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Title page

Induction of Metallothionein by Manganese Is Completely Dependent on Interleukin-6 Production

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IL-6-mediated induction of metallothionein by manganese

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ABBREVIATIONS:

MT, metallothionein; MTF-1, metal transcription factor-1; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IFN-γ, interferon-γ; STAT, signal transducer and activator of transcription; SAA, serum amyloid A; ALT, alanine aminotransferase; BUN, blood urea nitrogen; HPLC/ICP-MS, high performance liquid

chromatography/inductively coupled argon plasma-mass spectrometry; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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ABSTRACT

Metallothionein (MT) is a cysteine-rich protein that binds to and is inducible by heavy metals such as cadmium and zinc. However, the precise mechanism of MT induction by other metals remains unclear. In the present study, we investigated the mechanism of MT induction by manganese focusing on the involvement of cytokine production. Administration of manganese chloride ($MnCl_2$) to mice resulted in the induction of MT dose-dependently in the liver with little accumulation of manganese. Speciation analysis of metals in the liver cytosol showed that the major metal bound to the induced MT was zinc. Administration of $MnCl_2$ caused the increase in mRNA levels of interleukin-6 (IL-6) in the liver as well as the increase in serum levels of IL-6, but not those of other inflammatory cytokines. Subsequently, serum levels of serum amyloid A (SAA), an acute phase protein induced by IL-6, increased with a peak at 24 h. However, no increase in serum alanine aminotransferase activity was observed, suggesting that manganese enhanced the production of IL-6 and SAA without causing liver injury. In response to IL-6, the expression of a zinc transporter ZIP14 was enhanced in the liver, possibly contributing to the synthesis of hepatic zinc-MT. In IL-6 null mice, the induction of hepatic MT by treatment with $MnCl_2$ was completely suppressed to the control level. These results suggest that manganese is a unique metal that induces the synthesis of hepatic MT completely depending on the production of IL-6 without accompanying liver injury.

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Introduction

Metallothionein (MT) is a cysteine-rich low-molecular-weight protein with a high affinity for heavy metals. Physiological roles of MT display a broad spectrum including detoxification of heavy metals, scavenging of free radicals, regulation of cell growth, and the maintenance of homeostasis of trace metals such as zinc and copper (Suzuki et al., 1993). The most prominent characteristic of MT is its inducibility not only by metals but also by various factors such as hormones, cytokines, organic chemicals, starvation and physical stress (Kägi, 1993). It is generally conceived that the expression of MT gene in response to a metal load is regulated by metal-responsive transcription factor (MTF-1) that binds to metal response elements of the promoter region of MT genes (Heuchel et al., 1994). The mechanisms underlying the activation of MT gene through the interaction of MTF-1 and metal response elements have been investigated extensively (Andrews, 2001; Otsuka, 2004), but the precise mechanism of MT induction by non-zinc metals including cadmium has been poorly understood (Daniels et al., 2002; Wang et al., 2004).

In addition to potent MT-inducing metals such as zinc, cadmium, copper, mercury, silver, and bismuth, other metals such as chromium, iron, cobalt, nickel, arsenic, and manganese can also induce MT, but to the lesser levels (Fleet et al., 1990; Albores et al., 1992). These weak MT-inducing metals can not bind to MT protein. It has been considered that MT induction by these metals is mediated by inflammation or stress responses, but the exact mechanisms have not yet been fully elucidated.

Several metals are known to induce inflammatory cytokines in animals. For instance, cadmium induces tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6

and interferon (IFN)-γ (Kayama et al., 1995). TNF-α, IL-1β, IL-6 and IFN-γ are well-known inducers of hepatic MT (De et al., 1990). IL-6 induces MT-I and MT-II also in the brain (Penkowa and Hidalgo, 2000). The involvement of a signal transducer and activator of transcription (STAT) in the expression of MT gene in response to IL-6 stimulation has been postulated (Lee et al., 1999). Recently, we have demonstrated that trivalent cerium (Kobayashi et al., 2005) and pentavalent vanadium (Kobayashi et al., 2006) are rather potent MT inducers, and the production of IL-6 by these metals are, at least in part, involved in MT induction. However, quantitative analyses on the involvement of IL-6 in the induction of MT by other metals have not been examined.

An early study by Yoshikawa (1970) showed that pretreatment with manganese protected against lethal toxicity of cadmium. Goering and Klaassen (1985) also reported the reduction of cadmium-induced liver damage by pretreatment with manganese, and suggested that the protective action of manganese might be explained by MT induction. Several studies have shown that manganese induced MT synthesis in the liver of animals (Suzuki and Yoshikawa, 1976; Eaton et al., 1980; Waalkes and Klaassen, 1985). On the other hand, Bracken and Klaassen (1987) reported that manganese did not induce MT in primary cultured rat hepatocytes, suggesting that the manganese-induced hepatic MT synthesis is caused by an indirect mechanism. However, the mechanism of MT induction by manganese has not yet been elucidated.

In the present study, we investigated the mechanism of MT induction by manganese in mice, and found that manganese has an ability to induce hepatic MT but does not bind to the induced MT. Determination of cytokine production and utilization of IL-6 null mice revealed that hepatic MT synthesis by manganese was completely

dependent on IL-6 production. This is the first report demonstrating that the induction of MT by a metal compound is exclusively mediated by the production of IL-6.

Materials and Methods

Animals. Male ICR mice were purchased from Charles River Japan, Inc. (Atsugi, Japan), and used at the age of seven weeks. IL-6 null mice, and B6J129Sv mice as wild-type controls, were purchased from Jackson Laboratories (Bar Harbor, ME), and bred in the National Institute for Environmental Studies (NIES; Tsukuba, Japan). Male IL-6 null and wild-type mice at age of 10 weeks were used for experiments. All animal experiments were conducted according to the NIES guidelines for animal welfare and treatment.

