Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 1053–1056, February 1984
Biochemistry

Induction of mouse metallothionein-I mRNA by bacterial endotoxin is independent of metals and glucocorticoid hormones

(acute-phase response/lipopolysaccharide/interleukin 1)

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Contributed by Earl P. Benditt, November 4, 1983

ABSTRACT **Induction of metallothionein-I (MT-I) mRNA by bacterial endotoxin (LPS) was examined. A single injection of LPS induced MT-I mRNA accumulation in both liver and kidney comparable to that induced by heavy metals. Maximal message levels were achieved 6 hr after LPS administration, prior to any increase in either hepatic or renal Zn or Cu. Experiments in which LPS was administered to transgenic mice harboring recombinant genes made by fusing the MT-I gene promoter to the herpes simplex virus thymidine kinase structural gene revealed that the response to LPS is independent of glucocorticoid hormones. These experiments begin to define the region of the MT-I gene promoter required for the LPS response.**

It has been well documented that the synthesis of metallothionein (MT) is regulated by both heavy metals, such as Cd and Zn, and glucocorticoid hormones (1–4). We have shown both *in vivo* and in cultured cells that the regulation of the mouse MT-I gene occurs primarily at the level of gene transcription for both classes of inducers (5–7). Definition of the region(s) of the MT-I gene promoter necessary for regulation by heavy metals has been approached by fusing deleted variants of the MT-I gene promoter to easily assayable genes, such as the herpes simplex virus (HSV) thymidine kinase (TK) gene, and analyzing metal regulation after the fused genes were introduced into either tissue culture cells or mouse eggs (8, 9). These studies indicate that the region of the mouse MT-I promoter necessary for metal regulation maps within 90 nucleotides 5′ of the normal MT-I transcription initiation site. Similar analysis of the region important for glucocorticoid regulation has not been possible because the mouse MT-I promoter is not responsive to glucocorticoids when transferred to other cells (9–11).

In addition to being regulated by metals and glucocorticoids, MTs are induced by a variety of stresses, including heat and cold exposure, strenuous exercise or tissue injury resulting from the injection of turpentine, carbon tetrachloride, or bacterial endotoxin (lipopolysaccharide, LPS) (12). The induction of MTs by tissue injury makes them similar to a group of serum proteins collectively called acute-phase proteins. This group includes such proteins as C-reactive protein, serum amyloid A protein, fibrinogen, α_1 antitrypsin, and α_1-acid glycoprotein (for a review, see ref. 13). Acute-phase proteins are typically serum proteins synthesized in liver. MTs differ in both of these respects because they are not secreted proteins and they are synthesized in a variety of tissues in addition to liver, including kidney and intestine (1).

The mechanisms by which MTs or other acute-phase proteins are induced by stresses have not been well defined. Like MT, some acute-phase proteins are regulated by elevated glucocorticoid levels resulting from stresses such as surgical trauma, pain, apprehension, and exposure to heat and cold (14–17). However, for MTs the response to inflammatory agents does not appear to be glucocorticoid-mediated because it has been recently shown that the injection of turpentine induced comparable amounts of hepatic MT in adrenalectomized and control rats (18). This suggests that there may be several mechanisms regulating MT synthesis that are operative during different types of stress and implies that perhaps a third class of regulatory molecule can affect MT-I gene transcription. Here we present several lines of evidence that rule out metal or glucocorticoid involvement in the response to the potent inflammatory agent LPS and thus support the notion that a third class of molecules may mediate MT-I gene expression during stress.

MATERIALS AND METHODS

MT-I mRNA Accumulation Time Course. Male Swiss–Webster mice, 14–17 g, were injected subcutaneously with $ZnSO_4$ (5 mg/kg) or intraperitoneally with LPS (3 mg/kg; Difco; lipopolysaccharide W, *Salmonella typhosa* 0901). Tissues were removed and MT-I mRNA levels were measured in total nucleic acid samples as described (19). Three mice were analyzed per time point; tissue samples were pooled for analysis.

Zn and Cu Uptake Measurements. Zn and Cu levels were measured by atomic absorption using an Instrumentation Laboratory atomic absorption spectrophotometer model 551. The instrument was used in the standard flame mode (acetylene/air) and calibrations were made against certified Zn and Cu standards (Fisher). The values are expressed as microgram of metal per gram of wet weight of liver or kidney.

