Endotoxin-induced inflammation does not cause hepatic zinc accumulation in mice lacking metallothionein gene expression

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The action of endotoxin lipopolysaccharide (LPS) on hepatic Zn uptake was examined in mice lacking expression of metallothionein (MT)-I and MT-II genes. Hepatic Zn concentrations, which in normal control mice increased by a mean 29 % (MT elevated 20-fold) 16 h post-LPS exposure, did not increase in MT-null mice. Plasma Zn fell by 68 % in controls and 32 % in MT-null mice. The time course of LPS action in normal mice was characterized by a rapid reduction (–74 % at 4 h, –81 % at 8 h) and partial recovery (–39 % at 24 h) in plasma Zn, with a progressive increase over 24 h in hepatic concentrations of MT

(by 36-fold) and Zn (by 40 %). In contrast, the MT-null mice had a linear decrease in plasma Zn (–15 % at 8 h, –41 % at 24 h) and early loss of Zn from the liver. The Zn changes seen in MT-null mice were largely attributable to LPS-associated anorexia. Food deprivation (20 h) alone caused respective 14 % and 30 % decreases in hepatic and plasma Zn concentrations and a 27 % reduction in total liver Zn reserves, whereas fasted normal mice conserved Zn with a 4-fold increase in hepatic MT. This study confirms that MT synthesis is essential for endotoxin-induced liver Zn accumulation.

INTRODUCTION

Metallothioneins (MTs) are highly conserved 60–62-amino-acid intracellular metal-binding proteins. The disposition of the 20 cysteine residues responsible for the incorporation of bivalent zinc and monovalent copper into thiolate clusters is invariant in mammals [1], suggesting that MT has a fundamental role in the intracellular regulation of these metals. Research into this and other biological properties of MT has been extensively reviewed [2–4].

There has been considerable interest in the part played by MT in the control of Zn redistribution within the body during inflammation. As Zn is essential for the function of more than 300 enzymes [5] as well as being an integral component of DNA-binding-protein motifs, it has been assumed that Zn uptake into the liver and tissues that combat infection has been necessary to support increased metabolic and synthetic activity [6]. We have previously used adjuvant-induced arthritis in rats as an inflammatory model [7], where the Zn content of the liver can double (from 8 % to 16 % of total body Zn) within 16 h of adjuvant injection. The hepatic Zn increment was wholly incorporated into MT [8]. The observed decrease in plasma Zn to one-third of its initial value [7] indicates that the redistribution of body Zn is pulled by hepatic uptake, rather than pushed by release of Zn from donor tissues. Hepatic MT synthesis thus appears to drive the redistribution of Zn, and is in turn triggered by inflammation-associated cytokine and hormone action [9,10], as well as by Zn itself.

Inhibitors of gene expression which block MT synthesis have been shown to impair hepatic Zn uptake [11,12], but are not specific for MT gene action. Mouse models either overexpressing or not expressing genes coding for specific proteins [13] are now commonplace, and both exist for MT [14–16]. The present study used mice lacking MT-I and MT-II [16], the only mouse hepatic MT isoforms. The aim was to determine, *in vivo*, the extent to which hepatic Zn uptake and accumulation is affected by the inability to synthesize MT in the liver, using physiological (food

deprivation) and pathological (endotoxin-induced inflammation) stressors known to induce hepatic MT synthesis in mammals.

MATERIALS AND METHODS

MT-I- and MT-II-deficient (MT–/–) mice were produced by Michalska and Choo [16]. The mice were in a mixed genetic background of OLA129 and C57BL6 strains. Control (MT+/+) mice were C57BL6 supplied by the Animal Resource Centre, Canning Vale, Western Australia. All mice were housed on sawdust in stainless steel or plastic cages in an animal house at 22 °C with a 14 h light/10 h dark cycle and were fed a commercial pelleted diet [10].

