Protective Effect of Zinc against Lipopolysaccharide/D-Galactosamine-Induced Lethality

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(Received October 7, 2002; Accepted October 11, 2002)

Zinc is an essential and multifunctional element for all cells. Metallothionein (MT) is a low molecular weight, cystein-rich, metal-binding protein that is involved in zinc homeostasis. During the acute-phase reaction, hepatic MT production is induced and hepatic zinc accumulation is stimulated. We previously reported that MT-I and II-deficient (MT-null) mice are highly sensitive to the lethal effects of lipopolysaccharide (LPS) plus D-galactosamine (GalN). The sensitization may relate to attenuatoin of α_1-acid glycoprotein (AGP) expression in MT-null mice. In the present study, we hypothesized that MT-induced hepatic zinc accumulation promotes AGP expression and prevents LPS/GalN-induced lethality. To determine whether zinc reduces LPS/GalN toxicity, zinc was administered to mice. Simultaneous administration of zinc and LPS/GalN showed no effect on the lethality of LPS/GalN in mice. Zinc administration at 3 hr prior to LPS/GalN challenge reduced LPS/GalN-induced death. However, zinc pre-administration at 24 hr before LPS/GalN challenge did not reduce LPS/GalN-induced death. The expression of AGP mRNA was elevated at 3 hr after zinc administration, 24-hr pretreatment was ineffective. The protective effect of zinc was observed in both wild-type and MT-null mice. These results show that the protective effects of zinc were not caused by MT induction, but by AGP expression. We suggest that MT-induced hepatic zinc accumulation may promote AGP expression and thus prevent LPS/GalN-induced lethality.

Key words —— metallothionein, zinc, lipopolysaccharide, D-galactosamine

INTRODUCTION

Zinc is an essential and multifunctional element for all cells, and confers structural and/or catalytic function upon some 300 enzymes involved in various aspects of metabolism and cell growth.[1,2] During the acute-phase response, blood zinc levels decrease and hepatic zinc levels increase.[3–5] This elevation in the levels of hepatic zinc at the onset of inflammation may facilitate the numerous enzymatic and transcriptional processes necessary for mounting the acute-phase response.

Metallothionein (MT) is a low molecular weight, cysteine-rich, metal-binding protein, and is involved in zinc homeostasis.[6,7] During the acute-phase response, hepatic MT production is induced in response to interleukin (IL)-6 and subsequently promotes hepatic zinc accumulation.[8–10] Philcox *et al.* reported that lipopolysaccharide (LPS) did not induce hepatic zinc uptake in mice lacking expression of MT-I and MT-II genes.[11] MT may have a role in maintaining hepatic and blood glucose levels during endotoxemia.[12]

We previously reported that MT-I and II-deficient (MT-null) mice are highly sensitive to the lethal effects of LPS/D-galactosamine (GalN).[13] α_1-Acid glycoprotein (AGP) protects against LPS/GalN-induced death.[14] The expression of AGP mRNA was lower in MT-null mice than in wild-type mice. This suggested that MT might inhibit the lethal effects of LPS/GalN by stimulating AGP expression. Furthermore, we showed that MT can activate MRE-binding transcription factor (MTF)-1, which is also activated by zinc during the acute-phase reaction.[15] The activation of MTF-1 may lead to an increase in AGP expression.[16] We therefore hypoth-

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esized that MT-induced hepatic zinc accumulation stimulates AGP expression and may be a key factor in preventing LPS/GalN-induced death.

In the present study, we investigated whether zinc inhibits the lethal effects of LPS/GalN. We demonstrated that the administration of zinc does inhibit LPS/GalN-induced death, which suggests that hepatic zinc accumulation promotes AGP expression. The present results also suggest that hepatic zinc accumulation is a key factor in MT-mediated protection against LPS/GalN-induced death.

