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Original Article

Effect of metallothionein on ethanol-induced hepatotoxicity in mice

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ABSTRACT — Metallothionein (MT) is a small, metal-binding protein that can act as a scavenger of free radicals. To determine whether MT is involved in ethanol-induced hepatotoxicity, which is known to occur through oxidative stress, we studied sensitivity to hepatotoxicity caused by ethanol in MT-null mice genetically deleted for *MT-I* and *MT-II*. MT-null mice and wild-type mice were i.p. administrated with ethanol (99.5%, 2.0 g/kg). The increase in GPT, GOT, and LDH activities in the serum of MT-null mice was significantly higher than in the wild-type mice 24 hr after ethanol treatment. Histopathological examination in the liver of ethanol-treated MT-null mice demonstrated vacuolar degeneration. In contrast, histopathologic change was less prominent in the liver of ethanol-treated wild-type mice. Moreover, ethanol increased lipid peroxidation levels only in the liver of MT-null mice. These results indicate that deletion of MT is associated with ethanol-induced severe hepatotoxicity through oxidative stress.

Key words: Ethanol, Metallothionein, Liver, Oxidative stress

INTRODUCTION

Ethanol is primarily metabolized in the liver (Lieber, 1997). Following metabolization, ethanol induces the generation of reactive oxygen species (ROS) in the liver, leading to oxidative injury (Kurose *et al.*, 1996; Nordmann *et al.*, 1992). Ethanol is metabolized through three major pathways with different subcellular locations: alcohol dehydrogenase in the cytosol, aldehyde dehydrogenase in the mitochondrion, and the microsomal ethanol-oxidizing system in the endoplasmic reticulum (Lieber, 1997). All of these pathways are closely associated with ROS generation. Several studies have suggested that ethanol-induced hepatotoxicity is diminished by antioxidants including glutathione precursors, vitamin E, vitamin C, and some trace elements (Nordmann, 1994; Zhou *et al.*, 2002, 2005).

Metallothionein (MT) is a cysteine-rich low molecular weight protein with a high affinity for various metals and having cytoprotective effects (Klaassen *et al.*, 1999). MT also acts as a scavenger of oxygen free radicals (Sato and Bremner, 1993; Cai *et al.*, 1999). In mammals, MT exists in four isoforms: MT-I and MT-II are expressed in almost all tissues, MT-III is mainly expressed in the brain, and MT-IV is expressed in stratified squamous epithelia (Vasak and Meloni, 2011). MT-null mice, which are deficient in MT-I and MT-II, are highly susceptible to oxidative stress induced by substances such as paraquat (Sato *et al.*, 1996), ultraviolet (Hanada *et al.*, 1998a, 1998b), carbon tetrachloride (Liu *et al.*, 1998), acetaminophen (Rofe

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et al., 1998; Zhang *et al*., 1999; Liu *et al.*, 1999), cisplatin (Satoh *et al.*, 1997, Liu *et al.*, 1998), doxorubicin (Kimura *et al.*, 2000), and ionizing radiation (Shibuya *et al.*, 2008).

MT was previously shown to have a cytoprotective effect on ethanol-induced gastroduodenal mucosal injuries (Takano *et al.*, 2000). However, its role in ethanol-induced hepatotoxicity remains unclear. We therefore examined the effect of endogenous MT on ethanol-induced hepatotoxicity using MT-null mice.

MATERIALS AND METHODS

Animals

MT-null mice whose *MT-I* and *MT-II* genes had null mutations and wild-type mice were kindly provided by Dr. K.H.A. Choo (Murdoch Institute for Research into Birth Defects, Royal Children's Hospital, Parkville, Australia) (Michalska and Choo, 1993). The mice were of a mixed genetic background of 129 Ola and C57BL/6 strains. F1 hybrid mice were mated with C57BL/6J mice (CLEA Japan, Tokyo, Japan) and their offspring were backcrossed to C57BL/6J for six generations. Both MT-null mice and wild-type mice were generated by the mating of heterozygous ($MT^{+/-}$) mice. The mice were routinely bred in the vivarium of the National Institute for Environmental Studies (Tsukuba, Japan), and were observed to reproduce normally, and had no overt abnormalities in physical state or behavior.