Treatment of Mice with $MnCl_2$. Male ICR mice were treated subcutaneously (s.c.) with $MnCl_2$ dissolved in saline at doses of 150, 300, 450 and 600 μmol/kg. Control mice were given saline. The animals were sacrificed at 24 h after $MnCl_2$ treatment, and blood was collected under anesthesia. The serum was separated by centrifugation. The activity of alanine aminotransferase (ALT) and the concentration of blood urea nitrogen (BUN) were determined using automatic analyzer (Model 7150; Hitachi Co., Tokyo, Japan). The concentration of serum amyloid A (SAA) was determined using Cytoscreen ELISA kit (BioSource International, Camarillo, CA). The liver and kidney tissues were stored frozen at –80 °C until subsequent analyses of metal and MT concentrations. In a time-course experiment, male ICR mice were sacrificed at 0, 1.5, 3, 6, 12, 24 and 48 h after treatment with $MnCl_2$ (300 μmol/kg, s.c.). Serum samples of animals were collected and used for analyses of TNF-α, IL-1β, IL-6, IFN-γ and SAA. The liver was removed for the measurement of manganese. The serum concentrations of cytokines and SAA were measured using Cytoscreen ELISA kit (BioSource International). To determine dose-dependent changes of serum IL-6

concentrations by $MnCl_2$ administration, male ICR mice were treated s.c. with $MnCl_2$ at doses of 0, 50, 100, 150, 300, 450 and 600 μmol/kg. Animals were sacrificed at 6 h after treatment, and serum IL-6 concentration was determined. To obtain more direct evidence for the role of IL-6 in the induction of MT by manganese, male IL-6 null mice and wild-type mice were treated s.c. with $MnCl_2$ at doses of 0, 150 and 300 μmol/kg. Animals were sacrificed 24 h after the treatment, and serum concentration of SAA was determined. The liver was removed and the concentration of MT was measured.

Determination of Metals and MT in Tissues. The liver and kidney samples were digested with nitric acid. Concentration of manganese in each sample was determined using atomic absorption spectrophotometer (SPECTRAA 400, Varian, Inc., Palo Alto, CA). Concentration of MT was determined by Hg-binding assay (Himeno et al., 2000) as modified from the original ^{203}Hg-binding assay (Naganuma et al., 1987). Mercury bound to MT was measured by atomic absorption spectrophotometry using mercury analyzer (RA-2A, Nippon Instruments, Tokyo, Japan) after digestion with nitric acid. The MT content was expressed as nanomoles of Hg bound.

Distribution Profiles of Metals in Liver Cytosol. The distribution profiles of metals in the soluble fraction of the liver of mice were analyzed using high performance liquid chromatography/inductively coupled argon plasma-mass spectrometry (HPLC/ICP-MS) as described by Suzuki (1991) with a modification. Portions of liver samples from three mice obtained 24 h after the treatment with $MnCl_2$ (300 μmol/kg, s.c.) were pooled and homogenized in four volumes of saline. The homogenate was centrifuged at 4 °C for 1 h at 105,000 *g*. The liver of mice obtained 24 h after the treatment with cadmium chloride ($CdCl_2$; 10 μmol/kg, s.c.) was prepared in the same

manner as a positive control. An aliquot (40 μl) of the supernatant was applied to a TSK gel G3000SW column (7.5 × 600 mm with a 7.5 × 75 mm guard column, Tosoh, Tokyo, Japan). The loaded sample was eluted with 50mM Tris-HCl (pH 8.6 containing 0.1% sodium azide) at a flow rate of 0.8 ml/min on an HPLC Instrument (HP1100; Yokogawa Analytical Systems, Musashino, Japan). The eluate was monitored at 280 nm, and introduced directly into the nebulizer capillary of an ICP-MS apparatus (HP4500; Yokogawa Analytical Systems). The distribution profiles of manganese, cadmium, zinc and copper were determined at the mass numbers of 55, 111, 66 and 63, respectively.

Determination of mRNA Levels of IL-6 and ZIP14. Male ICR mice were sacrificed 0, 1, 3, 6, 12 and 24 h after the treatment with $MnCl_2$ (300 μmol/kg, s.c.). Total RNA was extracted from the liver and kidney tissues of mice using guanidium thiocyanate-phenol-chloroform extraction method as described by Chomczynski and Sacchi (1987). Reverse transcription (RT) reaction was performed in a mixture containing 50 mM Tris-HCl pH 8.3, 70 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1mM each of dNTP, 4 units of RNase inhibitor, 2 μg of total RNA, 0.5 μM $Oligo(dT)_{15}$ primer (Promega, Madison, WI), and 5 units of reverse transcriptase in a total volume of 20 μL. The reaction was carried out at 37 °C for 1.5 h. The RT reaction mixture was used directly for PCR amplification. Quantitative real-time RT-PCR was performed using a TaqMan probe according to the procedure recommended by the manufacturer (Applied Biosystems, Foster City, CA). For cDNA synthesis, 450 ng of total RNA was used. The forward and reverse primers and TaqMan probes for IL-6, ZIP14 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were supplied by TaqMan

Assay-on-Demand Products (Applied Biosystems). PCR amplifications were always performed using universal temperature cycles: 10 min at 94 °C, followed by 35-45 two-temperature cycles (15 sec at 94 °C and 1 min at 60 °C). Fluorescence of PCR products was detected using an ABI Prism 7300/7500 Sequence Detector System (Applied Biosystems).

Statistical Analysis. Statistical significance was determined by using either one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. If the data were not normally distributed, we used the Kruskal-Wallis test (nonparametric analysis of variance) followed by Dunn's multiple comparison test. Differences between groups were considered significant at $P < 0.05$.

