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SHORT COMMUNICATION

METALLOTHIONEIN INDUCTION BY RESTRAINT STRESS: ROLE OF GLUCOCORTICOIDS AND IL-6

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Restraint stress increased liver metallothionein-I (MT-I) mRNA and MT-I+II protein levels. The glucocorticoid receptor antagonist RU 486 decreased this response. In contrast, adrenalectomy only decreased MT-I+II protein levels. Moreover, corticosterone or progesterone did not reverse the effect of RU 486. These results suggest that glucocorticoids are important for MT-I+II protein synthesis but not for MT-I mRNA accumulation during restraint stress, and that other factors must be involved in this process. Interleukin-6 (IL-6) deficient mice showed a significant decrease of restraint stress-induced liver MT-I mRNA levels (~30% of IL-6+/+ mice) up to ~4–5 hours after the onset of stress. Western blotting of hepatic nuclear proteins showed that the IL-6 responsive transcription factor Stat3, which has been shown to mediate MT induction by inflammation, was also activated by restraint stress. Results after extended periods of restraint stress indicate that IL-6 participates early and transiently in the process. The analysis of the expression of the acute phase plasma protein serum amyloid A suggests that restraint stress elicits an acute phase response similar to that caused by inflammation.

Although many factors induce liver metallothionein (MT) synthesis,[1] it is believed that inflammation and physical/psychological stress are the major physiological inducers. In a recent study,[2] we have demonstrated that IL-6 mediates liver MT induction by endotoxin. Glucocorticoids can induce mouse MT-I and -II expression,[3] but the results in rats do not support a mediating role of glucocorticoids during stress (see ref. 4 for a review). However, a recent report[5] has demonstrated that liver MT-I+II induction by restraint stress is decreased by the glucocorticoid receptor antagonist RU 486 in mice. RU 486 is an antagonist of the progesterone receptor too,[6] and, therefore, it seems important to confirm the results obtained with RU 486 in adrenalectomized mice. The cytokines are a group of essential mediators of the immune system, and also of other physiological systems.[7] Some reports suggest that circulating IL-6 levels are increased during stress,[8,9] and, thus, it seems plausible that the hepatic MT response to restraint stress could be mediated by restraint stress-induced cytokine release. We have studied this possibility by employing knock-out mice homozygous for a null mutation in the IL-6 gene.[10]

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RESULTS

Role of glucocorticoids

Liver MT-I mRNA and MT-I+II protein levels were significantly increased by restraint stress, and this induction was decreased by RU 486 (Fig. 1). In contrast to RU 486, adrenalectomy only decreased

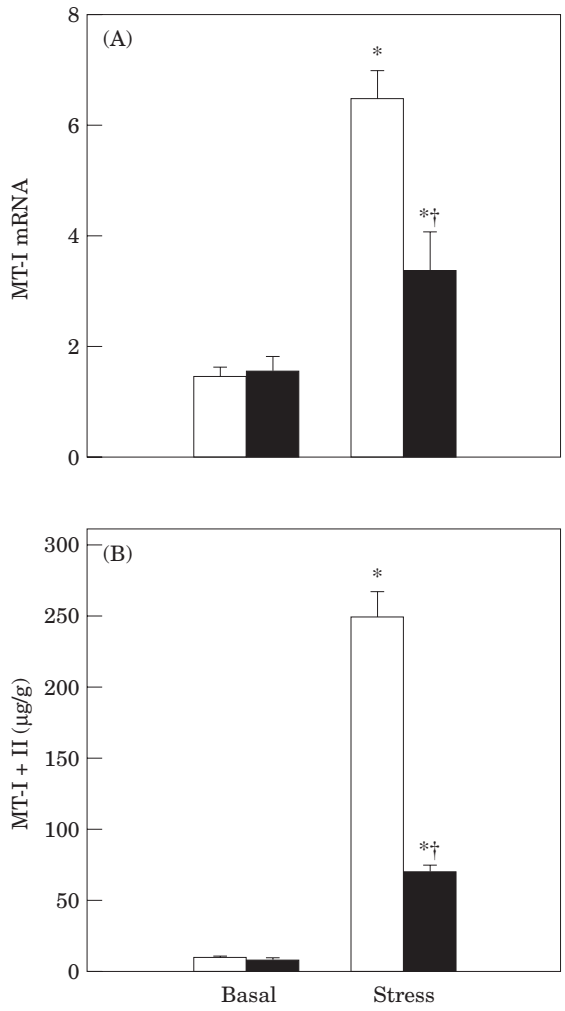

Figure 1. Effect of the glucocorticoid receptor blocker RU 486 on (A) liver MT-I mRNA and (B) MT-I+II protein levels on control and stressed mice.

