

Induction of hepatic metallothioneins determined at isoprotein and messenger RNA levels in glucocorticoid-treated rats

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Induction of metallothionein-I (MT-I) and metallothionein-II (MT-II) by glucocorticoids was determined by h.p.l.c. analysis of proteins and Northern-blot analysis of MT mRNAs. Rats were injected with dexamethasone (0.03–10 $\mu\text{mol/kg}$) and hepatic concentrations of MTs were determined 24 h later. In control rats, only MT-II was detected ($9.4 \pm 2.5 \mu\text{g/g}$ of liver), whereas the hepatic concentration of MT-I was below the detection limit ($5 \mu\text{g}$ of MT/g). Dexamethasone did not increase MT-I above the detection limit at any dosage tested, but MT-II increased to 2.5 times control values at dosages of 0.30 $\mu\text{mol/kg}$ and higher. Time-course experiments indicated that MT-II reached a maximum at 24 h after a single dosage of dexamethasone and returned to control values by 48 h. To determine whether dexamethasone increased MT-I in liver, samples were saturated with ^{109}Cd , after which the amount of ^{109}Cd in MT-I and MT-II was determined. Results indicated that, by this approach, MT-I and MT-II could be detected in control rats, and there was approx. 1.8 times more ^{109}Cd in MT-II than in MT-I. At 24 h after administration of dexamethasone (1 $\mu\text{mol/kg}$), there was a small increase in the amount of ^{109}Cd bound to MT-I, whereas the amount of ^{109}Cd bound to MT-II increased to more than 2 times control values. Northern-blot hybridization with mouse cRNA probes indicated that MT-I and MT-II mRNAs increased co-ordinately after administration of dexamethasone. Thus, although glucocorticoids increase both MT-I and MT-II mRNAs, MT-II preferentially accumulates after administration of dexamethasone.

INTRODUCTION

Metallothioneins (MTs) are a family of cysteine-rich metal-binding proteins that are widely distributed among plant and animal species (Kagi *et al.*, 1984). In mammals, there are two major classes of MTs, referred to as MT-I and MT-II (Nordberg & Kojima, 1979). These isoforms differ slightly in amino acid composition (Webb, 1979) and isoelectric points (Nordberg *et al.*, 1972; Cherian, 1974). In human tissues, several subtypes of the two major isometallothioneins have been identified (Kagi *et al.*, 1984; Karin *et al.*, 1984), whereas in rodents only two forms of MT are found (Webb, 1979; Searle *et al.*, 1984). MTs are also inducible proteins. Exposure to a wide variety of heavy metals, such as Cd, Zn, Cu and Hg, increases tissue concentrations of MTs. As a result, it has been suggested that MTs function in the homeostasis of essential metals (Cousins, 1983) and in the detoxication of toxic heavy metals (Leber & Miya, 1976).

MTs are also induced by a variety of stressful conditions, including hot and cold temperature, strenuous exercise (Oh *et al.*, 1978), food restriction (Bremner & Davies, 1975; Klaassen, 1981), bacterial infection (Sobocinski *et al.*, 1978) and exposure to alkylating agents (Kotsonis & Klaassen, 1979). Induction of hepatic MTs by these stressful conditions implicated glucocorticoid-hormone involvement in regulating tissue contents of MTs, and indeed it has been established that glucocorticoid hormones are primary inducers of MTs (Karin *et al.*, 1980*a,b*). In human tissues, MT genes are regulated

differentially by glucocorticoids, as the MT-II_A gene is maximally induced by dexamethasone, whereas the MT-I_A gene is only weakly induced (Richards *et al.*, 1984). Moreover, the human MT-I_F gene lacks a glucocorticoid-receptor-binding site (Varshney *et al.*, 1986), which may explain the relative insensitivity of MT-I genes to glucocorticoids. In contrast, the MT-I and MT-II genes in mice appear to be co-ordinately regulated by glucocorticoids (Yagle & Palmiter, 1985). Although these experiments evaluated the increase in MT-I and MT-II mRNAs, it was not possible to determine simultaneously the increase in hepatic concentrations of MT-I and MT-II proteins. However, a method combining h.p.l.c. with atomic-absorption spectrophotometry has been developed to quantify the isoforms of MT (Lehman & Klaassen, 1986), thereby allowing for the rapid, accurate, determination of tissue concentrations of isometallothioneins. Therefore the purpose of the present study was to evaluate the induction of MTs at the isoprotein and mRNA levels in glucocorticoid-treated rats.