Results

Accumulation of Manganese and Induction of MT in the Liver and Kidney. Hepatic and renal concentrations of MT and manganese in ICR mice treated with $MnCl_2$ are shown in Fig. 1. The administration of $MnCl_2$ resulted in a dose-dependent increase in MT levels in the liver. On the other hand, no increase in MT level was detected in the kidney. Contrary to MT induction, renal manganese concentrations increased markedly and dose-dependently, but hepatic manganese concentrations hardly changed from the control level (Fig. 1B). Thus, MT was induced by the administration of $MnCl_2$ with little accumulation of manganese in the liver, while MT was not induced in the kidney though high concentration of manganese was deposited.

Distribution Profiles of Manganese in Liver Cytosol. To examine whether manganese was bound to the MT in the liver, distribution profiles of manganese, zinc and copper in soluble fraction of the liver of $MnCl_2$-treated mice were analyzed by a speciation analysis using HPLC/ICP-MS. The column used for the analysis (TSK gel G3000SW) can separate MT-I and MT-II isoforms in a single elution (Suzuki, 1991). As shown in Fig. 2, manganese was detected mainly in the high-molecular-weight fraction. On the other hand, two clear peaks of zinc (retention time; 22.1 min and 23.6 min) were detected in the liver cytosol of $MnCl_2$-treated mice. These peaks corresponded to the two peaks of cadmium (retention time; 22.0 min and 23.5 min) in the liver cytosol of $CdCl_2$-treated mice used as a positive control. These data indicate that the major metal bound to the MT induced by the administration of $MnCl_2$ was zinc, but not manganese.

Changes in Biochemical Markers of Tissue Injury after $MnCl_2$

Administration. To explore possible involvement of tissue injury caused by $MnCl_2$ in MT induction, we determined biochemical markers for liver and kidney injury. However, no increase in the activity of ALT or the concentration of BUN was observed. On the other hand, the concentration of SAA, an acute phase protein, increased markedly and dose-dependently 24 h after the treatment with $MnCl_2$ (Fig. 3A). These data suggest that the administration of $MnCl_2$ did not cause hepatic and renal injury, but enhanced the serum levels of SAA.

Time-dependent Changes in Cytokine-related Markers. We next examined the time-course of changes in serum cytokine concentrations as well as tissue manganese concentrations after the treatment with $MnCl_2$. In this experiment, we used the dose of 300 μmol/kg $MnCl_2$ since the induction of MT and SAA reached the plateau levels at the dose of 300 μmol/kg (Figs. 1A and 3A, respectively). As shown in Fig. 4A, hepatic manganese concentrations increased quickly and reached a maximum level at 1.5 h after the $MnCl_2$ administration, and then decreased rapidly. The serum IL-6 concentrations also increased after $MnCl_2$ administration, with a peak at 6 h (Fig. 4B). However, little or no changes in the levels of serum TNF-α, IL-1β, or IFN-γ were found after the $MnCl_2$ administration (data not shown). The concentrations of SAA began to increase at 12 h after the $MnCl_2$ administration, and reached a peak level at 24 h (Fig. 4C). To examine the dose-dependency of IL-6 production by $MnCl_2$ administration, serum IL-6 concentration was measured at 6 h after the treatment with $MnCl_2$ (0-600 μmol/kg). The serum IL-6 levels were increased dose-dependently by $MnCl_2$ administration (Fig. 5).

mRNA Levels of IL-6 and ZIP14. In order to confirm the expression of IL-6 gene

in the liver, the mRNA levels of IL-6 in the liver of $MnCl_2$-treated mice were evaluated by a real-time RT-PCR. As shown in Fig. 6A, the levels of IL-6 mRNA increased with a peak at 3 h after the $MnCl_2$ administration. No increase in IL-6 mRNA was found in the kidney (data not shown). Recently, IL-6-induced up-regulation of ZIP14, a zinc transporter responsible for the incorporation of zinc from the serum to the tissue, was reported (Liuzzi et al., 2005). As shown in Fig. 6B, administration of $MnCl_2$ markedly enhanced the mRNA levels of ZIP14. The maximum levels were observed 6-12 h after the $MnCl_2$ administration, suggesting that this increase is caused by the expression of IL-6 gene in the liver. However, no apparent increases in ZIP14 mRNA levels were observed in the kidney (data not shown). Thus, it seems likely that the administration of $MnCl_2$ induced the expression of IL-6 in the liver, and then the IL-6 induced the expression of both MT and ZIP14 genes, leading to the synthesis of zinc-MT in the liver.

MT Induction by Manganese in IL-6 null Mice. To explore the direct evidence for the involvement of IL-6 in the induction of MT by manganese, we compared the MT induction between IL-6 null and wild-type mice. The induction of hepatic MT by the treatment with $MnCl_2$ was completely suppressed in IL-6 null mice (Fig. 7). This is not caused by the suppression of manganese accumulation in IL-6 null mice since the peak levels of hepatic manganese 1 h after the treatment with $MnCl_2$ (300 μmol/kg) were about the same between IL-6 null mice (58.1 $\pm$ 5.9 μg/g) and wild-type mice (69.6 $\pm$ 3.9 μg/g). Furthermore, the injection of human recombinant IL-6 (250 μg/kg) together with $MnCl_2$ (300 μmol/kg) significantly recovered the induction of MT (35.7 $\pm$ 14.7 nmol Hg bound/g) in the liver of IL-6 null mice. These data further support the

notion that IL-6 plays a crucial role in hepatic MT induction by $MnCl_2$ treatment. Also, serum levels of SAA were completely suppressed in IL-6 null mice, suggesting that both hepatic MT induction and SAA production by the treatment with $MnCl_2$ were completely dependent on IL-6. This is the first observation that the induction of MT by a metal compound is exclusively mediated by IL-6 production.