MT-null mice and wild-type mice were housed in cages in ventilated animal rooms at a controlled temperature (23 ± 1ºC) and relative humidity (55 ± 10%) and a 12-hr light/dark cycle. They were maintained on standard laboratory chow and tap water *ad libitum* and received human care throughout the experiment according to the guidelines of the National Institute for Environmental Studies.

Treatment

Eight-week-old male MT-null mice and wild-type mice were randomized into control and experimental groups (four mice per group). Mice were given i.p. a single injection of ethanol (99.5%, 2.0 g/kg). Twenty-four hr after ethanol treatment, the liver and blood were removed under ether anesthesia.

MT determination

The amount of MT in the liver was determined using radioimmunoassay as previously described with some modifications (Nishimura *et al.*, 1989; Tohyama and Shaikh, 1981). The MT level was determined at the point of ethanol treatment.

Hepatotoxicity evaluation

As an indicator of hepatotoxicity, the activities of glutamic oxaloacetic transaminase (GOT), glutamate pyruvate transaminase (GPT), and lactate dehydrogenase (LDH) in the serum were determined using an automatic dry-chemistry analyzer system (Spotchem SP-4410; Arkray, Kyoto, Japan).

Lipid peroxidation measure

The level of malondialdehyde (MDA), which is an indicator of lipid peroxidation, was measured in the liver using the Lipid Peroxidation Assay Kit (Wako Pure Chemical Industries, Osaka, Japan).

Histochemical staining

The liver was fixed in 10% buffered formalin (pH 7.4) and embedded in paraffin. Deparaffinized liver sections (thickness, 5 μm) were stained with hematoxylin and eosin for histopathological analysis.

Statistical analysis

Statistical analyses were undertaken using Student's *t*-test ($P < 0.05$).

RESULTS

The protein level of MT in the liver was determined at the point of ethanol injection. The protein level of MT in the wild-type mice was 2.37 ± 0.61 μg/g tissue; as expected, that of the MT-null mice was under the detection limit (< 0.2 μg/g tissue). The activities of GPT, GOT, and LDH were significantly increased in the serum of wild-type mice treated with ethanol compared with untreated mice (Fig. 1). Moreover, the activities of GPT, GOT, and LDH were escalated more significantly by ethanol in the serum of MT-null mice than in wild-type mice (Fig. 1). Ethanol treatment slightly triggered vacuolar degeneration in the liver of wild-type mice (Fig. 2B), while untreated MT-null mice showed the same liver morphology as untreated wild-type mice (Figs. 2A, C). Ethanol-treated MT-null mice had more prominent vacuolar degeneration in the liver than ethanol-treated wild-type mice (Fig. 2D).

Because ethanol induces oxidative stress in the liver, we next examined hepatic lipid peroxidation level. MDA level in the liver of ethanol-treated wild-type mice was the same as in untreated wild-type mice (Fig. 3). However, MDA level in the liver of MT-null mice was significantly increased by ethanol compared with untreated MT-null mice (Fig. 3).

