Involvement of Oxidative Stress in the Synthesis of Metallothionein Induced by Mitochondrial Inhibitors

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We previously reported that synthesis of metallothionein (MT) was induced by mitochondrial inhibitors such as 2,4-dinitrophenol (DNP) or antimycin A (Kondoh *et al.*, 2001), which are potent inhibitors of mitochondrial respiration. Although the inhibitors are known to be radical generators in mitochondria, the involvement of oxidative stress in the synthesis of MT induced by mitochondrial inhibitors and the biological functions of MT remain obscure. In this study, therefore, we examined the involvement of oxidative stress in MT synthesis induced by mitochondrial inhibitors and the biological functions of MT. In cultured mouse fibroblast cells, the addition of DNP increased both MT concentration and MT mRNA level. Administration of DNP to L-buthionine-SR-sulfoximine (BSO)-pretreated mice increased hepatic lipid peroxidation and induction of MT synthesis. In addition, vitamin E prevented induction of MT synthesis as well as lipid peroxidation in the liver of mice caused by administration of DNP. Administration of mitochondrial inhibitor to mice elevated the levels of lipid peroxidation in the liver and mitochondria, and MT in the liver, indicating the generation of mitochondrial oxidative stress. These data suggest that the induction of MT synthesis by mitochondrial inhibitors is correlated with generation of oxidative stress in mitochondria. Furthermore, the level of DNP-induced alanine aminotransferase (ALT) activity, reflecting hepatic damage, was greater in MT-null mice than in wild-type mice, and intracellular accumulation of reactive oxygen species (ROS) caused by the action of mitochondrial inhibitors was greater in MT-null fibroblast cells than in wild-type cells. The results suggest that MT plays a role as a radical scavenger of intracellular ROS produced in mitochondria. Taken together, the results suggest that mitochondrial oxidative stress induces the synthesis of MT, which may contribute to regulation of mitochondrial ROS production.

Key words metallothionein; 2,4-dinitrophenol; mitochondria; reactive oxygen species

Mitochondria are a major source of reactive oxygen species (ROS) and are produced in mammalian cells as a by-product of the mitochondrial energy production pathway, oxidative phosphorylation (OXPHOS). During OXPHOS, between 0.4 and 4% of the oxygen consumed is reduced to form superoxide anion.^{1–5} This superoxide anion is reduced to hydrogen peroxide (H₂O₂) by mitochondrial manganese superoxide dismutase (MnSOD). Most of the ROS in the cells is produced in mitochondria. Much of the ROS produced in mitochondria is toxic and the production of ROS results in oxidation of mitochondrial lipids, proteins, and DNA (mtDNA).⁶ Recent studies have indicated the involvement of mitochondrial ROS in aging⁷ and in various diseases such as diabetic hyperglycemia⁸ and Parkinson's disease.⁹ Therefore, increasing attention has been paid to the production of ROS in mitochondria and to the defense systems against mitochondrial ROS production.

Metallothionein (MT) is a small cysteine-rich protein induced by various stimuli such as cytokines, hormones, metals and ROS generators.¹⁰ ROS generators, which are potent MT inducers, are classified into two groups depending on the localization of ROS production: 1) paraquat and doxorubicin, which produce ROS in the cytosol, and 2) cisplatin, which produces ROS in the nucleus.^{11,12} The biological functions of MT induced by ROS are thought to be protection of DNA, lipids and proteins from oxidative injury.¹³ In fact, it has been shown that MT-overexpressing transgenic mice are highly resistant to doxorubicin-induced cardiotoxicity.^{14,15}

that preinduced MT decreased paraquat lethality *via* inhibition of pulmonary lipid peroxidation due to administration of paraquat,¹⁶ and that the sensitivity of cisplatin renal toxicity increased in MT-null transgenic mice with a null mutation of MT-I and MT-II genes.¹⁷ MT is localized mainly in the cytosol of cells and is transferred into the nucleus in partial hepatectomy, and its localization is known to be important for its biological functions such as scavenging ROS.^{18–23} Recently, Ye *et al.*²⁴ reported that MT functions as a modulator of respiration, a function that is dependent on the localization of MT in mitochondria. MT synthesis is known to occur in response to oxidative stress in the cytosol¹¹ and nucleus,¹² but it has not been determined whether MT synthesis is induced by oxidative stress in mitochondria, a major source of ROS in cells.