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Discussion

In the present study, we demonstrated that administration of $MnCl_2$ induced MT in the form of zinc-MT in the liver of mice, and this induction is completely dependent on the IL-6 production. Although some metal compounds have been shown to elicit cytokine production through the activation of inflammatory responses, manganese is unique in that it does not cause liver injury.

To elucidate the mechanism of MT induction by metal compounds, many studies have been focusing on the role of the transcription factor MTF-1 and metal response elements (Andrews, 2001; Otsuka, 2004). The role of zinc ions in the activation of MTF-1 has been well characterized (Andrews, 2001; Jiang et al., 2003). However, potent MT-inducing metals such as cadmium or copper did not activate the DNA-binding activity of MTF-1 in a cell-free system (Bittel et al., 1998), suggesting that these metals activate MTF-1 indirectly via the release of zinc ions from intracellular zinc stores (Zhang et al., 2003). In addition, cadmium induces oxidative stress and produces several kinds of cytokines, both of which are known to induce the synthesis of MT. This suggests that other mechanisms than MTF-1 activation may also be involved in the induction of MT by cadmium (Chu et al., 1999). Thus, the molecular mechanisms of MT induction by non-zinc metal compounds still remain unclear.

Among MT-inducing metals, iron, chromium, cobalt, nickel, arsenic, and manganese are incapable of binding to MT protein. It has been postulated that these metals are indirect MT inducers and that the MT induction by these metals is mediated primarily by inflammation or stress responses. Since inflammatory cytokines such as IL-6, IL-1β and TNF-α are known MT inducers (De et al., 1990; Penkowa and Hidalgo,

2000), and some metal compounds elicit allergic reactions, it is reasonable to assume that these cytokines are involved in the MT induction by the non-specific MT-inducing metals. However, the actual roles of these cytokines in the MT induction by metal compounds have been poorly understood.

In the previous studies, we have demonstrated that administration of trivalent cerium compound, $CeCl_3$ (Kobayashi et al., 2005), and pentavalent vanadium compound, ammonium metavanadate (Kobayashi et al., 2006), induced MT in the liver of mice, and this induction was significantly suppressed in IL-6 null mice compared with the wild-type mice, indicating that IL-6 production by these metals are the major cause for the MT induction. Similar to manganese, cerium and vanadium compounds increased serum levels of IL-6, but not those of IL-1β or TNF-α. In the present study, however, the administration of $MnCl_2$ even at the highest dose did not increase serum activity of ALT (Fig. 3B), whereas the administration of $CeCl_3$ or ammonium metavanadate in the previous studies caused dose-dependent increases in serum AST and ALT activities. Thus, the treatment of mice with $MnCl_2$ enhanced the expression of IL-6 in the liver (Fig. 6A) without causing liver injury. Furthermore, the MT induction by the administration of $MnCl_2$ was completely suppressed in IL-6 null mice (Fig. 7A), while the MT induction by cerium and vanadium compounds in IL-6 null mice were reduced to about a half of that in the wild-type mice (Kobayashi et al., 2005; Kobayashi et al., 2006). These results suggest that other factors than IL-6 production are also involved in the MT induction by the hepatotoxic metals such as cerium and vanadium, while MT induction by non-hepatotoxic manganese in the liver is mediated exclusively by the production of IL-6.

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It has been known that manganese is rapidly excreted into the bile from the liver when injected to animals (Klaassen, 1974). In the present study, we found little increase in hepatic manganese concentration at 24 h after the administration (Fig. 1B), but the time-course experiment showed that high concentration of manganese existed in the liver with a peak at 1.5 h (Fig. 4A). Probably, this transient increase in manganese concentration in the liver caused the expression of IL-6 gene in the liver with a peak at 3 h (Fig. 6A) either in Kupffer cells (Busam et al., 1990) or parenchymal hepatocytes (Saad et al., 1995). Subsequently, serum levels of IL-6 increased with a peak at 6 h (Fig. 4B), and then the enhanced production of SAA (Fig. 4C) and MT synthesis were followed in response to IL-6 stimulation. On the other hand, little increase in MT concentration was detected in the kidney even though high concentrations of manganese were accumulated in the kidney (Fig. 1). This may be due to the overwhelming abundance of IL-6 receptor in the liver compared with the kidney (Castell et al., 1988).

It has been well known that serum levels of SAA, an acute phase protein produced in the liver, are increased by inflammatory stimuli (Uhlar and Whitehead, 1999). Expression of SAA gene is regulated primarily by the inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Uhlar and Whitehead, 1999). In the present study, serum levels of IL-6 increased with a peak at 6 h after $MnCl_2$ administration, and then the SAA levels increased and reached a maximum level at 24 h (Fig. 4). Furthermore, the increase in SAA levels by $MnCl_2$ administration was completely suppressed in IL-6 null mice (Fig. 7B). These results suggest that the production of SAA by manganese is mediated solely by IL-6 production and is independent of liver injury.

Recently, several studies have demonstrated that IL-6 null mice exhibited more severe liver damage after the administration of lipopolysaccharide (Inoue et al., 2005), concanavalin A (Klein et al., 2005), and carbon tetrachloride (Kovalovich et al., 2000) than the wild-type mice. These findings suggest the roles of IL-6 or its downstream products in triggering the protective process against liver injury. Earlier studies have shown that treatment with manganese protected against hepatotoxicity induced by cadmium (Yoshikawa, 1970; Goering and Klaassen, 1985), suggesting that the MT induced by manganese is responsible for the attenuation of hepatotoxicity. However, we cannot exclude a possibility that manganese-induced IL-6 expression itself or its downstream products other than MT triggered the protection against hepatotoxicity. It is noteworthy that ME3738, an agent that induces IL-6 in the liver, protected against concanavalin A-induced hepatotoxicity (Kuzuhara et al., 2006). Further studies are warranted to clarify whether MT is the primary downstream product responsible for the IL-6-triggered cytoprotection or other non-MT factors induced by IL-6 are more important.

