

Induction of Hepatic Metallothionein by Mitochondrial Oxidative Stress in Mice

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The effects of three inhibitors of mitochondrial respiratory functions on the induction of metallothionein (MT) in the liver were investigated in mice. C57BL/6 mice were injected subcutaneously with sodium azide, atractyloside, or malonic acid and were analyzed for the hepatic levels of thiobarbituric acid-reactive substances (TBARS) and MT. Sodium azide and atractyloside increased the hepatic MT level transiently around 6 and 12 hr, respectively, after the injection. Malonic acid significantly increased the MT level even at 3 hr and the increase continued until at least 24 hr after the injection. In all three cases, the changes in hepatic MT level showed almost the same patterns as those of TBARS with respect to both kinetics and magnitude. The increases in both TBARS and MT in the liver due to malonic acid were inhibited by about 90% by preinjection of succinic acid. These results suggest that the inhibitors of mitochondrial respiratory functions directly affect the mitochondria of hepatic cells, causing the production of reactive oxygen species, and consequently resulting in the induction of MT in mice.

Key words — metallothionein, mitochondria, oxidative stress, atractyloside, malonic acid

INTRODUCTION

The mitochondrion is a major physiologic source of reactive oxygen species (ROS), which can be generated during mitochondrial respiration. Superoxide radicals, formed by minor side reactions of the mitochondrial electron transport chain or by an NADH-independent enzyme, can be converted to H₂O₂ and to a powerful oxidant, the hydroxyl radical.¹⁾ Thus mitochondria are continually exposed to ROS, which cause the peroxidation of membrane lipids, cleavage of mitochondrial DNA, and impairment of ATP generation, with resultant irreversible damage to the mitochondria. Mitochondrial dysfunction might contribute to aging and various diseases such as Parkinson's, Alzheimer's, and Huntington's disease.^{2–4)} On the other hand, low levels of ROS generated in mitochondria can act as signaling molecules under physiologic conditions. ROS produced in mitochondria can activate transcription factors such as nuclear factor-kappa B (NFκB) and activat-

ing protein 1 (AP-1),⁵⁾ and can function as signals in apoptosis that is induced by tumor necrosis factor-alpha (TNF-α)⁶⁾ or chemical hypoxia.⁷⁾

Metallothionein (MT) is a small, cysteine-rich protein induced by various stimuli such as cytokines, hormones, metals, and ROS-generating agents.^{8,9)} The biological functions of MT induced by ROS-generating agents are thought to include the protection of DNA, lipid, and proteins from oxidative injury.

We previously reported that the subcutaneous injection of antimycin A and 2,4-dinitrophenol (DNP), which are an inhibitor of mitochondrial respiratory enzyme complex III and an uncoupler of the electron transport from ADP, respectively, both of which are known to be ROS generators, induced MT in mice.¹⁰⁾ However, the origin of the oxidative stress in mitochondria remains unclear, because antimycin A and DNP have a variety of actions. Therefore in this study, to clarify whether systemically administered mitochondrial inhibitors generally induce oxidative stress resulting in MT induction in the liver, we investigated the effects of various chemicals that inhibit different mitochondrial respiratory functions on the hepatic levels of ROS and MT protein in mice.

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MATERIALS AND METHODS

Chemicals — Malonic acid disodium salt, atractyloside potassium salt, and succinic acid disodium salt were purchased from Sigma Chemical (St. Louis MO, U.S.A.). Sodium azide (NaN_3) was obtained from Wako Co. (Tokyo, Japan). Sodium pentobarbital was from Dainippon Pharmaceutical Co. (Osaka, Japan).

Animals and Treatments — Six-week-old male C57BL/6J mice were purchased from Clea Japan Inc. (Tokyo, Japan). They were housed in a plastic cage at 23°C on a 12-hr light and dark cycle. Mice were inoculated subcutaneously on the back with various chemicals. Three to 24 hr after injection, the mice were anesthetized with sodium pentobarbital and killed by cervical dislocation, and the livers were removed to measure the MT and thiobarbituric acid-reactive substances (TBARS) levels. In the case of combined treatment, succinic acid was injected intraperitoneally 1 hr before the malonic acid injection. All experiments were approved by the Animal Ethics Committee of Tokushima Bunri University.