MATERIALS AND METHODS

General

Male Sprague–Dawley rats (Sasco, Omaha, NE, U.S.A.) weighing 225–275 g were used. Rats were housed under a 12 h-light/12 h-dark cycle and allowed free access to water and food (Purina Laboratory Rodent Chow; Ralston–Purina Co., St. Louis, MO, U.S.A.). Dexamethasone sodium phosphate was generously given

Abbreviation used: MT, metallothionein.

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by Merck, Sharp and Dohme Research Laboratories (West Point, PA, U.S.A.). Corticosterone was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Administration of steroids

Dexamethasone sodium phosphate was dissolved in 0.9% NaCl and administered (subcutaneously) to rats in dosages of 0.03, 0.10, 0.30, 1, 3 or 10 $\mu\text{mol/kg}$. Corticosterone was dissolved in corn oil (Fisher Scientific Co., Fairlawn, NJ, U.S.A.) and administered (subcutaneously) to rats in dosages of 3, 30 or 300 $\mu\text{mol/kg}$. Control rats for studies with dexamethasone or corticosterone received saline or corn oil respectively. All treatments were injected in a volume of 2 ml/kg. For dose-response experiments, livers were removed for analysis of MT content 24 h after administration of the steroids. In a time-course experiment, rats were injected with dexamethasone (1 $\mu\text{mol/kg}$) and hepatic MT content was determined 3, 6, 12, 24 and 48 h later.

Analysis of MT-I and MT-II by h.p.l.c./atomic-absorption spectrophotometry

MT-I and MT-II were determined in liver by this method described by Lehman & Klaassen (1986). Chromatography was performed on an anion-exchange column (DEAE-5PW, 7.5 cm \times 7.5 mm; Waters Associates, Milford, MA, U.S.A.). MT-I and MT-II were eluted with a linear gradient of Tris/HCl mobile phase (solvent A, 10 mM-Tris/HCl, pH 7.4 at room temperature; solvent B, 200 mM-Tris/HCl, pH 7.4) from 0 to 40% B in 12 min at a flow rate of 1 ml/min. The atomic absorption of Cd in Cd-saturated MT-I and MT-II was determined by connecting the outlet of the u.v. detector of the h.p.l.c. system to the nebulizer uptake capillary of an atomic-absorption spectrophotometer (model 2380; Perkin-Elmer, Norwalk, CT, U.S.A.). Integration of Cd-containing peak areas was performed with an IBM computer (model 9000) and the Chromatography Applications Program (Version 1.3; IBM, Danbury, CT, U.S.A.).

Hepatic MT-I and MT-II were purified from Cd-treated rats by the method of Wong & Klaassen (1979) and used as standards for the h.p.l.c. assay. Freeze-dried MTs were reconstituted in distilled water, and protein concentrations were determined by the Kjeldahl method for the determination of N as described by Lang (1958). To saturate the MT standards with Cd, 50 μl of Cd (1000 $\mu\text{g/ml}$) was added to 950 μl of MT solutions (approx. 1000 $\mu\text{g/ml}$) and incubated at room temperature for 15 min. A 100 μl portion was injected on to the anion-exchange column and the resulting areas of the Cd-saturated peaks were determined.

To prepare cytosols for h.p.l.c. analysis, tissues were homogenized in Tris/HCl buffer (10 mM, pH 7.4 at room temperature; 1 g/2 ml). Cytosols were prepared by centrifugation at 10000 g for 15 min, after which the resulting supernatant was centrifuged at 100000 g for 1 h. Cytosols were saturated with Cd as described previously for MT standards. Cd-saturated cytosols were heat-denatured in boiling water for 1 min, and denatured proteins were removed by centrifugation at 10000 g for 3 min. A 500 μl portion of supernatant was applied to the anion-exchange column, and Cd-saturated MT-I and MT-II were eluted as described previously. Recovery of MT-I and MT-II is very quantitative by this method, exceeding 95% for the isoproteins in control and treated

rats (Lehman & Klaassen, 1986). Under the conditions employed to saturate the MTs with Cd, no Zn or Cu was detected in the MT peaks (results not shown).

