cerebrals durant l'estrès o inflamacions.

En aquest sentit se sap que l'estrès incrementa el mRNA de la IL-1β (Minami *et al.*, 1991) i de la IL-6 (Shizuya *et al.*, 1997) en l'hipotàlam de rata i també es coneix que les citocines poden activar l'eix HPA (Turnbull and Rivier, 1995), sent aquesta relació immune-endocrina essencial en la resposta a l'estrès (Harbuz and Lightman, 1992). Si bé aquestes dades conjuntament amb els resultats observats al fetge

podríen fer pensar en un paper similar en el cervell, els resultats ens mostren que en el ratolí IL-6$^{-/-}$ i el ratolí IFN-γR$^{-/-}$ no es van observar diferències en els nivells basals dels nivells proteics de la MT-(I+II). Es va incrementar la MT-(I+II) després de l'estrès en el cervellet, l'hipocamp i la medulla+pons mentre que no va augmentar en el còrtex i l'hipotàlam. Cap diferència es va observar en els nivells de MT-(I+II) del ratolí IL-6$^{-/-}$ i IFN-γR$^{-/-}$ respecte els respectius controls. Malgrat això, i si bé hem de dir que la IL-6 i l'IFN semblen no tenir un paper rellevant en la regulació de les MTs cerebrals durant l'estrès, seran necessaris més estudis per evaluar el possible paper de la IL-6, l'IFN i altres citocines com el TNF o la IL-1 en la regulació de les MT cerebrals durant l'estrès, sobretot a nivell de mRNA mitjançant hibridació *in situ*, ja que les concentracions al cervell són molt més petites que al fetge i els possibles canvis també. Diem això en referència a un experiment mostrat en el treball 4, que si bé no entrem a valorar atès que aquesta part ha estat portada a terme per un altre dels autors de la publicació, podem dir que l'IFN-γ afecta a nivell basal al mRNA de la MT-I en dues àrees com són el gir dentat i la habenula. Cap diferència es veu en els nivells basals de mRNA de la MT-III.

La regulació de les MTs cerebrals ha estat també estudiada durant inflamacions. Són varis els experiments que han mostrat la regulació de la endotoxina sobre les MTs cerebrals. Es conegut l'increment de la expressió del mRNA de la MT-(I+II) en cervell de ratolí i rates injectades amb endotoxina (Searle *et al.*, 1984; De *et al.*, 1990; Palmiter *et al.*, 1992; Choudhuri *et al.*, 1993). En anteriors estudis en rates s'havia observat que els nivells proteics de la MT-(I+II) no eren induïts 8 h després d'administrar LPS (Gasull *et al.*, 1994b). Aquí hem mostrat per primera vegada la inducció dels nivells proteics de les MT-(I+II) en cervell de rata 24 h després d'administrar LPS. Així la MT-(I+II) incrementa els seus valors proteics en la medula+pons i el cervellet, d'acord amb resultats anteriors en rata on s'ha mesurat el mRNA (Itano *et al.*, 1991). En altres experiments hem analitzat els nivells de mRNA de la MT-I 6 h després d'administrar endotoxina i els nivells de MT(I+II) 24 h després, en còrtex i cervellet de ratolí. Com s'esperava, els nivells de mRNA s'incrementen significativament en el cervell, així com els nivells de la proteïna (De *et al.*, 1990; Zheng *et al.*, 1995). Tanmateix hem observat d'acord amb els treballs fets amb mRNA a ratolí, com la endotoxina incrementa els nivells proteics de MT-(I+II) en totes les àrees estudiades, hipotàlam, hipocamp, cervellet, còrtex i medul.la+pons. Per tant, si bé la resposta observada al fetge pot comparar-se amb la resposta cerebral, la hepàtica és més ràpida i més elevada.

Respecte a la MT-III, en l'experiment estudiat els nivells de mRNA es van veure disminuïts respecte els controls, indicant així una regulació diferenciada respecte les MT-(I+II).

Per últim, hem estudiat el paper de les citocines en la regulació de les MTs cerebrals. En aquest sentit hem vist com el ratolí IFN-$\gamma R^{-/-}$ ha mostrat uns nivells més incrementats de proteïna en l'hipocamp i la medul.la+pons dels animals tractats amb LPS, indicant un paper inhibidor com el vist al fetge. Les desconegudes vies a través de les quals funciona l'IFN duen estar funcionant tant al cervell com al fetge.

Gràcies a un altre ratolí transgènic hem estudiat l'efecte de la sobreexpressió de la IL-6 sota el control del promotor del GFAP en el cervell de ratolí. Hem estudiat dues línies del ratolí GFAP-IL6, una denominada G16 (expressió de IL-6 baixa) i una altra denominada G36 (expressió de IL-6 alta). Aquest ratolí és un model de gliosi cerebral amb clars símptomes de neurodegeneració i ens ha estat molt útil per a estudiar la regulació de les MTs en aquestes condicions.