Animals were killed 8 h (for mRNA analysis) or 18 hours (for protein analysis) after the onset of restraint stress along with unstressed mice. RU 486 (100 mg/kg) was given orally as a suspension in aqueous solution containing 0.25% carboxymethyl cellulose and 0.2% Polysorbate 80, in a volume of 10 ml/kg. RU 486 was administered twice, approximately 20 and 2 h before subjecting the animals to restraint stress. Control animals always received the vehicle. Stress was caused by wrapping the animals in a metallic net. Results are mean ± SE (n=9). Two-way ANOVA with restraint stress and RU 486 as main factors indicated that restraint stress increased liver MT-I mRNA and MT-I+II levels (*P<0.001), and that RU 486 decreased them (†P<0.05). (□), Vehicle; (■), RU 486.

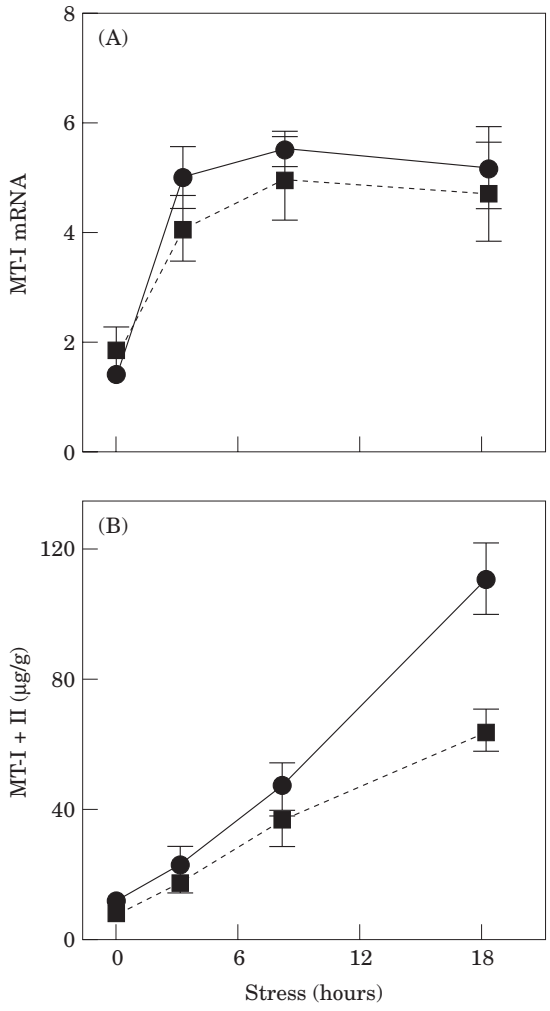

Figure 2. Effect of adrenalectomy on liver MT response to restraint stress.

(A) MT-I mRNA and (B) total MT-I+II protein levels were measured in control and restraint stress conditions. Mice were bilaterally adrenalectomized (ADX) or sham-operated (sham-ADX) under ether anesthesia. ADX animals were given saline to drink, and the experimental procedures were started 8 days after surgery. Results are mean ± SE (n=6–8). Two-way ANOVA with restraint stress and adrenalectomy as main factors indicated that restraint stress increased liver MT-I mRNA and MT-I+II protein levels (P<0.001), and that ADX reduced the latter (P<0.025) but not the former. (●), Sham-ADX; (■), ADX.

MT-I+II protein levels during stress (Fig. 2). Neither corticosterone nor progesterone reversed the effect of RU 486 on liver MT induction by restraint stress (Table 1). Dexamethasone only caused a minor reversing effect.

Role of IL-6

The IL-$6^{-/-}$ mice responded less to restraint stress than did any of the control strains examined (Fig. 3). The cytokine was also important in unstressed mice.