Experiment 1

Male mice, six per weight-matched group (24.3 ± 0.4 g), were used to evaluate the effects of MT gene deletion on Zn homeostasis after food deprivation or intraperitoneal (ip) injection of lipopolysaccharide (LPS). The LPS (from *Escherichia coli* 0111:B4; Sigma Chemical Company, St. Louis, MO, U.S.A.) was administered at a dosage of 1 µg/g body weight in 0.15 ml of 0.85 M saline. This dose resulted in only moderate physical changes with no deaths, but elicited a reproducible, strong MT response in the MT+/+ mice. The MT–/– and MT+/+ mice (36 in all) were evenly divided between the two treatments and an *ad libitum* fed control group. Food intake and weight changes were monitored. At the end of the experiment, 20 h after food withdrawal or 16 h after ip LPS injection, the mice were anaesthetized with halothane and 1 ml of blood was withdrawn by cardiac puncture. The mice were then killed by cervical luxation and the liver, kidneys, spleen and epididymal fat removed and weighed.

The liver was homogenized in four volumes of 0.01 M Tris/HCl buffer, pH 8.2, using a motor-driven Potter–Elvehjem homogenizer. An aliquot (1 ml) of homogenate was dried for 48 h at 70 °C prior to nitric acid digestion for Zn analysis, and the remainder centrifuged at 20000 g for 30 min. The supernatant

Abbreviations used: IL, interleukin; ip, intraperitoneal; LPS, bacterial endotoxin lipopolysaccharide; MT, metallothionein; TNF α, tumour necrosis factor α.

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was separated and heat treated at 95 °C for 2 min and centrifuged at 14000 g for 5 min prior to Cd-haem assay of Zn-MT [17,18] on the resulting supernatant. Cu and Zn were analysed by atomic absorption spectroscopy.

Experiment 2

Male MT^{-/-} and MT^{+/+} mice (29.6 ± 0.5 g) were used to establish a time course of the effect of MT on LPS-induced hepatic Zn uptake. Six mice (three MT^{-/-}, three MT^{+/+}) were killed at 4 h, 8 h, 16 h and 24 h after ip LPS injection. Other experimental conditions were as above. The zero-time-point data were taken from experiment 1.

Note about units used

MT refers specifically to hepatic Zn-MT as nmol of Cd bound per g of liver wet weight and is referred to in the text as 'nmol of Cd/g' for brevity. The Cd-haem assay used has 1:1 stoichiometry between the Zn incorporated into MT and the displacing Cd.

For comparative purposes, total hepatic zinc has been normalized to a 25 g mouse weight, i.e. total Zn (nmol/liver) = [Zn (wet) concentration (nmol/g) × liver weight (g) × 25] ÷ mouse weight (g). The mouse weight at the time of death was used in the calculation.

All experimentation was approved by the IMVS Animal Ethics Committee.

Results are reported as mean ± S.E.M. Significance was determined by Student's *t*-test for independent samples.

RESULTS

Experiment 1

Differences between MT^{-/-} and normal mice

The first column of data in Table 1 compares measurements in fed, unstressed MT^{+/+} and MT^{-/-} mice. Plasma concentrations of both Zn and Cu were respectively 31 % and 39 % higher in the MT^{-/-} mice. Mean (dry) hepatic Zn concentrations and total

liver Zn levels were not shown to be significantly different between groups, although the MT^{-/-} mice had a 12 % higher mean hepatic (wet) Zn concentration.

Hepatic MT in MT^{-/-} mice assayed at 2.0 ± 0.2 nmol of Cd/g (*n* = 31) averaged over all experiments, including those where strong MT induction occurred in normal mice. As this is at the level of non-specific binding in the Cd-haem assay used, individual MT concentrations from the MT^{-/-} mice have not been reported.

The inability of MT^{-/-} mice to synthesize MT was further substantiated by an absence of Zn uptake into the hepatic cytosol following ip administration of 5 µg/g of Zn as ZnSO₄, a dose that doubled cytosolic Zn concentrations in normal mice, with all absorbed Zn bound to MT. Liver cytosolic preparations from ip Zn-injected MT^{-/-} mice showed no Zn in the Sephadex G75 fractions normally associated with MT (results not included).