An early study reported that the MT induced by manganese in rat liver was co-eluted with zinc in the gel filtration chromatography of liver supernatant (Suzuki and Yoshikawa, 1976). Waalkes et al. (1984) showed that manganese has no affinity for MT by using equilibrium dialysis *in vitro*. In the present study, the results of HPLC/ICP-MS analyses of liver cytosol showed more clearly that manganese was not bound to the $MnCl_2$-induced MT and zinc was the major binding metal to the MT (Fig. 2). Recently, Liuzzi et al. (2005) demonstrated that IL-6 induces hypozincemia through the activation of zinc transporter, ZIP14, which is responsible for the incorporation of zinc

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into the liver from the serum. As shown in Fig. 6B, the expression of ZIP14 was enhanced after the peak induction of IL-6 by $MnCl_2$ treatment, suggesting that the enhanced incorporation of zinc from the extracellular pool via the IL-6-activated ZIP14 expression might have contributed to the synthesis of zinc-MT in the liver. In support of this notion, very little increase in ZIP14 expression was detected in the liver of IL-6 null mice treated with $MnCl_2$ (data not shown).

In conclusion, our study demonstrated that manganese induces hepatic MT synthesis completely depending on the production of IL-6 without accompanying liver injury. Further studies are warranted to clarify the mechanism underlying the specific activation of IL-6 gene by manganese.

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Footnotes

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Legends for figures

Fig. 1. Dose-dependent changes in MT induction (A) and manganese accumulation (B) in the liver and kidney of mice. Mice were treated s.c. with $MnCl_2$ at the indicated doses and the liver and kidney tissues were removed 24 h after the $MnCl_2$ treatment. Concentrations of MT and manganese were determined by the Hg-binding assay and atomic absorption spectrophotometry, respectively. Values are mean ± S.D. for five mice. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Fig. 2. HPLC/ICP-MS profiles of metals in the liver cytosol of mice. Mice were treated s.c. with $MnCl_2$ (300 μmol/kg) or $CdCl_2$ (10 μmol/kg) and sacrificed 24 h after the administration of metal compounds. A portion (40 μl) of the supernatant of the liver was applied to a TSK gel G3000SW column and the distribution profiles of metals were determined by ICP-MS connected directly to HPLC. The mass numbers of 55, 111, 66 and 63, were used for manganese, cadmium, zinc and copper, respectively.

Fig. 3. Dose-dependent changes in SAA concentrations (A), ALT activities (B) and BUN concentrations (C) in serum of mice. Mice were treated s.c. with $MnCl_2$ at the indicated doses and the blood was collected 24 h after the $MnCl_2$ treatment. The activity of ALT and concentration of BUN were determined using the automatic analyzer. SAA concentration was determined using a commercial ELISA kit. Values are mean ± S.D. for five mice. **, $P<0.01$; ***, $P<0.001$.

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Fig. 4. The time-course of changes in manganese concentrations in the liver (A), and serum concentrations of SAA (B) and IL-6 (C) in mice. Mice were treated s.c. with $MnCl_2$ (300 μmol/kg) and the blood was collected at the indicated time points after the treatment with $MnCl_2$. Concentrations of SAA and IL-6 were determined using commercial ELISA kits. Values are mean ± S.D. for five mice. **, $P<0.01$; ***, $P<0.001$.

Fig. 5. Dose-dependent changes in serum concentrations of IL-6 in mice. Mice were treated s.c. with $MnCl_2$ at the indicated doses and the blood was collected 6 h after the $MnCl_2$ treatment. Concentrations of IL-6 were determined using a commercial ELISA kit. Values are mean ± S.D. for five mice. **, $P<0.01$; ***, $P<0.001$.

Fig. 6. The time-course of changes in mRNA levels of IL-6 (A) and ZIP14 (B) in the liver of mice. Mice were treated s.c. with $MnCl_2$ (300 μmol/kg), and sacrificed at the indicated time points. Total RNA was extracted from the liver, and mRNA levels of IL-6 and ZIP14 were determined by quantitative real-time RT-PCR. The relative ratios of mRNA levels of IL-6 and ZIP14 normalized by GAPDH were expressed as the fold-increase compared with the control value. Values are mean ± S.E. for five mice. *, $P<0.05$

Fig. 7. Concentrations of MT in the liver and concentrations of SAA in the serum of IL-6 null and wild-type mice. IL-6 null (open columns) and wild-type (closed columns) mice were treated s.c. with $MnCl_2$ and the liver and blood samples were collected 24 h

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after the $MnCl_2$ treatment. Concentrations of MT were determined by the Hg-binding assay and concentrations of SAA were determined using a commercial ELISA kit. Values are mean ± S.D. for five mice. ***, $P<0.001$ versus control mice. $^{###}$, $P<0.001$ versus wild-type mice.