A comparison was made of the induction of hepatic MT and serum amyloid A (SAA) during restraint stress and turpentine-induced inflammation (Fig. 4). Liver MT-I and SAA mRNA levels were significantly increased in a time-dependent manner by both restraint stress and inflammation, and the functional deficiency of IL-6 decreased both responses. MT-I induction by restraint stress was decreased by IL-6 deficiency at early (up to ~5 h) but not at later times.

TABLE 1. Effect of glucocorticoid and progesterone therapies on RU 486-induced changes on mouse liver MT levels

	Liver MT-I+II (μg/g)	
	Vehicle	RU 486
Experiment A		
Control		
Saline	19.5 ± 1.96	12.2 ± 1.53
Corticosterone (20 mg/kg)	37.4 ± 2.72†	15.6 ± 2.77
Progesterone (2 mg/kg)	18.5 ± 2.09	12.4 ± 2.57
Stress		
Saline	261 ± 16.1*	71.6 ± 10.5*
Corticosterone (20 mg/kg)	248 ± 32.9*	97.8 ± 27.3*
Progesterone (2 mg/kg)	270 ± 34.0*	70.4 ± 5.24*
Experiment B		
Control		
Saline		18.3 ± 4.07
Stress		
Saline		38.0 ± 3.79*
Dexamethasone (20 mg/kg)		58.0 ± 9.30*†
Progesterone (20 mg/kg)		44.0 ± 4.41*

Results are means ± SE (n=6–7 in both experiments). In experiment A, the stressed mice were subjected to 18 h of restraint, and the animals received three injections at 0, 5 and 8 h after the onset of restraint stress at the indicated dosages. In experiment B, the mice were subjected to 8 h of restraint and were injected with the hormones with a similar protocol but at the dosages stated. RU 486 decreased the responses as revealed by the proper ANOVA (not shown). The other significances are as stated: *P<0.05 vs control mice, and †P<0.05 vs the proper saline mice.

Effect of restraint stress on Stat3 activity in the liver

IL-6 activation of acute-phase gene expression involves the nuclear translocation of Stat3.[11] We have recently demonstrated that this transcription factor is involved in endotoxin-induced MT-I gene transcription.[2]. Figure 5 shows that Stat3 levels were significantly increased by restraint stress in a clear, temporal manner.

DISCUSSION

Stress and inflammation are two major physiological inducers of MT synthesis in the liver. The teleological reason for such upregulation of these proteins is probably related to their significant antioxidant properties.[12,13] The factors involved in the control of MT genes during stress and inflammation, however, are poorly known. The mouse MT genes display a number of control sequences including GRE, Sp1, MTF1 and MLTF/ARE.[3,14–16] A recent elegant report[5] demonstrated that restraint stress increased MT gene transcription and that the glucocorticoid receptor blocker RU 486 inhibited this induction significantly. We herewith confirm those results. However, RU 486 binds not only to the glucocorticoid but also to the progesterone receptor,[6] and it has been suggested that progesterone induces MT synthesis[17] and that indeed this could be mediated by the GRE.[18]