No nutritional problems were apparent in MT-deficient mice reared under controlled conditions; they appeared able to absorb enough Zn from their diet for normal growth and maintenance of body Zn.

Response to food deprivation

In the absence of food intake, however, as shown in the second data column of Table 1, clear differences between normal mice and the MT-deficient animals emerged. A 20 h fast resulted in a 27 % fall in total hepatic Zn in the MT^{-/-} mice, in contrast to the MT^{+/+} controls in which hepatic MT increased 4-fold, sequestering Zn in the liver. Note that although the fasting plasma Zn concentrations of both groups of mice were similar, they represented decreases from normal, fed values of only 1.7 nmol/ml in the MT^{+/+} mice, but a greater 5.3 nmol/ml in the MT^{-/-} mice. Fasting was also associated with pronounced ketonuria (Ames Ketostix) and apparent hypometabolism in the MT^{-/-} mice. While their MT^{+/+} counterparts were alert and actively foraging at the end of the 20 h period, the MT^{-/-} mice were inactive and slower to respond. They lost 29 % less

Table 1 Response in hepatic Zn uptake of normal and MT-null mice to food deprivation and inflammation

MT^{+/+} and MT^{-/-} mice (six per group) were either fed *ad libitum*, fasted for 20 h or injected ip with LPS 16 h prior to death and measurement of reported parameters (see Experiment 1). Significance *†, *P* < 0.05; **, †† *P* < 0.01; ***, ††† *P* < 0.001. First significance (*, **, ***) represents difference from fed state, second significance (†, ††, †††) represents difference between MT^{+/+} and MT^{-/-} mice. Abbreviations: nd, not done; na, not applicable.

Parameter	Mice	Treatment		
		Fed	Fasted (20 h)	LPS-injected (16 h)
Plasma Zn (nmol/ml)	MT ^{+/+}	13.7 ± 0.45	12.0 ± 0.35*	4.4 ± 0.36***
	MT ^{-/-}	17.9 ± 1.03†	12.6 ± 0.50**	12.2 ± 0.56**, †††
Plasma Cu (nmol/ml)	MT ^{+/+}	9.2 ± 0.59	nd	11.0 ± 0.22**
	MT ^{-/-}	12.7 ± 0.66†		16.0 ± 0.70**, ††
Hepatic Zn (nmol/g dry wt.)	MT ^{+/+}	1430 ± 30	1410 ± 42	1970 ± 34***
	MT ^{-/-}	1462 ± 9	1175 ± 43**, ††	1430 ± 38 †††
Hepatic Zn (nmol/g wet wt.)	MT ^{+/+}	472 ± 13	495 ± 6	609 ± 6***
	MT ^{-/-}	527 ± 10†	451 ± 13**, †	467 ± 9**, †††
Total liver Zn (nmol)	MT ^{+/+}	565 ± 29	520 ± 16	770 ± 14***
	MT ^{-/-}	625 ± 32	455 ± 22**, †	612 ± 22†††
Hepatic MT (nmol of Cd/g wet wt.)	MT ^{+/+}	3.0 ± 0.6	11.8 ± 2.0***	61 ± 2.3***
Weight loss (%)	MT ^{+/+}	na	13.0 ± 0.8	9.3 ± 0.3
	MT ^{-/-}		9.2 ± 0.3††	7.1 ± 0.8†
Epididymal fat (% of body wt.)	MT ^{+/+}	1.20 ± 0.12	0.64 ± 0.09**	1.08 ± 0.12
	MT ^{-/-}	1.61 ± 0.27	1.05 ± 0.16	1.15 ± 0.14

Figure 1 Time course of response to LPS over 24 h in hepatic MT, hepatic Zn and plasma Zn concentrations in MT+/+ and MT-/- mice

Open squares and solid lines represent hepatic Zn in nmol/g of (wet) liver, solid squares and lines represent hepatic MT in nmol of Cd bound/g of (wet) liver and solid circles and broken lines depict plasma Zn in nmol/ml (right-hand y-axis). $n = 6$ for zero time point, $n = 3$ for all other time points. Full details of procedure are given in the Materials and methods section.

weight than did the controls although their epididymal fat pad reduction was no different, at 0.56% of body weight.