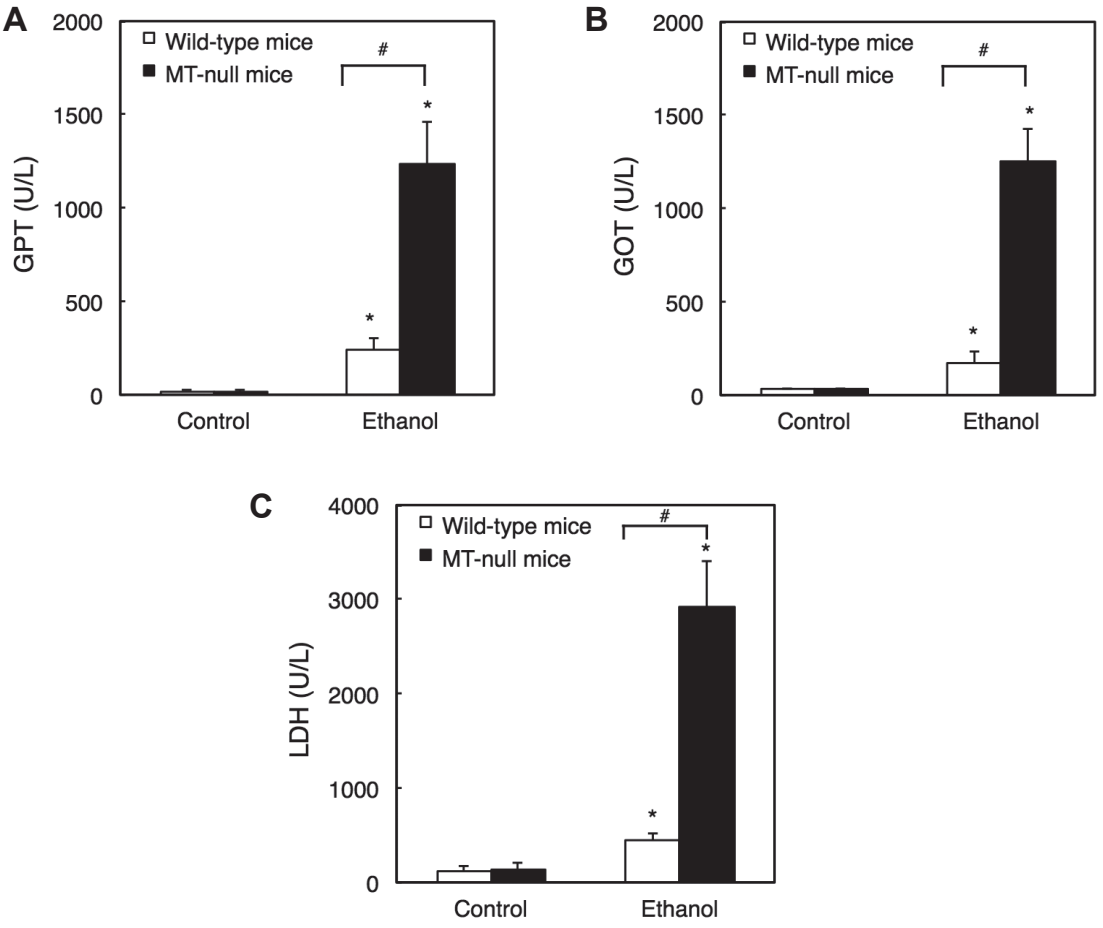

Fig. 1. Ethanol-induced hepatotoxicity in the wild-type mice and MT-null mice. (A) Serum GPT activity in the wild-type mice and MT-null mice 24 hr after ethanol injection. (B) Serum GOT activity in the wild-type mice and MT-null mice 24 hr after ethanol injection. (C) Serum LDH activity in the wild-type mice and MT-null mice 24 hr after ethanol injection. Values are the means ± S.D. (n = 4). *Significantly different from the control group, $P < 0.05$. # $P < 0.05$.

DISCUSSION

The present study demonstrated that ethanol caused more severe hepatotoxicity in the MT-null mice compared with wild-type mice. Previously, MT-null mice were found to be more sensitive to ethanol-induced gastroduodenal mucosal injury than wild-type mice (Takano *et al.*, 2000). Together, these results indicate that MT is deeply involved in the prevention of ethanol toxicity not only in the gastroduodenal mucosa but also in the liver.

MT can effectively scavenge oxygen free radicals such as hydroxyl radical and superoxide anion, and protect various tissues from oxidative stress (Sato and Bremner, 1993; Cai *et al.*, 1999; Satoh, 2004). MT-null mice are a simple experimental model to elucidate the role of MT as an antioxidant. MT-null mice have previously shown an increased sensitivity to oxidative liver injury caused by carbon tetrachloride (Liu *et al.*, 1998), acetaminophen (Rofe *et al.*, 1998; Zhang *et al*., 1999; Liu *et al.*, 1999), and paraquat (Sato *et al.*, 1996). In the present study, ethanol markedly caused lipid peroxidation in the liver of MT-null mice, suggesting that MT plays a protective role against oxidative liver injury caused by several substances including ethanol.

In conclusion, endogenous MT may participate in hepatic cytoprotection against ethanol, presumably as an antioxidant. The induction of MT expression may therefore prevent hepatotoxicity caused by oxidative stress.