In a separate experiment, cytosols were saturated with Cd by the addition of 50 μl of Cd (1000 $\mu\text{g/ml}$, containing ^{109}Cd ; 0.05 $\mu\text{Ci}/\mu\text{g}$; New England Nuclear, Boston, MA, U.S.A.). Cytosols were prepared as described previously for h.p.l.c. analysis, and 500 μl of Cd-saturated sample was injected on to the anion-exchange column. Fractions were collected at 15 s or 30 s intervals and analysed for ^{109}Cd content with a Packard Autogamma scintillation spectrometer (model 5130).

Preparation of hybridization probes

Anti-sense RNA (cRNA) was synthesized from mouse MT-I and MT-II cDNAs kindly provided by Dr. Richard Palmiter (University of Washington, Seattle, WA, U.S.A.). Briefly, MT-I and MT-II cDNAs were subcloned into Riboprobe vectors pSP64 and pSP65 (Promega Biotec, Madison, WI, U.S.A.) respectively. Recombinant plasmids were linearized, and single-stranded ^{32}P -labelled anti-sense RNA probes were synthesized with SP6 RNA polymerase and [^{32}P]GTP (800 Ci/mmol; New England Nuclear) as described by Green *et al.* (1983). The MT-I probe was complementary to the entire mouse MT-I mRNA sequence of 380 bases, including 3' and 5' untranslated sequences. The MT-II probe (158 bases) was complementary to the 3' half of mouse MT-II mRNA including about 65 bases of 3' untranslated sequence.

Northern-blot hybridization analysis of MT mRNAs

Total RNA was extracted from livers by a modification of the SDS/buffer/phenol/chloroform procedure described by Andrews & Teng (1979). RNA was denatured in 50% (w/v) formamide/2.2 M-formaldehyde, and RNAs were subjected to electrophoresis on 1.5% agarose gels containing 2.2 M-formaldehyde (Lehrach *et al.*, 1977). RNAs were then transferred to nitrocellulose (Thomas, 1980) and pre-washed for hybridization by the procedure described by Andrews *et al.* (1984). Filters were hybridized with ^{32}P -labelled cRNA probes for MT-I and MT-II mRNAs. After hybridization, the filters were washed in successively lower concentrations of SSC at 65 $^{\circ}\text{C}$ (3 M-NaCl/0.3 M-sodium citrate), with the final wash being 0.1 \times SSC (Andrews *et al.*, 1984). Autoradiograms were developed by exposing X-ray film (XAR-05; Kodak, Rochester, NY, U.S.A.) in conjunction with a high-plus intensifying screen (Dupont, Wilmington, DE, U.S.A.) for 6–24 h at -70°C . The location of the RNA-RNA hybrid was identified, and hybrids were cut from the nitrocellulose to determine the amount of ^{32}P in each hybrid by liquid-scintillation spectrometry.

Specificity of hybridization probes

To determine the specificity of hybridization of the mouse MT cRNA probes, mouse MT-I and MT-II cDNAs were transferred to nitrocellulose (Southern, 1975) and hybridizations were carried out as described above. The results established that the mouse MT cRNA probes selectively hybridized with their respective cDNAs, as homologous hybrids were at least 10 times more intense than heterologous hybrids when judged by autoradiography (results not shown). These results are in good agreement with those of Searle *et al.* (1984), who