Response to LPS injection

The third data column of Table 1 represents the response to LPS-induced inflammation, 16 h after ip LPS injection. (The mice also did not eat.) In MT+/+ mice, a 20-fold rise in MT resulted in 29% and 36% increases in (wet) concentration and total Zn in the liver respectively. The MT-/- mice, on the other hand, had a significant reduction in hepatic Zn (wet) concentration, but sustained their total liver Zn through an increase in liver weight as a proportion of total body weight. LPS administration resulted in plasma Zn reductions of 5.7 nmol/ml (32%) in MT-/- mice and 9.3 nmol/ml (68%) in MT+/+ mice.

Plasma Cu concentrations 16 h after ip LPS were increased respectively by 20% and 26% in MT+/+ and MT-/- mice. The physical condition of the MT-/- mice 16 h and 24 h (see Experiment 2) after LPS injection was better than after the 20 h fast. They showed a similar degree of piloerection and anorexia to the MT+/+ LPS controls and were more active and alert than their fasted counterparts.

Experiment 2

Time course of Zn changes

Figure 1 illustrates the action of LPS on plasma and hepatic Zn concentrations at 4 h, 8 h, 16 h and 24 h post ip LPS injection in MT+/+ and MT-/- mice. A clear distinction in Zn handling was apparent between the MT+/+ mice, which demonstrated a abrupt fall and then recovery in plasma Zn accompanied by a progressive MT-associated hepatic Zn increase, and the MT-/- mice, which showed a steady decline in plasma Zn and early reduction in hepatic Zn concentrations. Total liver Zn (625 ± 32 nmol at 0 h) fell rapidly to 436 ± 22 nmol (near 20 h fasting values) by 8 h before stabilizing at 16 h (470 ± 6 nmol) and recovering partially, but significantly ($P < 0.02$), at 24 h (536 ± 8 nmol). This may place a different interpretation on the apparent constancy of total liver Zn in MT-/- mice 16 h after LPS exposure in experiment 1, where liver Zn may have initially fallen but rebounded earlier and more completely than in

experiment 2. Hepatic Zn concentrations (nmol/g dry wt.) steadily declined from 1462 ± 9 (0 h) to 1300 ± 20 (8 h) and 1230 ± 17 (24 h) in the MT-/- mice (8 h, $P < 0.01$; 24 h, $P < 0.001$).

Plasma Cu concentrations of MT-/- and MT+/+ mice increased by 7.1 and 5.7 nmol/ml ($P < 0.05$; $P < 0.001$) respectively, in the 16 h period from 8–24 h post-LPS treatment. In this experiment the 8 h plasma Cu concentrations were 41% higher in the MT-/- mice than in the MT+/+ controls ($P < 0.02$).

MT-/- mice lost weight at only 70% of the rate of MT+/+ mice for the first 16 h after LPS treatment ($P < 0.05$), consistent with experiment 1. Over the next 8 h, however, the MT-/- mice suffered a greater weight loss than the controls, so that at 24 h there was no significant difference between the two groups. Epididymal fat pads at 4 h and 8 h were larger in MT-/- than MT+/+ mice ($1.81 \pm 0.10\%$ versus $1.30 \pm 0.14\%$ body weight, $P < 0.05\%$).

DISCUSSION

It is self evident that MT is not obligatory for prenatal development and post-natal growth of MT-null mice. Absorption from the diet is adequate to maintain normal or supra-normal plasma and hepatic Zn concentrations, despite the absence of MT.