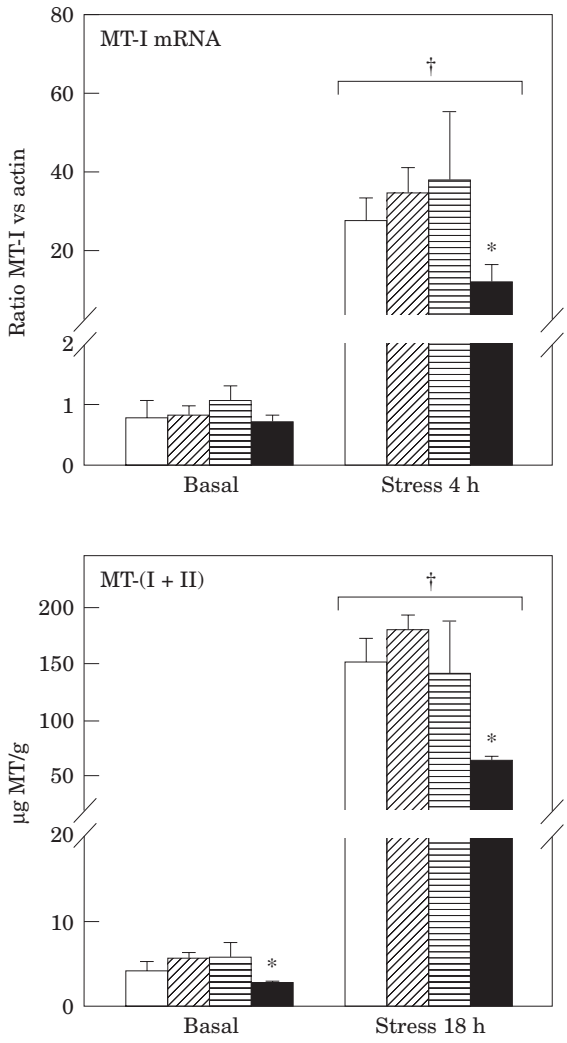

Figure 3. Effect of IL-6 deficiency and of genetic background on (A) liver MT-I mRNA and (B) MT-I+II protein levels.

Animals of the indicated strains were killed 4 h (for mRNA analysis) or 18 h (for protein analysis) after the onset of restraint stress along with unstressed mice. Results are mean ± SE (n=5–8). †P<0.001 vs unstressed, control mice. *P<0.05 vs control (C57BL/6, 129/Sv, C57BL/6 × 129/Sv) mice. (□), C57BL/6; (▨), 129/Sv; (▤), B57BL/6 × 129/Sv; (■), IL-6$^{-/-}$.

Results in adrenalectomized mice clearly indicate that glucocorticoids are not needed for the increase of MT-I mRNA levels during restraint stress, although they are for that of MT-I+II protein levels. Neither glucocorticoids nor progesterone produced significant reversing effects on the blocking action of RU 486. Taken together, these results question that either of these hormones is important in the control of liver MT gene transcription during restraint stress, and suggest that the glucocorticoid receptor, which is binding actively to the GRE of the MT gene promoter during restraint stress,[5] is acting independently of the physiological ligand, corticosterone. Glucocorticoid-independent glucocorticoid receptor