Transgenic Mice. The transgenic mice used were offspring of the mice described by Palmiter *et al.* (10).

HSV TK Measurements. TK activity was assayed as described by Brinster *et al.* (8) and is expressed as the amount of thymidine monophosphate formed per minute per microgram of wet weight of liver minus the endogenous activity measured in the presence of an antibody specific for HSV TK.

RESULTS

Comparison of the Induction of MT-I mRNA by LPS and Zn. We initiated our study by comparing the induction of MT-I mRNA accumulation by LPS to that of Zn, a metal known to be a potent inducer of MT-I mRNA synthesis (5). Fig. 1*A* shows the kinetics of MT-I mRNA accumulation in liver over a 9-hr period after a single injection of either LPS or Zn. It is apparent that, although LPS and Zn induce almost identical amounts of MT-I mRNA during the first 4 hr,

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Abbreviations: MT, metallothionein; HSV, herpes simplex virus; LPS, lipopolysaccharide, also called bacterial endotoxin; IL 1, interleukin 1; bp, base pair(s); TK, thymidine kinase.

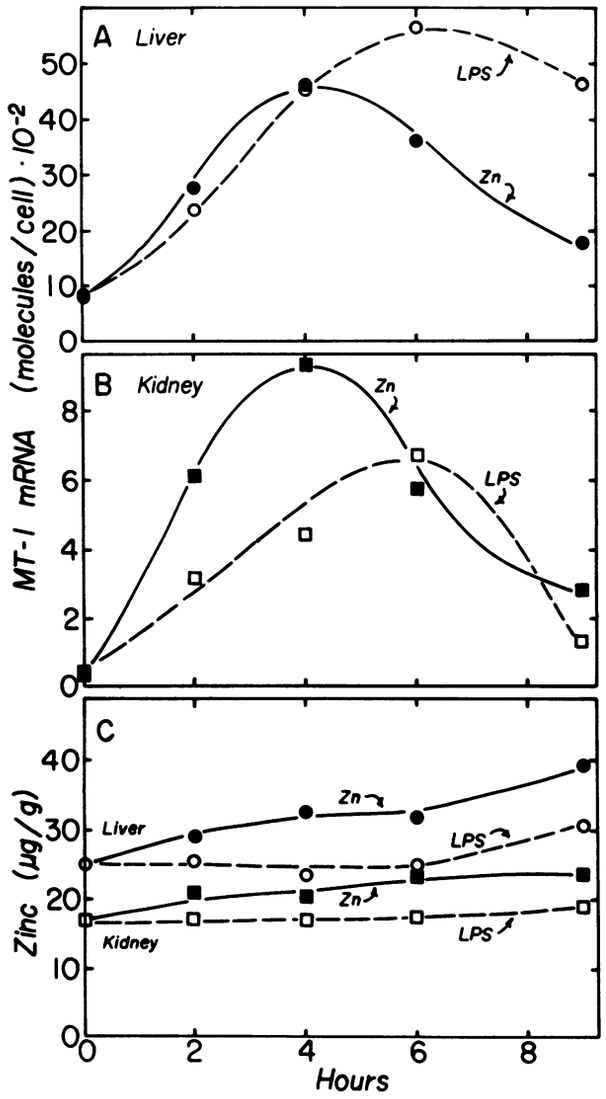

FIG. 1. Induction of hepatic and renal MT-I mRNA by Zn and LPS. MT-I mRNA levels in liver (*A*) and kidney (*B*) were measured 2, 4, 6, and 9 hr after either $ZnSO_4$ (5 mg/kg) or LPS (3 mg/kg) administration. Untreated mice were used for 0-hr measurements. (*C*) Tissue Zn levels measured in the same tissues used for the mRNA measurements. ● and ○, Liver measurements; ■ and □, kidney measurements; ---, LPS inductions; ——, Zn inductions.

LPS continues to increase MT-I mRNA levels for about 2 hr longer than Zn, reaching a peak of induction about 6 hr after injection. Because Zn and other metals are also known to be potent inducers of renal MT-I mRNA, we measured MT-I mRNA in kidney over the 9-hr time course (Fig. 1*B*). LPS was also an effective inducer of renal MT-I mRNA. The kinetics of induction in kidney by both LPS and Zn are identical to that in the liver, but the amount of renal MT-I mRNA induced by LPS was only 80% of that induced by Zn in this experiment. Other experiments have revealed that LPS induces MT-I mRNA in heart and brain but not in intestine or testes.