We previously reported the induction of MT synthesis by an uncoupler of OXPHOS, 2,4-dinitrophenol (DNP), and by an inhibitor of electron transport chain complex III, antimycin A.²⁵ However, the involvement of mitochondrial oxidative stress in the induction of MT synthesis by mitochondrial respiratory inhibitors remains unclear. In this study, therefore, we examined whether oxidative stress is involved in MT synthesis induced by DNP *in vivo* and *in vitro*. Furthermore, to investigate the role of MT induced by mitochondrial oxidative stress in mitochondria, we compared the intracellular ROS accumulation in MT-null fibroblasts and that in wild-type fibroblasts. We investigated the possibility that MT prevents liver damage caused by ROS generated in mito-

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chondria.

MATERIALS AND METHODS

Chemicals DNP and ($\pm$)- α -tocopherol acetate (vitamin E) were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). Antimycin A was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-Buthionine-SR-sulfoximine (BSO) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). DNP (0.4 g) was placed in 30 ml of ultrapure water adjusted to pH with 10 M sodium hydroxide (NaOH) and allowed to dissolve, and the pH was adjusted to 7.8 by dropwise addition of 1 M hydrochloric acid (HCl). The solution was then diluted to a volume of 200 ml with 0.1 M potassium phosphate buffer, pH 7.8. BSO was dissolved in 0.15 M NaOH, and the pH of the solution was adjusted to 7.0 with 1 M HCl. Vitamin E was dissolved in corn oil.

Animals and Treatment 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-h light/dark cycle and were given laboratory food (Clea Japan Inc., Tokyo) and tap water *ad libitum*. Three mice were randomly assigned to each treatment group, and additional experiments were carried out as needed. The mice received s.c. 35 mg/kg of DNP 8 h before the second injection of DNP at 45 mg/kg (DNP treatment group) or a sham injection of an equal volume of a vehicle (control group for DNP treatment). Since TBARS levels after single injection of DNP were different in the experiment, DNP was administered twice. Amount of DNP incorporated into mitochondria or extent of diffusion of the formed H_2O_2 from mitochondria to cytosol and nucleus may be different. BSO (6 mmol/kg) or saline was administered i.p. at a volume of 15 ml/kg to these mice 2 h before the DNP administration. The mice were anesthetized with sodium pentobarbital at 12 h after the second DNP administration. Vitamin E (0.56 mmol/kg) or corn oil was administered *p.o.* at a volume of 30 ml/kg to these mice 50, 25, 3 h prior to the DNP injection. Then the livers were removed for determination of MT and TBARS levels and stored at -80 °C before use.

In other studies, 129Sv MT-null- and wild mice (Jackson Laboratory, Bar Harbor, ME, U.S.A.) were injected with 30 mg/kg of DNP after the injection of DNP at 45 mg/kg. ALT levels were determined 12 h later using a commercially available kit (GPT-Test Wako, Wako Pure Chemicals Co., Ltd., Osaka, Japan).

Measurement of Hepatic MT Liver tissue was homogenized in Tris buffer (50 mM Tris-HCl (pH 8.0)), and measurement of MT was performed by the Cd-heme method.^{26,27} DNP, and BSO treatment did not alter the liver weight; therefore, the amounts of MT were expressed in terms of micrograms per gram of wet tissue.

Measurement of Lipid Peroxidation The degree of lipid peroxidation was estimated by monitoring the formation of thiobarbituric acid reactive substances (TBARS). The use of TBARS as a measurement of MDA has been shown to correlate closely to other measures of lipid peroxidation such as chemiluminescence, ethane production and fluorescent products.²⁸ The TBA method for MDA was performed as described previously.²⁹ Mitochondria liver fractions were prepared as follows. Liver tissue was homogenized in 9 vol

of 0.25 M sucrose solution. The homogenate was centrifuged at 800 *g* for 5 min at 4 °C, and the supernatant was further centrifuged at 6000 *g* for 20 min at 4 °C. The resultant precipitate was washed with an assay buffer (1.15% KCl and 3 mM Tris-HCl, pH 7.4) and resuspended in the assay buffer for the measurement of lipid peroxidation. A protein assay was performed using a commercially available kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) using bovine serum albumin as a standard.