Measurement of Hepatic MT — Liver tissue was homogenized in 50 mM Tris-HCl (pH 8.0) on ice, and the MT level in the cytosol was estimated using the Cd-hem method as described.¹¹⁾

Measurement of TBARS — As a relative measure of the total amount of ROS in liver tissue, TBARS was estimated using the method of Mihara and Uchiyama.¹²⁾

Statistical Analysis — Statistical significance was determined using Student's *t*-test.

RESULTS

First, the kinetic changes in the hepatic levels of TBARS and MT protein after subcutaneous injection of inhibitors of mitochondrial respiratory functions were examined in mice. The doses used below are around one-third of LD_{50} values after subcutaneous injections. In mice injected with NaN_3 , which is an inhibitor of complex IV in the mitochondrial respiratory chain enzyme, the hepatic TBARS level was significantly elevated at 6 hr (109.2 ± 11.1 nmol/g tissue) as compared with that in the untreated controls (time 0 hr, 65.2 ± 10.1 nmol/g tissue) and then it decreased to the control level at 12 hr (Fig. 1a). The hepatic MT level was also significantly augmented 3–6 hr after the injection and decreased at 12 hr (Fig. 1b). The level at 6 hr was about 3-fold

higher than the control level (45.4 ± 6.6 vs. 14.7 ± 2.0 $\mu\text{g/g}$ tissue). In the case of atractyloside, an inhibitor of the adenine nucleotide transporter in mitochondrial membrane, neither the TBARS nor the MT level was significantly increased even 6 hr after the injection, but at 12 hr, they were about 8- and 7-fold higher than the respective control levels (Fig. 1c and 1d). Thereafter, the levels decreased to the control levels 24 hr after the injection. The maximum levels of TBARS and MT induced by atractyloside (878 ± 23.1 nmol/g tissue and 97.3 ± 21.8 mg/g tissue, respectively) were higher than those induced by NaN_3 . In mice administered malonic acid, an inhibitor of the mitochondrial respiratory chain enzyme complex II, both the TBARS and MT levels continued to increase until at least 24 hr (Fig. 1e and 1f). The maximum levels of TBARS and MT (both 24 hr after injection) were 944 ± 81.3 nmol/g tissue and 97.4 ± 6.6 $\mu\text{g/g}$ tissue, respectively, which were almost the same as the levels induced by atractyloside.

We next estimated the effect of pretreatment with succinic acid, which is a substrate for complex II in the mitochondrial respiratory chain, on the induction of MT and TBARS by malonic acid. As shown in Fig. 2a, 24 hr after the malonic acid injection, the TBARS level was elevated to 11.4-fold the control level (656.3 vs. 57.3 nmol/g tissue), while the level of elevation decreased to 1.6-fold the control (92.9 nmol/g tissue) with the pre-injection of succinic acid (Fig. 2a). Almost the same result was obtained for the MT level: the 11.3-fold increase in the hepatic MT level by malonic acid was reduced to a 2.2-fold increase with succinic acid (Fig. 2b). Succinic acid alone had no effect on either the TBARS level or MT level.

DISCUSSION

We demonstrated a significant induction of TBARS and MT protein in the liver by systemic subcutaneous injection of mitochondria inhibitors. The three inhibitors used here all caused induction of both TBARS and MT in the liver, but with different patterns. NaN_3 augmented them transiently, with the most rapid kinetics and the lowest magnitude of induction (Fig. 1a and 1b). Malonic acid caused continuous elevation of MT until 24 hr after the injection (Fig. 1e and 1f). Although the precise reason for these differences is not clear, they might be dependent on the differences of their blood clearance