One proposed function of MT is to conserve body Zn reserves during periods of food restriction, and the association between starvation and increased hepatic MT has long been recognized [19,20]. The main hormonal response to fasting is a decrease in the insulin:glucagon ratio to enhance glucagon-mediated effects on the liver (e.g. mobilization of glycogen stores and stimulation of gluconeogenesis). Glucagon has also been shown to induce severalfold increases in hepatic MT [21,22]. Recently, rats deprived of food for 36 h were shown to have reductions in total liver Zn of up to 23% despite increased hepatic Zn concentrations [23]. These findings are reflected in our observation in normal mice that a 20 h fast resulted in 3–4-fold increases in MT, sufficient to sustain hepatic Zn concentrations, but not total liver Zn, which fell by 9.5%. There was a minor reduction in plasma Zn. The MT-/- mice, on the other hand, had pronounced decreases in liver and plasma Zn concentrations and a large reduction (27%) in liver Zn reserves. It is clear that MT synthesis is vital for hepatic Zn sequestration in starvation. Catecholamine release [24], accentuated by handling stress, may have also contributed to MT synthesis in the normal mice.

The hepatic MT response to LPS is evoked chiefly by cytokines. Interleukin (IL)-1 α , IL-1 β and tumour necrosis factor α (TNF- α) stimulate cytokine production in a range of tissues (TNF- α predominantly in the liver), ultimately leading to increased hepatic IL-6, which acts directly to induce MT mRNA [25]. Cytokines induce MT independent of the action of glucocorticoids [26] and other counter-regulatory hormones, including those that participate in the fasting response. What the present study has shown is that a range of effectors, which are triggered by the presence of LPS to stimulate liver Zn accumulation, cannot do so in the absence of MT, i.e. their demonstrated enhancement of MT synthesis is wholly responsible for hepatic Zn accretion.

Although Zn accumulation was not seen in MT-/- mice, LPS-associated factors may have influenced hepatic Zn in a more subtle manner. Comparison of total liver Zn values in MT-/- mice following 20 h of food deprivation with those 16 h (experiment 1) and 24 h (experiment 2) after LPS injection (also no food intake), shows that LPS inflammation ameliorated the

loss of liver Zn caused by fasting. It is likely that in the absence of MT induction, but with appropriate stimulus, hepatocellular uptake (or conservation) of Zn is possible, but at a rate sufficient only to meet immediate cellular requirements. If absorption from the diet is maintained, Zn needs may be met, but the ability to move Zn from other tissues to build hepatic reserves is lost. We have previously seen, in rats with mammary adenocarcinomas, rapid tumour growth and consequent high Zn uptake in the presence of low tumour MT concentrations [18]. The rats were eating a diet with more than adequate Zn to maintain body Zn levels as well as to provide for tumour growth. It is only when Zn conservation or redistribution is necessary in the absence of dietary Zn intake that the role of MT becomes paramount in preserving and (re)allocating existing pools of this important cation. MT thus appears to have a conserving function as an intracellular Zn buffer, maintaining a large Zn content in a non-toxic, non-ionized form in times of reduced intake and/or increased intracellular demand. In the unstressed state, however, MT seems to have a very minor role, accounting for less than 1 % of hepatic Zn in this study.

There were no apparent defects in inflammatory response to LPS in MT^{-/-} mice in relatively short-term experiments. The presence of LPS, of course, does not constitute a true infection, but only a signal to the body's defences that one exists. The physiological changes caused by injection of LPS, resulting from cytokine (TNF, IL-1, IL-6) release and action [27], appeared to be unimpaired in the MT^{-/-} mice which had the same degree of piloerection, anorexia and plasma Cu increase as MT^{+/+} control mice. Further evaluation, involving direct measurement of cytokines and acute-phase proteins, is required. One suspects that, when exposed to a real infection, the MT^{-/-} mice, with their potential for Zn loss, would not fare as well. The acute lowering of plasma Zn, which did not occur in the MT^{-/-} mice, has been said to benefit the infected host, by both removing nutrient Zn from the external milieu of the invading pathogens and by enhancing migration and activity in macrophages and neutrophils [28,29]. Without MT synthesis, the host would be unable to marshal or even maintain Zn reserves during cytokine-induced anorexia, further favouring the infective agents. Longer-term experiments using live bacteria are needed to better understand the effects of MT deletion on inflammation/infection.

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