MATERIALS AND METHODS

Reagents —— LPS from *Escherichia coli* O26 : B5 was obtained from Difco Laboratories (Detroit, MI, U.S.A.). Recombinant mouse tumor necrosis factor alpha (TNF-α) was obtained from Pepro Tech, Inc. (Canton, MA, U.S.A.). GalN was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).
Animals —— MT-null mice (129/Sv)[17] provided by the Jackson Laboratory (Bar Harbor, ME, U.S.A.) were crossed with C57BL/6J mice, supplied by Clea Japan (Osaka, Japan). The resulting heterozygous litters were used for two further cycles of inbreeding at our laboratory. Offspring (129/Sv × C57BL/6J) from heterozygous matings were genotyped by PCR, and then 8- to 12-week-old males were used in the experiments. All mice were housed under conditions of controlled temperature (23–24°C) and light (12 hr light and 12 hr dark). Food and tap water were provided *ad libitum*.
Animal Experiments —— All experiments were conducted in accordance with the institutional guidelines of Osaka University. C57BL/6J mice were intraperitoneally administered with LPS (5 μg/kg) and GalN (700 mg/kg). 129/Sv × C57BL/6J-derived F2 (wild-type and MT-null) were intraperitoneally administered with LPS (50 μg/kg) and GalN (700 mg/kg). Seventy to eighty percent of mice died of these treatments. The survival rate of mice over 24 hr post-administration was recorded. To clarify the protective effect of zinc, mice were subcutaneously administered with zinc (20 mg/kg).
Determination of Hepatic Zinc Concentration —— Livers were perfused with saline under anesthesia and liver samples were excised. The liver samples were solubilized with nitric acid, and the zinc concentration was analysed using an atomic absorption spectrophotometer.
Northern Hybridization —— Total RNA isolated from wild-type and MT-null mice before and after the administration of LPS/GalN was resolved by electrophoresis using a 1.0% agarose/10% formaldehyde gel and transferred onto a nylon membrane (Amersham) in 20 X SSC. The RNA was subjected to Northern hybridization with a ^{32}P-labeled AGP-specific probe.[18] The membrane was reprobed with a ^{32}P-labeled DNA probe for mouse 18S rRNA, which served as an internal control.
Statistical Analysis —— Differences in survival rates were analyzed by the Kaplan-Meier (Product-Limit) method and log rank test. Differences in the other data were analyzed by one-way analysis of variance (ANOVA) and Fisher's PLSD test. Differences in survival rates were analyzed by the Kaplan-Meier (Product-Limit) method and log rank test. Differences between groups were considered significant at the $p < 0.05$ level.

RESULTS

To determine whether zinc reduces LPS/GalN-induced lethality, zinc was administrated to C57BL/6J mice. Simultaneous administration of zinc and LPS/GalN showed no effect on lethality (Fig. 1A). When zinc was administered at 3 hr prior to LPS/GalN, the lethal effects were reduced. Hepatic zinc levels 3 hr after administration of zinc were significantly higher than the in basal levels (354.9 ± 16, 417.3 ± 7.8 μg/g liver; $p < 0.05$). The protective effect of zinc was seen in MT-null mice (Fig. 1B). However, no reduction in lethality was observed when zinc was administered at 24 hr prior to LPS/GalN challenge (Fig. 1A).

The levels of AGP mRNA were determined by Northern blot analysis. Zinc administration induced AGP expression, mRNA levels began to increase at 3 hr post-administration, and continued increasing until 12 hr post-administration (Fig. 2).

DISCUSSION

During the acute-phase reaction, hepatic MT is induced and promotes hepatic zinc accumulation. However, the specific functions of induced MT and accumulated zinc remain to be elucidated. We showed that supplementation of zinc reduces the lethal effects of LPS/GalN. Taken together, the results reported here and previously strongly suggest that MT induction is a key process in suppressing

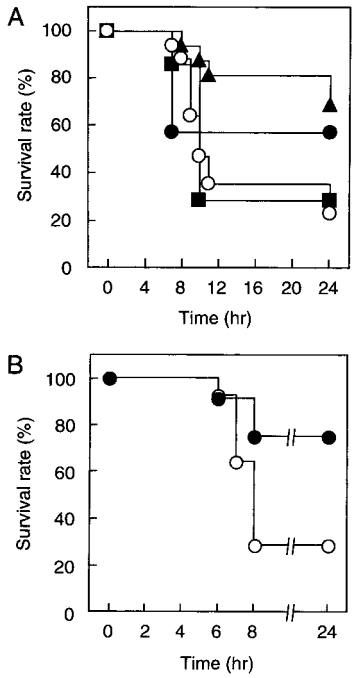

Fig. 1. Time Dependence of Zn-Induced Inhibition Against LPS/GalN-Induced Lethal Effects

(A) Wild-type mice were administered with zinc (20 mg/kg, s.c.) at –24 hr (filled circles; $n = 7$), –3 hr (filled triangles; $n = 16$), and 0 hr (filled squares; $n = 7$). Control mice were administered vehicle (open circles; $n = 17$). At 0 hr, all mice were administered a single i.p. dose of LPS/GalN. Survival rates in controls and mice given zinc at –3 hr were significantly different ($p < 0.01$). (B) MT-null ice were administered zinc (filled circles; $n = 9$) or vehicle (control, open circles; $n = 7$) at –3 hr. At 0 hr, all mice were administered a single i.p. dose of LPS/GalN. Survival rates of control mice and those administered zinc were significantly different ($p < 0.05$).