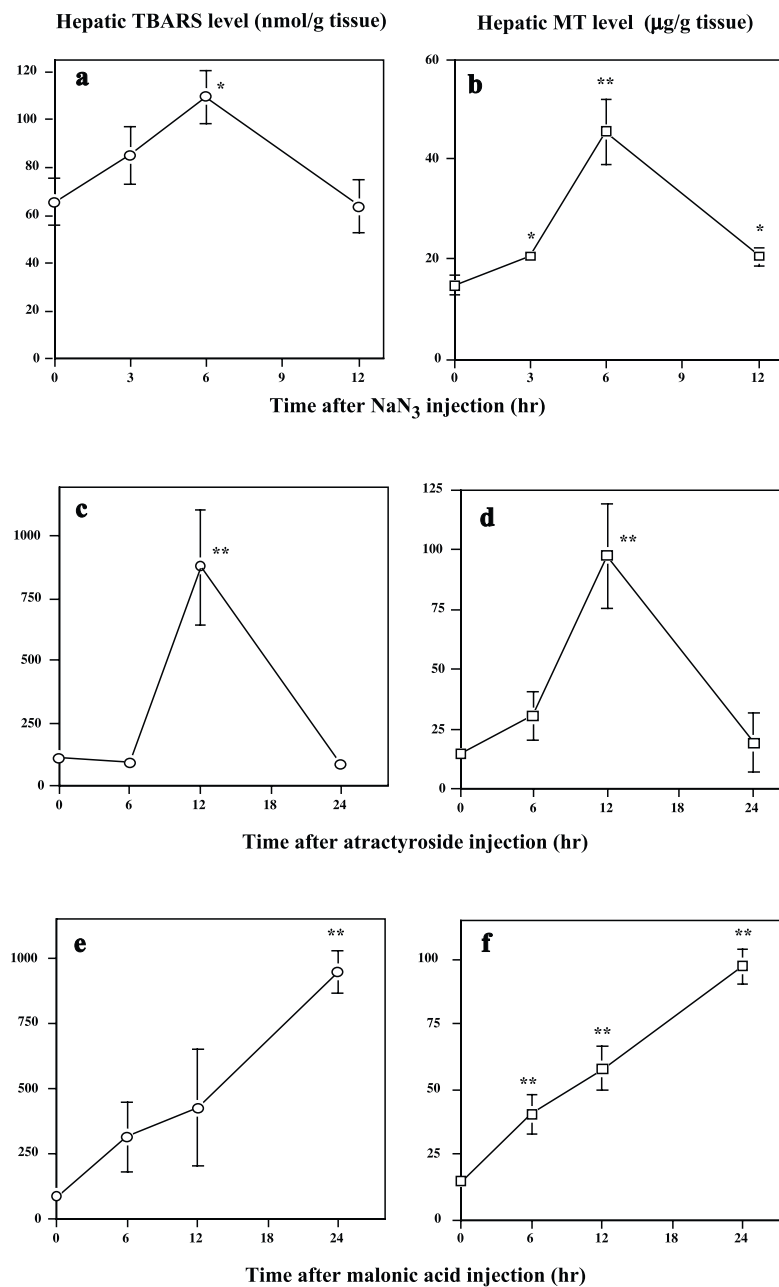


Fig. 1. Effects of Mitochondrial Inhibitors on Hepatic Levels of TBARS and MT

Mice were injected subcutaneously with sodium azide 2 mg/kg (a and b), atractyloside 150 mg/kg (c and d), or malonic acid 2.8 g/kg (e and f). At the indicated times after the treatment, mice were killed to measure the hepatic levels of TBARS (a, c, e) and MT (b, d, f), as described in MATERIALS AND METHODS. The value at time 0 hr is that of untreated mice. Values are means \pm SEM of 3–9 mice. * $p < 0.05$, ** $p < 0.01$ vs. 0 hr control.

rates, at least in part. It is noteworthy that the administration of each of the three mitochondrial inhibitors showed very close correlations between the hepatic levels of TBARS and MT with respect to both the kinetics and magnitude of the induction. If these chemicals indirectly induced MT *via* MT-inducible cytokines such as interleukin (IL)-1 and IL-6, or glucocorticoid produced by some organ other than the liver, the kinetics of MT induction would

be different from those of TBARS induction. Further, the addition of DNP to the medium directly augmented the MT-II mRNA level in a cultured fibroblast cell line (data not shown), indicating that MT induction is also likely to occur without the contribution of cytokines in the liver. This was further confirmed by the competitive inhibition by succinic acid of the MT induction by malonic acid (Fig. 2), because succinic acid is the specific substrate of suc-