En conjunt, els nivells de MT-(I+II) es troben induïts en el cervell dels animals GFAP-IL6, essent aquesta inducció específica de moltes àrees com cervellet, medul.la+pons, hipotàlam i còrtex, mentre l'hipocamp no mostra cap canvi. Aquesta important inducció es veu ben correlacionada amb la resposta inflamatòria observada en aquestes àrees cerebrals (Campbell *et al.*, 1993; Chiang *et al.*, 1994) i d'acord amb l'efecte inductor de la IL-6 en altres teixits (De *et al.*, 1990; Schroeder and Cousins, 1990). Aquesta inducció no es va veure reflexada en els nivells de mRNA, ja que aquests mostraven uns increments molt lleugers i es suggeria l'existència d'algun tipus de control post-trancripcional (Hernández *et al.*, 1997b). Estudis posteriors ens han mostrat que aquesta discrepància era fruit de la normalització dels resultats amb els nivells de mRNA de γ-actina, ja que aquests es veuen afectats també en el ratolí GFAP-IL6. Així, Northerns posteriors i dades obtingudes amb hibridació in situ ens indiquen que efectivament els nivells de mRNA de MT-I es veuen incrementats de forma important en la majoria d'àrees d'acord amb els resultats de la proteïna.

La MT-III mostra un control diferencial respecte la MT-(I+II), mostrant una regulació a la baixa en els animals que tenen major neurodegeneració com són els G36 de 3 mesos.

Aquestes dades ens confirmen que, en efecte, es pot considerar a la MT-(I+II) com una proteïna *acute phase* o de fase aguda en el CNS. La MT-III té un patró totalment oposat a les MT-(I+II) durant l'inflamació, essent la seva expressió disminuïda. Altres estudis mostren que aquesta disminució de la MT-III canvia en el

temps per ser incrementada i se li atribueix a la MT-III un paper reparador de teixit (Anezaki *et al.*, 1995; Inuzuka *et al.*, 1996). Especulant, podriem dir que el paper de la MT-III difereix segons la situació fisiològica o patològica en què ens trobem, essent la seva regulació inicial decreixent en inflamacions, neurodegeneracions i AD, per a ser possiblement incrementada al llarg del temps, participant llavors en processos de reparació. Evidentment són necessaris més estudis per establir el paper de la MT-III al cervell.

En conjunt podem establir que les MTs, tant hepàtiques com cerebrals, en diverses situacions com estrès, inflamacions o neurodegeneracions mostren una expressió incrementada, essent aquesta regulada per diferents factors, com metalls, hormones i citocines. Aquesta multiregulació per part de l'organisme ha de respondre a la necessitat d'establir un sistema de protecció cel.lular. Aquests resultats impliquen a les MTs en aquest paper i es pot considerar a la MT com una proteïna de resposta de fase aguda en aquestes situacions.

Les MTs podríen tenir un paper protector de l'organisme, tant a fetge com a cervell, mantenint la integritat cel.lular envers radicals lliures i altres substàncies danyines. Per una altra banda, la MT-III podria participar a més en altres processos de reparació cel.lular.

Conclusions

CONCLUSIONS

1). En general, les metal.lotioneïnes hepàtiques i cerebrals es poden considerar com unes proteïnes de fase aguda o *acute phase* en les diverses situacions fisiològiques estudiades, com l'estrès i l'inflamació. La regulació que té lloc en ambdós casos és multifactorial. Així,

2). La mobilització de Zn durant l'estrès cap al fetge des d'altres teixits sembla contribuir a la síntesi hepàtica de la MT.

3). Els glucocorticoides intervenen en la resposta hepàtica de la MT durant l'estrès en ratolí, i no en rata. En cervell participen en la regulació dels nivells de mRNA de MT-I durant l'estrès, tant en rata com en ratolí, suggerint-se un patró diferencial específic segons l'àrea cerebral.

4). De les citocines estudiades en els ratolins modificats genèticament, només la IL-6 intervé significativament en la resposta de les MTs hepàtiques en situació basal i d'estrès, mentre que en cervell cap de les citocines han tingut un paper rellevant. Malgrat això, tant la IL-6 com la IL-1 indueixen els nivells de MT-(I+II) en diferents àrees cerebrals quan són administrades de forma aguda.

5). L'inflamació provoca un increment dels nivells de MT hepàtics i cerebrals. En un model d'inflamació crònica com és la sobreexpressió de IL-6 sota el control del promotor del GFAP en cervell de ratolí s'observa en conjunt un augment dels nivells de MT(I+II), correlacionant-se amb la resposta inflamatòria observada en aquests animals.

6). Els resultats amb els ratolins modificats genèticament durant inflamació aguda ens demostren que la IL-6 té un paper mediador de la resposta de les MTs hepàtiques. Els resultats observats en els ratolins deficients del TNFR1 (a nivell hepàtic) i el IFN-γR1 (a nivell hepàtic i cerebral) suggereixen que el TNF no té un paper mediador i que l'IFN inhibeix la síntesi de les MTs.

7). El mRNA de la MT-III segueix un patró d'expressió durant l'estrès diferencial respecte les isoformes MT-(I+II), decreixent a l'hipocamp i incrementat al reste d'àrees. Es suggereix un cert paper dels glucocorticoides durant l'estrès en l'expressió de la MT-III. La resposta de la MT-III a l'endotoxina es troba disminuïda en cervell de ratolí, i de la mateixa manera s'observa una regulació a la baixa en el model d'inflamació crònica del ratolí GFAP-IL6. En conjunt la MT-III mostra una regulació diferencial respecte a les MT-(I+II).

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