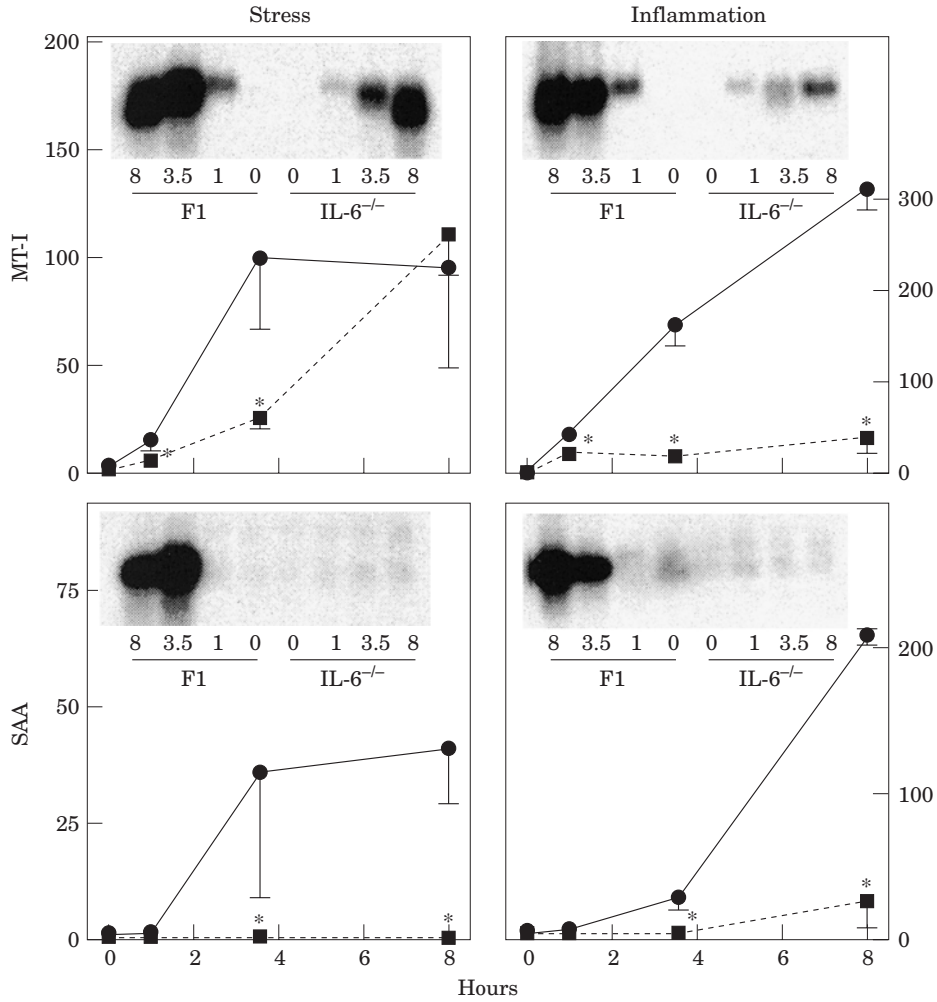

Figure 4. Effect of IL-6 deficiency on liver MT-I and SAA response to restraint stress and turpentine (50 µl, subcutaneously), measured by Northern blotting.

Results were analyzed with two-way ANOVA with restraint stress/inflammation and strains as main factors indicated that both restraint stress and inflammation dramatically increased MT-I and SAA mRNA levels, and that IL-6 deficiency significantly decreased these responses ($P<0.001$). Representative blots are shown within the plots. Results are mean ± SE ($n=3$–5). (——), control mice; (– – –), IL-6 deficient mice.

activation has previously been reported (see ref. 19 for a review).

The above results indicate that other factors in addition to the glucocorticoid receptor must participate in liver MT induction by stress. IL-6 has long been known to be an inducer of the hepatic MT genes in vitro[20] and in vivo,[21,22] is released during exposure to psychological and physical stressors,[8,9] and IL-6 responsive elements have recently been described in the MT gene promoters.[2,23] We recently demonstrated that IL-6 mediates endotoxin induction of liver MT through Stat1 and 3 and MTF-1 binding to the proximal MT-I promoter.[2] The results reported here clearly suggest that IL-6 elicits an acute-phase response during restraint stress and that it is a factor controlling MT expression in the liver during the early stages of

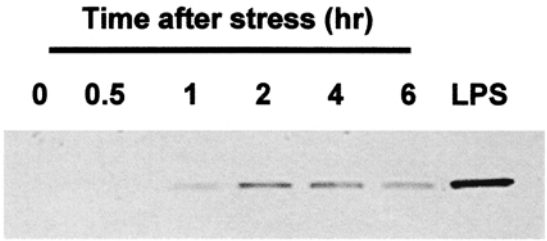

Figure 5. Western blot detection of nuclear Stat3 in the liver after immobilization restraint stress.

Liver nuclear extracts were prepared at the indicated time after restraint stress was initiated or 2 h after an injection of LPS (100 µg/mouse). Nuclear proteins (5 µg) were subjected to SDS-polyacrylamide gel electrophoresis and electroblotted to a nitrocellulose membrane. The membrane was incubated with rabbit anti-Stat3 antisera, followed by goat anti-rabbit IgG conjugated with horseradish peroxidase. Immunoblots were visualized by chemiluminiscence as described in Materials and Methods.

the restraint stress response. This is supported by the findings that SAA expression is also induced during restraint stress, that Stat3 levels in the nucleus are increased during restraint stress, and that IL-6 deficiency attenuates this response.

MATERIALS AND METHODS

Animals

In the experiments related to the role of glucocorticoids, adult male Swiss were used. In the experiments with the IL-6 deficient mice, three different controls were used: C57BL/6 (Jackson, Germany), 129/Sv, and the F_2 mice C57BL/6 × 129/Sv (provided by Biological Research Lab. Ltd., Basel, Switzerland).

mRNA analysis

Livers were removed and immediately snap-frozen in liquid nitrogen. MT-I mRNA levels were quantified by Northern blot and/or dot-blot analysis as previously described.[22,24]

MT-I+II protein assay

MT-I+II levels were measured by radioimmunoassay as described previously.[25]

Western blot detection of hepatic nuclear STAT 3

Stat3 activity was measured by western blotting in isolated nuclei from mice that were subjected to restraint stress for up to 6 h. The procedure has been described previously.[2]

Statistical assays

Results were analysed with one-, or two-way ANOVA. Logarithmic transformation of the data and post hoc comparisons of the means were applied when necessary.

Acknowledgements

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