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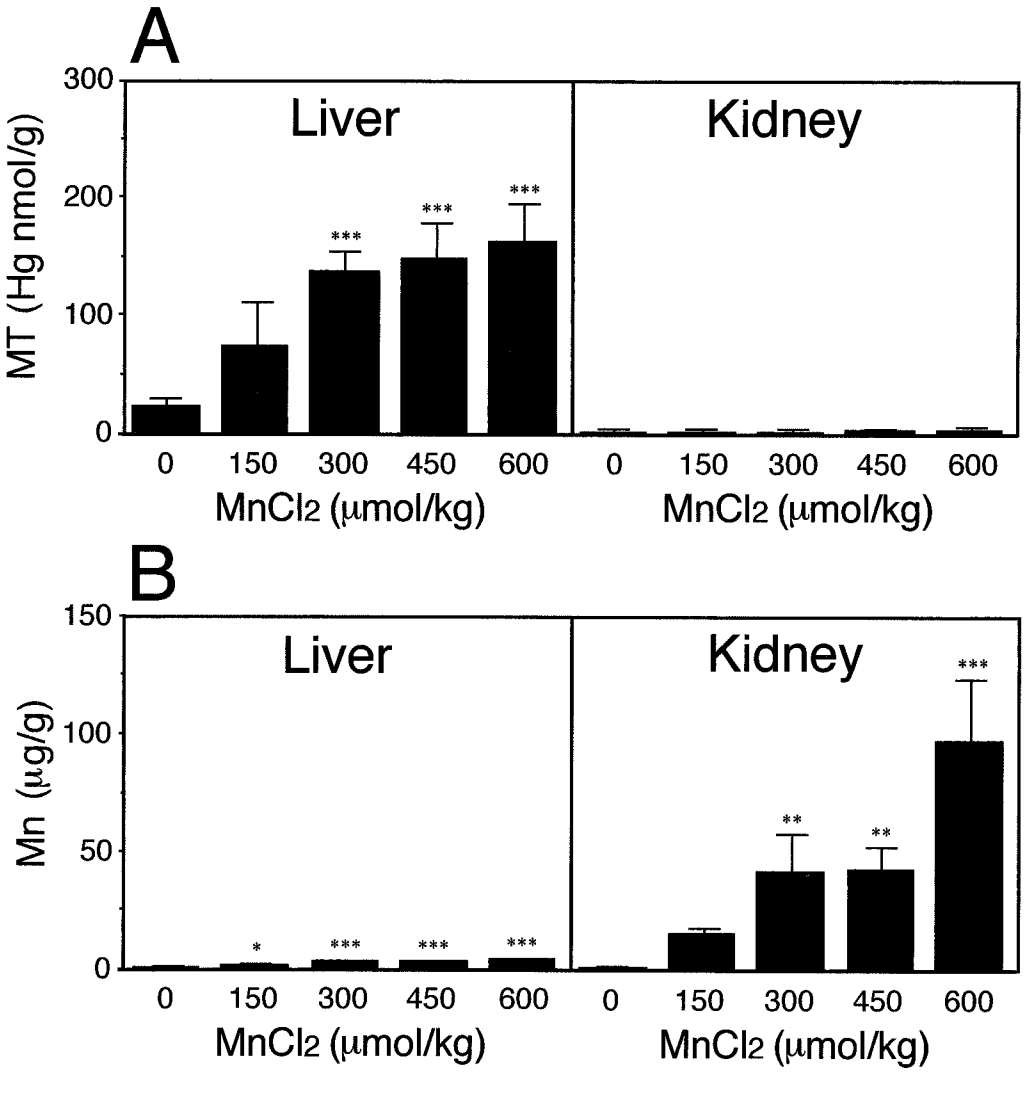

Fig. 1

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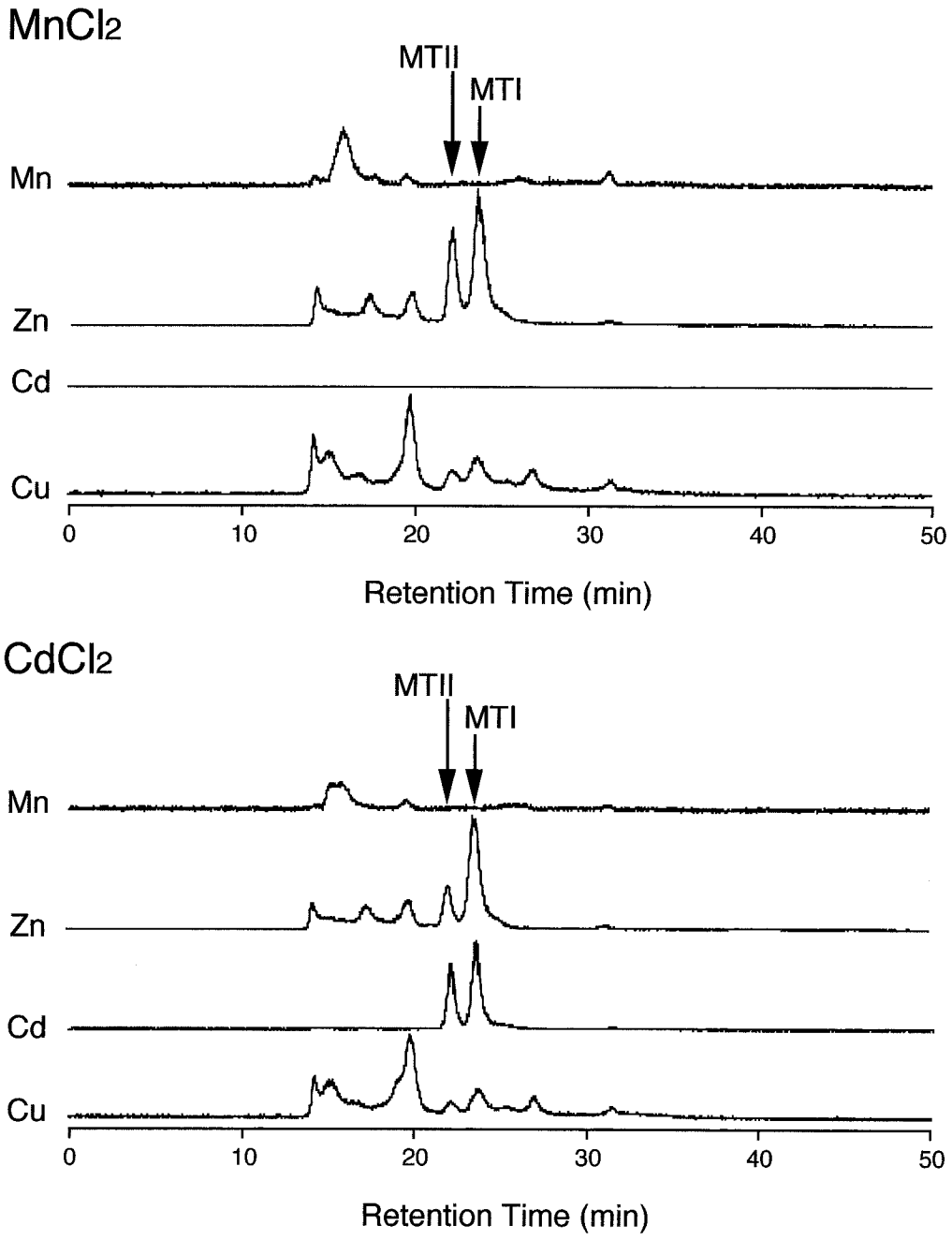