It has been proposed that LPS induces the uptake of Zn by the liver (20). We therefore measured the amount of Zn accumulated by the tissues during the 9-hr time course to discern whether LPS could be inducing MT-I mRNA accumulation by a Zn-mediated process. The results of these measurements are shown in Fig. 1*C*. Both liver and kidney in the animals injected with Zn showed a fairly steady increase in Zn throughout the time course. In contrast, the LPS-injected animals showed no measurable increase in Zn in either liver or kidney until about 9 hr after injection. These results correspond well with the results of Oh *et al.* (12), who found no significant change in hepatic Zn levels after a variety of types of stress, and suggest that LPS stimulation of MT-I mRNA levels is independent of changes in Zn levels. Cu levels in both liver and kidney remained constant after the administration of LPS (data not shown).

We next began an analysis of the effect of LPS administration on transgenic mice carrying the MK fusion gene (10). This gene was made by ligating the mouse MT-I promoter/regulatory region to the structural gene of HSV TK. The mice used in this study are offspring of mice that developed from eggs into which the MK gene had been microinjected. Analysis of transgenic mice offered two advantages over similar analyses on normal mice. First, the MK gene of transgenic mice responds as expected to heavy metal administration but, unlike the normal MT-I gene, is unaffected by glucocorticoid administration (10). This attribute of the MK gene allows us to unequivocally determine whether the response to LPS is independent of glucocorticoid hormones. Second, by studying transgenic mice that have deletions in the MT-I promoter/regulatory region we can begin to map the region essential for its regulation by LPS.

The transgenic mice used in this study are second-generation offspring of two transgenic mice, MyK 103 and MyK 84, which have been described previously (10). MyK 103 has 2 copies of the MK gene (unpublished data), including ≈1.7 kilobases of the sequence 5′ of the MT-I transcription start site. MyK 84 carries about 100 copies of the MK gene, including only ≈350 base pairs (bp) upstream of the transcription start site. Because offspring of these and other transgenic mice vary in the activity of their inherited MK genes, it was necessary to perform serial hepatectomies (several weeks apart) after various treatments rather than compare different offspring. These data are shown in Table 1. Control levels of HSV TK activity are low for all of the mice and are increased at least 10-fold by the administration of Cd. As had been documented previously (10), administration of dexamethasone (a synthetic glucocorticoid) had no effect on MK gene activity in these animals. LPS administration induced TK in the MyK 103 offspring at least as high as and in two cases much higher than those induced by Cd, indicating that the LPS response is independent of glucocorticoid hormones. MyK 84 offspring also showed inductions in response to LPS, but in general the response was smaller than the response to Cd. In contrast, two other transgenic mice

Table 1. Analysis of HSV TK activity in the liver of transgenic mice

	TK activity, cpm/μg per min			
Mouse	Control	With cadmium*	With dexamethasone†	With LPS‡
MyK 103-A	0.3	46.6	ND	40.2
MyK 103-B	0.7	8.3	0.0	27.0
MyK 103-C	0.3	4.0	0.0	34.3
MyK 84-D	16.5	200.0	ND	93.4
MyK 84-E	0.1	56.0	ND	15.0
MyK 84-F	0.2	178.0	ND	46.8

TK activity was measured as described (8) and is expressed as the difference in TK activity in the absence and presence of an antibody specific for HSV TK. The average endogenous TK activity (mean ± SEM) that was subtracted was 1.6 ± 0.2 cpm/μg per min. Control mice were untreated. ND, not determined.

*$CdSO_4$, 1 mg/kg, administered 18 and 4 hr prior to hepatectomy.

†Dexamethasone, 12 mg/kg, administered 18 and 4 hr prior to hepatectomy.

‡LPS, 1 mg/kg, administered 18 and 4 hr prior to hepatectomy.

that have only 185 bp of 5′ MT-I sequence responded well to Cd but not at all to LPS administration (data not shown). These data suggest that the region that allows LPS induction of the MT-I gene lies within the region 185–350 bp 5′ of the MT-I transcription start site.