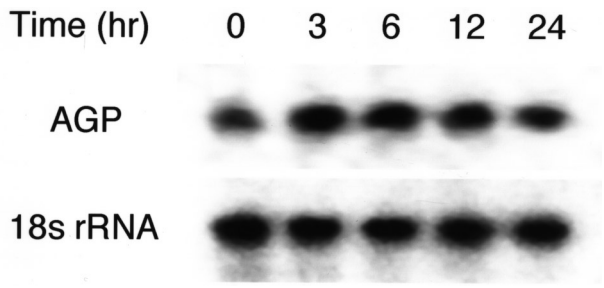

Fig. 2. Hepatic AGP mRNA Levels in Wild-Type and MT-null Mice before and after Administration of LPS/GalN

Experimental conditions were similar to those in Fig. 1. Total RNA was isolated and analyzed by Northern blotting. Nucleotides 451–613 of mouse AGP-1 cDNA sequence and nucleotides 165–203 of mouse rRNA functioned as mouse AGP and 18S rRNA probes, respectively.

harmful effects during the acute-phase reaction.

In this study, we showed that 3 hr pre-administration of zinc prevents LPS/GalN-induced death in C57BL/6J mice (Fig. 1A). The protective effect was observed in MT-null mice (Fig. 1B). Philcox *et al.* reported that hepatic zinc concentrations, which in wild-type mice increased after LPS injection, did not increase in MT-null mice.[11] At 7 hr after administration of LPS/GalN, hepatic zinc concentrations in MT-null mice were lower than that in wild-type mice (336.8 ± 102.7, 184.3 ± 73.2 μg/g liver; $p < 0.05$). We previously reported that MT-null mice are highly sensitive to the lethal effects of LPS/GalN.[13] These results suggested that supplementation of zinc increased the hepatic zinc concentration and reduced the lethal effects of LPS/GalN. It is suggested that zinc can make good the lack of MT after LPS/GalN administration in MT-null mice.

We attempted to determine whether the protective effects of zinc are related to AGP, which is one of the protective factors against LPS/GalN-induced lethality.[14] Since AGP was induced by zinc,[16] It is suggested that accumulated zinc increases AGP expression. In previous studies, we showed that AGP induction was decreased in MT-null mice 7 hr after administration of LPS/GalN.[13] Zinc administration induced AGP expression, mRNA levels began to increase at 3 hr post-administration (Fig. 2). On the other hand, simultaneous administration of zinc and LPS/GalN showed no effect on lethality (Fig. 1A). Pre-administration of zinc 24 hr prior to LPS/GalN injection also showed no effect on LPS/GalN-induced lethality. As shown in Fig. 2, an abrupt increase in AGP expression was seen at 3 hr after administration of zinc. The abrupt increase in AGP expression may be an important factor for zinc-mediated protection. Libert *et al.* showed that AGP should be administered 2–4 hr before TNF-α/GalN to confer protection[14] and that high constitutive expression of AGP does not protect against the lethal effects of TNF-α/GalN.[19] TNF-α appears to play a crucial role in the lethal effects of LPS/GalN. The present results support the findings of their study.

Since zinc prevents LPS/GalN-induced lethality, we may infer that hepatic zinc accumulation by MT promotes AGP expression and prevents the lethal effects of LPS/GalN. In 2001, the protective effects of zinc against TNF-induced death were reported,[20] where mice were treated with zinc for a period of 7 days, and then challenged with TNF. MT-null mice pretreated with zinc showed higher survival rates than untreated MT-null mice. Waelput *et al.* suggested the involvment of HSP70 in the zinc-induced protective effects against TNF, and that this protection is via an MT-independent mechanism. However, the experimental conditions of that study and the present study are significantly different, par-

ticularly relating to the zinc treatment. Therefore, the functions of zinc demonstrated in the two studies are likely to be distinct. Other investigators have also reported the effects of zinc against the lethal effects of LPS. Synder and Walker showed that zinc inhibited lethality,[21] while Shibayama *et al*. reported that zinc augmented LPS-induced death.[22] These effects of zinc need to be investigated further in order to be fully understood.

In conclusion, within the narrow limits of our experimental conditions, the mice injected with zinc 3 hr prior to LPS/GalN challenge were protected. The present results suggest that MT-induced hepatic zinc accumulation promotes AGP expression and prevents LPS/GalN-induced death. MT is suggested to regulate the distribution of zinc. These results demonstrate that this regulation of the harmful effects during the acute-phase reaction is a novel function of MT.

Acknowledgements This study was partly supported by a Grant-in-Aid for Scientific Research (No. 13376003) from Ministry of Education, Culture, Sports, Science and Technology.

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