Fig. 2

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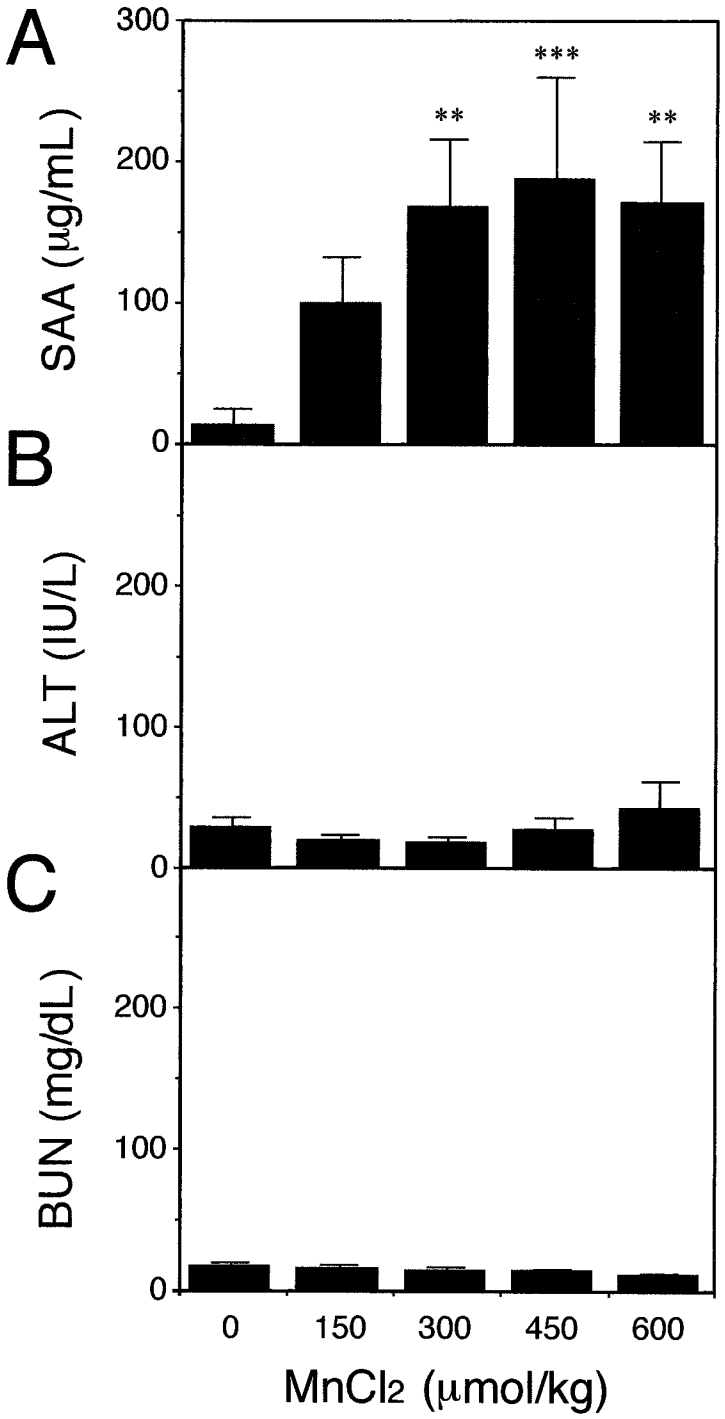