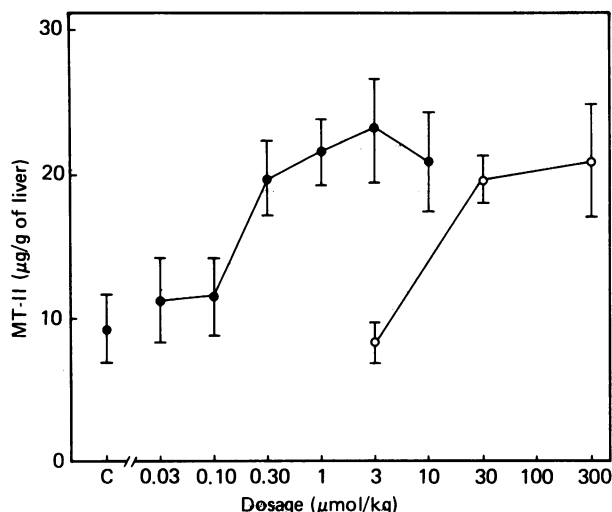


Fig. 1. Dose-response for induction of hepatic MT-II by dexamethasone (●) and corticosterone (○)

MT contents were determined 24 h after administration of glucocorticoids. Results are means \pm S.E.M. for four to six rats. Control rats (C) were injected with saline (dexamethasone control) or corn oil (corticosterone control) and, because there was no difference in the resulting MT contents, these groups were pooled ($n = 10$).

were unable to identify and clone MT-II genomic DNA with a nick-translated MT-I cDNA hybridization probe. Mouse MT-I and MT-II coding sequences are approx. 80% homologous, and thus do not cross-hybridize under stringent hybridization conditions reported by Searle *et al.* (1984). In contrast, mouse MT-I and rat MT-I coding sequences are approx. 97% homologous, and cross-species hybridization has been shown to occur (Andersen *et al.*, 1983). Therefore, with the conditions described for the hybridization procedures, cross-hybridization between mouse MT-I cRNA and rat MT-II mRNA or mouse MT-II cRNA and rat MT-II mRNA should be negligible (results not shown).

Statistics

Data were analysed by analysis of variance, followed by Dunnett's Multiple Comparison Test or Duncan's New Multiple Range test. Statistical differences between the concentrations of MT-I and MT-II were determined with Student's *t* test. The acceptable level of significance was set at $P < 0.05$ (Steel & Torrie, 1960).

RESULTS

The dose-response curves for induction of MTs by dexamethasone and corticosterone are shown in Fig. 1. In control rats, only MT-II was detected in liver, as MT-I was below the detection limit of 5 µg of MT/g of tissue for the h.p.l.c. assay (Lehman & Klaassen, 1986). Dosages of 0.3 µmol of dexamethasone/kg and higher increased hepatic concentrations of MT-II about 2.5-fold above control values. MT-I was not increased above the detection limit after any dosage of dexamethasone tested. Corticosterone also increased hepatic concentrations of MT-II about 2.5-fold above control values, but was approx. 100 times less potent than dexamethasone. Corticosterone did not increase MT-I above the detection

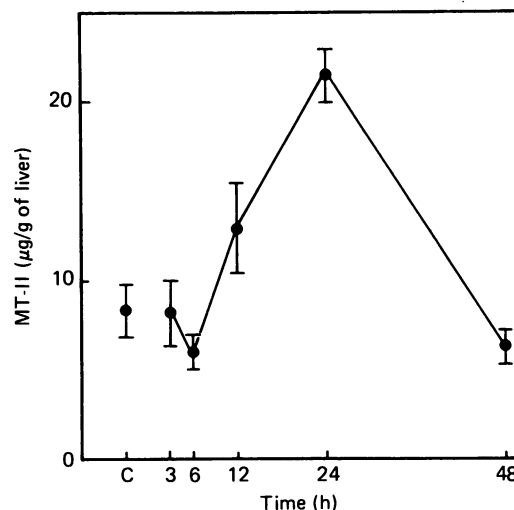


Fig. 2. Time course for induction of hepatic MT-II by dexamethasone

Hepatic concentrations of MTs were determined at various times after administration of dexamethasone (1 µmol/kg). Results represent means \pm S.E.M. for six rats.

limit of the h.p.l.c./atomic-absorption-spectrophotometry assay.

The time course for induction of hepatic MT-II by dexamethasone (1 µmol/kg) is indicated in Fig. 2. At 24 h after administration of dexamethasone, MT-II content was significantly higher than controls, reaching a maximum about 2.5 times control values. By 48 h after administration of dexamethasone, MT-II returned to control values. Again, MT-I was not detected.