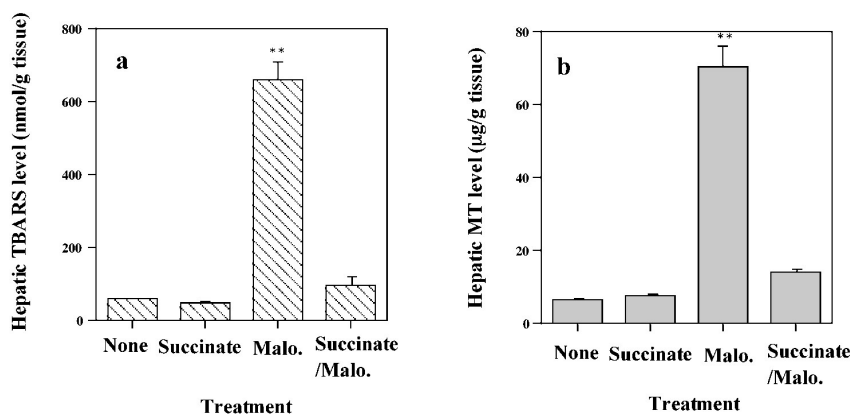


Fig. 2. Inhibitory Effects of Succinic Acid on Induction of Hepatic TBARS and MT by Malonic Acid

Mice were injected intraperitoneally with succinic acid (300 mg/kg) or saline (None), followed 1 hr later by subcutaneous injection with malonic acid (2.8 g/kg) or saline. Twenty-four hours after malonic acid administration, mice were killed to measure the hepatic levels of TBARS (a) and MT (b), as described in MATERIALS AND METHODS (mal, malonic acid). Values are means \pm SEM of 3–9 mice. ** $p < 0.01$ vs. None.

cinase dehydrogenase, which is inhibited by malonic acid in a competitive manner. As has been described,¹⁰ DNP, an uncoupler of electron transport in mitochondrial membrane, augmented hepatic TBARS and MT levels in the liver. This induction was also completely inhibited by preinjection of the antioxidant α -tocopherol in mice (data not shown). Taken together, these results imply that such induction of MT by the inhibitors of mitochondrial respiratory functions is highly dependent on the mitochondrial ROS production in the liver, irrespective of the target functions of the inhibitors.

What is the function of mitochondrial ROS production in the regulation of cellular homeostasis? Is it the same as the function of high levels of cytosolic ROS? MT is localized in the cytosol, nucleus, or both, and its antioxidative function is thought to be dependent on the subcellular localization.^{13,14} Zhang *et al.* demonstrated that the generation of superoxide in mitochondria leads to the generation of hydrogen peroxide by manganese-superoxide dismutase, and then the hydrogen peroxide diffuses into the cytosol, resulting in the activation of MT transcription *via* an increase in the pool of free zinc.¹⁵ MT has been proposed to have a role as a zinc transporter that mediates uptake into the mitochondria from the cytosol.¹⁶ Further, Ye *et al.* demonstrated the localization of MT in the mitochondria of the normal rat liver and the reactivation of zinc-inhibited mitochondrial respiration by unchelated MT (thionein).¹⁷ If the ROS signal specifically elevates the mitochondrial MT level, then MT will efficiently relieve mitochondrial oxidative stress. We have observed that the administration of DNP to MT-null

mice caused severe liver damage compared with the damage in wild-type mice (data not shown). Thus MT might substantially protect liver cells against the toxicity of ROS produced in the mitochondria. Since it has been reported that 1–2% of the oxygen consumed in mitochondria is converted to superoxide,¹⁸ which has been implicated in the aging process,¹⁹ MT may also modulate the aging process. However, the mechanisms of subcellular organelle-specific localization or trafficking of newly induced MT are not well known. Therefore the induction of MT by mitochondria-targeting oxidative stress demonstrated will be a good model for investigation of the mitochondria-specific antioxidative function of MT *in vivo*.

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