DISCUSSION

We have analyzed the regulation of the mouse MT-I gene after the administration of LPS in an attempt to determine whether the MT-I gene, which is known to be regulated by both heavy metals and glucocorticoid hormones, is responsive to yet another inducer during inflammatory stress. Our results show that LPS is a very effective inducer of MT-I mRNA, causing increases comparable to those obtained by metals in both liver and kidney. The induction of renal MT-I mRNA by LPS was unexpected because LPS has not been shown previously to affect the synthesis of renal proteins. This may reflect the fact that acute-phase proteins are thought to be liver-specific (13) and may be present in a transcriptionally inactive form in other tissues, whereas MT-I is transcriptionally active in many tissues, including kidney. However, such differences in transcriptional activity do not account for all of the tissue specificity of LPS because the MT-I gene, although transcriptionally active in testes and intestine, is unaffected by LPS.

It has been previously shown that serum Zn levels are decreased by a variety of stresses, including LPS injection (20, 21). Sobocinski *et al.* (20) suggested that this drop in serum Zn results from an uptake of Zn by the liver, which in turn results in the induction of hepatic MT. Our data do not support this hypothesis because we measured no change in either renal or hepatic Zn levels during the early phase of MT-I mRNA induction by LPS. Our results are in agreement with the work of Oh *et al* (12), who also found no increase in hepatic Zn levels after stress. The slight increase that we measured 9 hr after LPS administration probably results from the scavenging of Zn by newly synthesized MT. We measured a steady uptake of Zn in both liver and kidney after the injection of Zn (5 mg/kg); at peak induction, Zn levels were about 1.6-fold and 1.2-fold higher than either LPS or control levels in liver and kidney, respectively. We purposely used a suboptimal dose of Zn for these studies (5). If the increase in Zn measured here is not enough to give maximal induction, then it seems very unlikely that a much smaller increase in hepatic or renal Zn could account for the induction by LPS.

Further evidence that the response to LPS is independent of heavy metals comes from our analysis of the regions of the MT-I promoter that are necessary for regulation by LPS and metals. Previous studies have shown that the sequences required for heavy metal regulation map within 90 bp 5′ of the normal MT-I transcription start site (8). The studies with transgenic mice presented here show that the region responsible for regulation by LPS lies within the region 185–350 bp 5′ of the transcription start site. This suggests that the sequences necessary for LPS regulation lie upstream from the sequences necessary for heavy metal regulation.

Our data also demonstrate that this response to LPS is not mediated by glucocorticoid hormones. First of all, we measured an increase in renal MT-I mRNA levels that would not be predicted if the response were glucocorticoid-mediated because glucocorticoids do not affect renal MT-I mRNA levels (7). Second, the MK genes of transgenic mice have never been shown to be responsive to glucocorticoids (10), yet both MyK 103 and MyK 84 offspring respond well to LPS administration. Taken together, these data show that glucocorticoids, although probably responsible for MT induction in some types of stress, do not mediate the induction of MT-I by LPS.

If metals and glucocorticoids are not involved in the MT-I response to LPS, then how does LPS induce MT-I mRNA? Hepatic synthesis of certain acute-phase proteins (e.g., C-reactive protein and serum amyloid A) is thought to be mediated by the hormone interleukin 1 (IL 1), which is liberated by macrophages in response to LPS (22, 23). Because homogeneous preparations of IL 1 are not currently available, the role of this polypeptide in acute-phase protein induction has not been firmly established (24, 25). In one pilot experiment we measured MT-I mRNA levels in normal and transgenic mice after the administration of a commercially available human IL 1 (Genzyme, Norwalk, CT). Our results indicated that IL 1 induced MT-I mRNA in liver but only to levels about 30% of the level induced by LPS. IL 1 had no effect on renal MT-I mRNA levels. As a control, we measured serum amyloid A as described (26) and similarly observed induction to about 30% of that induced by LPS. The evidence suggests that the MT-I gene may be responding to the same inducer as acute-phase proteins, but whether this inducer is IL 1 or another activity remains to be established.

The data presented here clearly show that the MT-I gene is responsive to at least three types of inducers: heavy metals, glucocorticoid hormones, and the as yet unidentified mediator of the LPS effect. The fact that three different inducers affect MT synthesis in times of stress strongly suggests that the MT gene product must play an important role in mediating the response to stress. What this role is and how these regulators interact to insure the precise regulation of MT synthesis remain to be determined.

We thank our colleagues for critical review of the manuscript and Abby Dudley for her help in preparing this manuscript. This work was supported by grants from the National Institutes of Health.

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