Fig. 3

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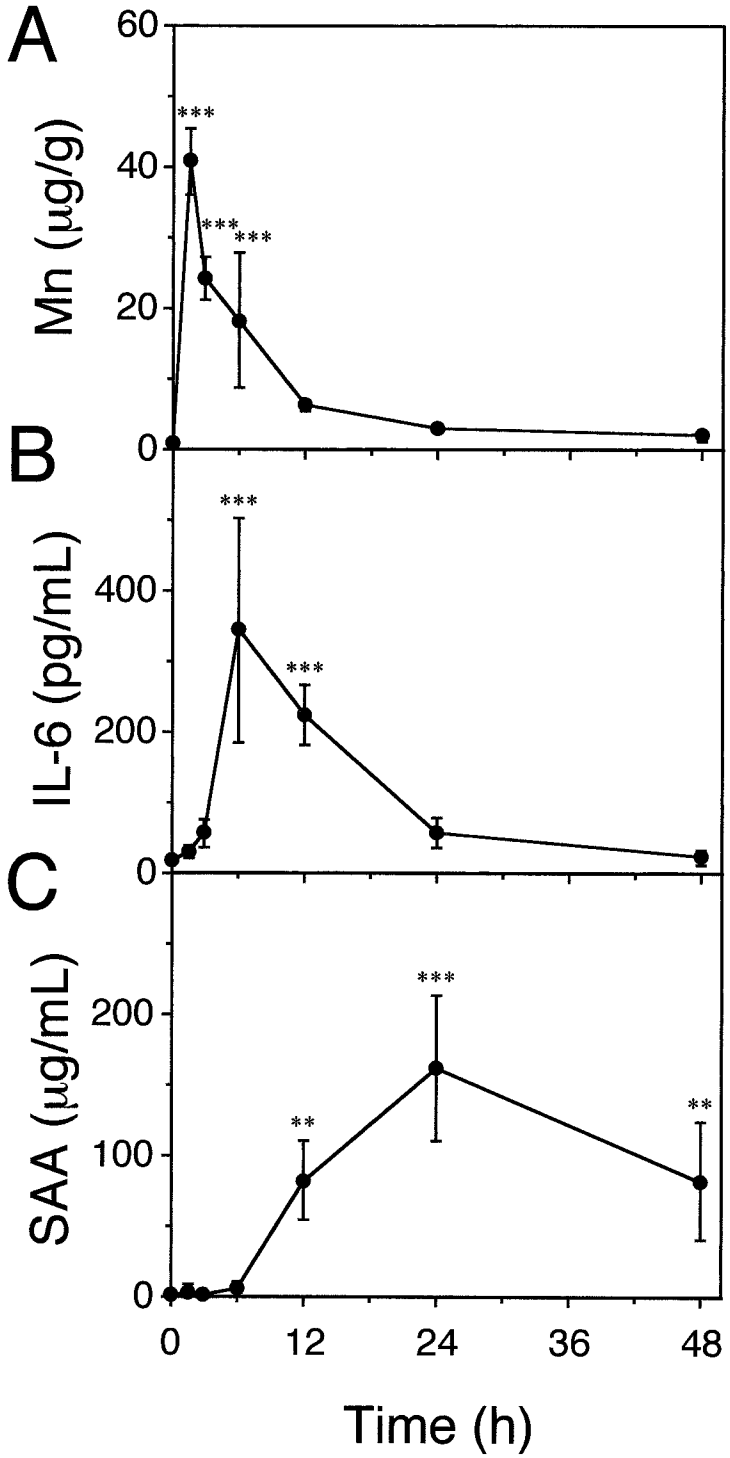

Fig. 4

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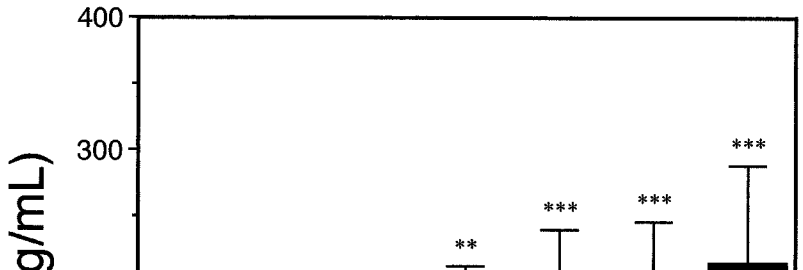

Fig. 5

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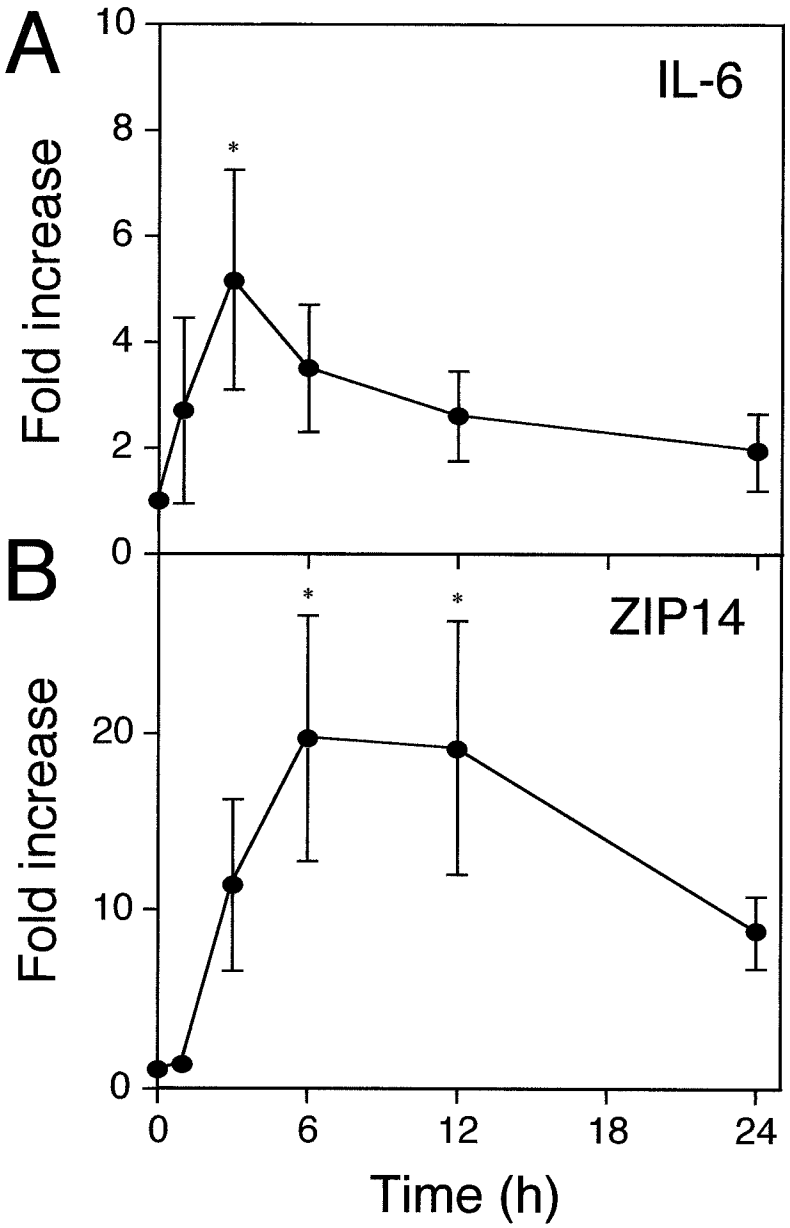

Fig. 6

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Fig. 7