To ascertain whether glucocorticoids increased MT-I in liver, it was necessary to modify the h.p.l.c. method so that MT-I could be detected. This was achieved by saturating liver cytosols with Cd mixed with ^{109}Cd and monitoring the elution of ^{109}Cd from the anion-exchange column (as described in the Materials and methods section). A representative radiochromatogram is shown in Fig. 3. ^{109}Cd was eluted with both MT-I (approx. 7.5 min) and MT-II (approx. 10 min) for control rat liver. However, 24 h after administration of dexamethasone (1 µmol/kg), there was a slight increase in ^{109}Cd in the MT-I peak, whereas there was a much larger increase in the amount of ^{109}Cd eluted in the MT-II peak. To compare the changes in MT-I and MT-II in control and dexamethasone-treated rats, the amount of ^{109}Cd eluted in the MT peaks was summed. In control rat liver, more ^{109}Cd was eluted in the MT-II peak than in the MT-I peak (740 ± 61 and 410 ± 52 c.p.m. of ^{109}Cd respectively; means of four determinations). After administration of dexamethasone, 580 ± 56 c.p.m. of ^{109}Cd was eluted in the MT-I peak, a 40% increase over control, whereas 1700 ± 160 c.p.m. of ^{109}Cd was eluted in the MT-II peak, a 230% increase over control.

Fig. 4 shows the results from Northern-blot hybridization of hepatic MT-I and MT-II mRNAs with mouse cRNA probes in untreated and dexamethasone-treated (1 µmol/kg) rats. The autoradiogram indicates that MT-I and MT-II mRNAs increased co-ordinately above control values at 3 and 6 h after dexamethasone, and were returning towards control values at 12 h. The amount of ^{32}P in each hybrid was determined, and the

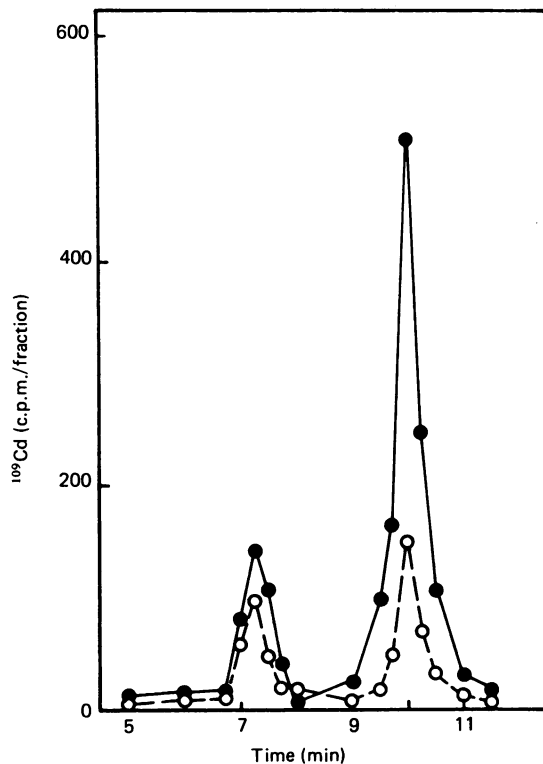


Fig. 3. Representative radiochromatogram of the elution of ^{109}Cd in MT-I and MT-II separated by anion-exchange h.p.l.c.

Cytosols, saturated with ^{109}Cd (as described in the Materials and methods section), were injected on to the anion-exchange column, and fractions were collected at 15 s or 30 s intervals. ^{109}Cd bound to MT-I was eluted in a peak between 7 and 8 min, whereas ^{109}Cd bound to MT-II was eluted in a peak between 9.5 and 10.5 min. ○, Control; ●, dexamethasone.

result indicated that, at the peak of induction (6 h), MT-I mRNA and MT-II mRNA hybrids represented 2228 ± 150 and 2275 ± 160 c.p.m. respectively (means of four determinations).

DISCUSSION

In the present study, the effect of glucocorticoids on MT isoprotein and mRNA contents was evaluated. The results indicate that the overall increase in MTs is small (about a 2.5-fold increase), and MT-II preferentially accumulates. At the mRNA level, glucocorticoids increased both MT-I and MT-II mRNAs.