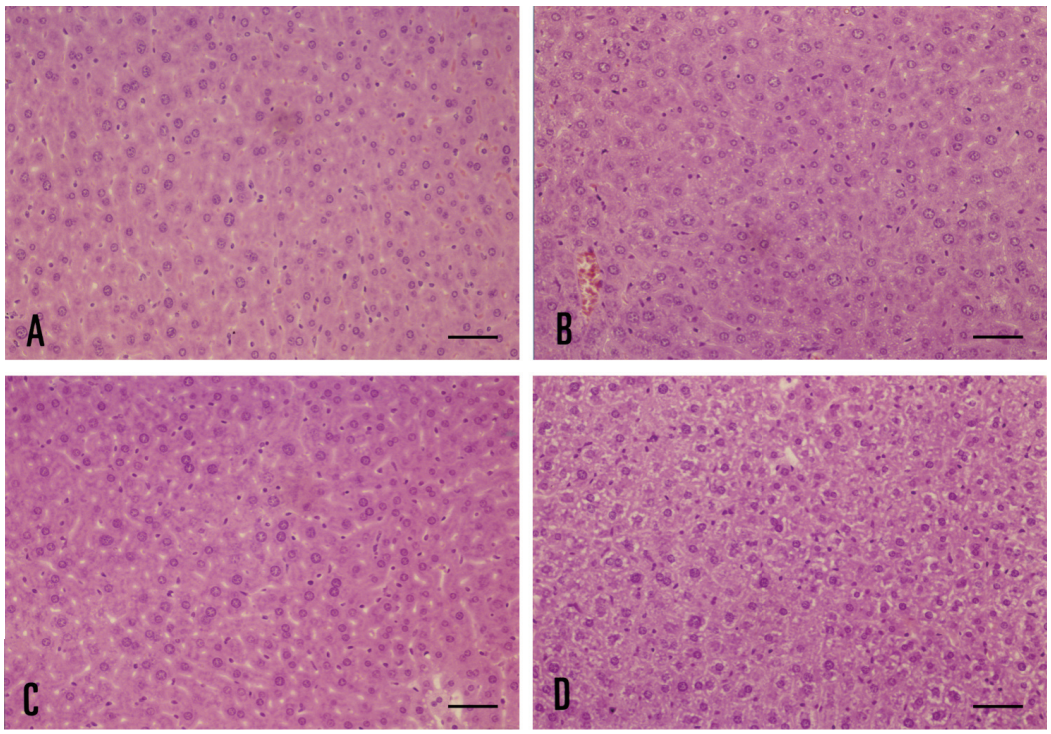

Fig. 2. Histopathological changes in the liver of wild-type mice and MT-null mice 24 hr after ethanol injection. (A) Untreated wild-type mice. (B) 99.5% ethanol-treated wild-type mice. (C) Untreated MT-null mice. (D) 99.5% ethanol-treated MT-null mice. Bar = 80 μm.

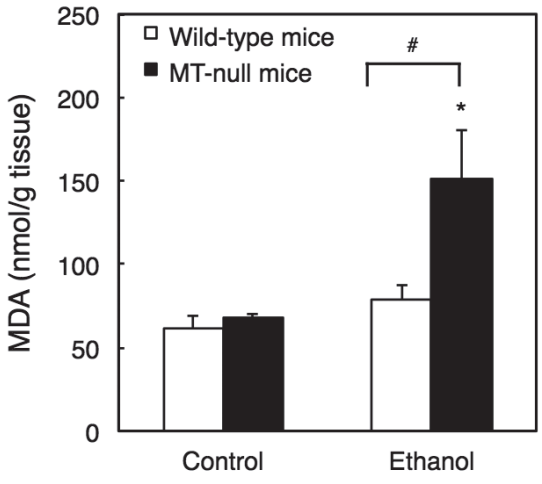

Fig. 3. Ethanol-induced lipid peroxidation in the liver of wild-type mice and MT-null mice. MDA level in the liver of wild-type mice and MT-null mice 24 hr after ethanol injection. Values are the means ± S.D. (n = 4). *Significantly different from the control group, $P < 0.05$. # $P < 0.05$.

Conflict of interest---- The authors declare that there is no conflict of interest.

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