The magnitude of the induction of the isoforms of MT reported here is in good agreement with previous reports in which total MTs have been quantified by less specific methods (Klaassen, 1981). The results, indicating that dexamethasone is a more potent inducer than corticosterone, correlate well with the observation that glucocorticoids induce MT through a glucocorticoid-receptor-mediated mechanism (Karin & Herschman, 1980). Time-course experiments indicated that MTs induced by glucocorticoids are relatively short-lived, an observation also reported for induction of MTs by glucocorticoids in cultured HeLa cells (Karin *et al.*, 1981).

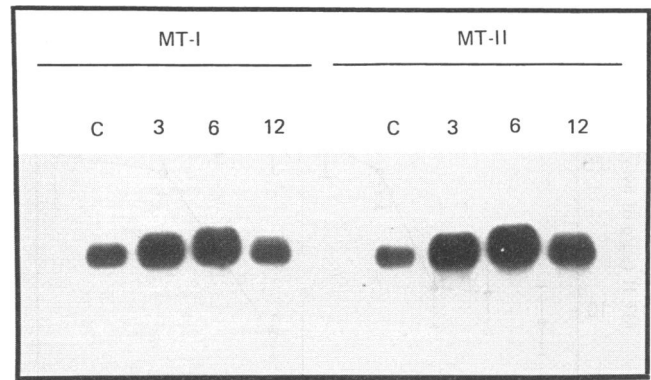


Fig. 4. Northern-blot hybridization analysis of MT-I and MT-II mRNAs in control (C) rats and 3, 6 and 12 h after administration of dexamethasone ($1 \mu\text{mol/kg}$)

Total cellular RNA ($6 \mu\text{g}$) was separated by electrophoresis on a 1.5% agarose gel, transferred to nitrocellulose and hybridized with ^{32}P -labelled mouse cRNA probes.

An interesting observation in this report is that, at the isoprotein level, MT-II, which is more abundant than MT-I in untreated rats, appears to be the predominant glucocorticoid-inducible MT in rat liver. It is also noteworthy that, in rats, MT-II is reported to be the predominant isoform induced by other stressful conditions, such as partial hepatectomy (Cain & Griffiths, 1984) and endotoxaemia (Suzuki & Yamamura, 1979).

There is, however, a discrepancy between the increase in both MT-I and MT-II mRNAs and the preferential accumulation of MT-II isoprotein in liver after dexamethasone treatment. Although the results of the present study suggest that glucocorticoid treatment increases hepatic contents of MT-I and MT-II mRNAs, the results also suggest that other, as yet undefined, events may be important in determining how the isoforms accumulate in tissues. Friedman & Stark (1985) have suggested that induction of MT-II mRNA by dexamethasone results from both increased transcription and post-transcriptional stabilization of mRNA. Perhaps, then, dexamethasone may preferentially stabilize MT-II mRNA.

At the protein level, reports in the literature suggest that MT-II has a longer half-life than MT-I (Andersen *et al.*, 1978; Cain & Holt, 1979; Cain & Griffiths, 1984; Lehman-McKeeman *et al.*, 1987). In contrast, synthesis of the two isoproteins occurs at similar rates in both control and Zn-treated rats (Lehman-McKeeman *et al.*, 1987). Taken together, it appears that MT-I has a more rapid turnover than MT-II. Given this difference in protein stability, a chemical which acts to stabilize MTs would cause the isoform that turns over less rapidly, MT-II, to accumulate preferentially. Thus one action that might explain, in part, the accumulation of MT-II by glucocorticoids and other stressful conditions, such as endotoxaemia or partial hepatectomy, is the stability of the isoproteins.

Recent interest in MTs has focused on the transcriptional regulation of the MT genes, with little effort directed toward gaining a better understanding of the functions of these proteins. The results of the present study indicate that, in rats, glucocorticoids increase MT-I and MT-II mRNAs co-ordinately. However, at the

protein level, MT-II appears to be the predominant glucocorticoid-inducible isoform.

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