Cell Culture MT-null and wild-type fibroblasts were kindly provided by Dr. Waalkes, M. P. (NIEHS, U.S.A.). Fibroblast cells were cultured in Dulbecco's modified Eagle's medium with 5% fetal bovine serum at 37 °C in an incubator under 5% CO_2 . The cells (1×10^6 cells) were treated with DNP (200, 400 μ M). These cultures were used for the Cd-heme assay and reverse transcriptase-polymerase chain reaction (RT-PCR) after 6–12 h of incubation with DNP.

Measurement of Intracellular MT Cultured fibroblast cells were collected using trypsin/EDTA, sonicated three times for 20 s in 300 μ l ice-cold Tris buffer (10 mM Tris-HCl (pH 7.4)). After centrifugation at 10000 *g* for 10 min, the supernatant was used for the estimation of MT using the procedure of Eaton and Toal.³⁰

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Total cellular RNA (20 μ g) was isolated using TRIzol reagent (Gibco-BRI, Gaithersburg, MD, U.S.A.). RT-PCR was performed using an Access RT-PCR system (Promega, Madison, WI, U.S.A.). The mRNA expression was studied by RT-PCR using specific oligonucleotides: 5'-ATG GAC CCC AAC TGC TCC TGC TCC ACC-3', and 5'-GGG TGG AAC TGT ATA GGA AGA CGC TGG-3' for MT-I; 5'-ATG GAC CCC AAC TGC TCC TGT GCC TCC-3' and 5'-GGT CTA TTT ACA CAG ATG TGG GGA CCC-3' for MT-II, and 5'-GTG AGG CCG GTG CTG AGT-3' and 5'-TTG CTG GGG TGG GTG GTC-3' for glyceraldehyde-phosphate dehydrogenase (GAPDH).

Measurement of ROS Intracellular ROS generation was assessed using the probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR, U.S.A.). DCFH-DA is membrane-permeable, and cellular esterases release the reduced form DCFH. Several ROS (such as hydrogen peroxide, hydroxyl radical and peroxynitrite) have been shown to oxidize DCFH to form the fluorescent species, 2',7'-dichlorofluorescein (DCF).^{38–40} The DNP-treated cells were incubated with DCFH-DA (5 μ M) for 30 min at 37 °C. Then the amount of fluorescence was detected by flow cytometry (Epics Elite, Coulter, Hialeah, FL, U.S.A.).

Statistical Analysis The data are expressed as means $\pm$ S.E.M. The significant difference was calculated using one-way ANOVA followed by Bonferroni multiple comparisons test, or where applicable, by Student's *t* test. The level of significance was set at $p < 0.05$.

RESULTS

Mitochondrial Inhibitor Induces Oxidative Stress Since oxidative stress induces lipid peroxidation, which is generally evaluated by TBARS formation,³⁴ we examined TBARS levels as a marker of lipid peroxidation. Administration of DNP elevated hepatic TBARS and mitochondrial TBARS levels (Figs. 1A, B), suggesting the induction of

Fig. 1. Levels of TBARS in the Liver Homogenates and Mitochondria of DNP-Treated Mice

Mice were injected with DNP (45 mg/kg, s.c.) after DNP (35 mg/kg, s.c.) administration, and hepatic TBARS levels were determined 12 h later in hepatic homogenates (A) and mitochondria (B). Values are means $\pm$ S.E.M. of 6–12 mice. *, ** Denote significantly different from control ($p < 0.05$, $p < 0.01$).

Fig. 2. Hepatic Contents of TBARS and MT in DNP-Treated Mice with or without BSO Pretreatment

The mice received s.c. 35 mg/kg DNP 8 h before the second injection of DNP at 45 mg/kg. BSO (6 mmol/kg, i.p.) was injected 2 h before the second DNP injection (45 mg/kg, s.c.). The livers were removed 12 h after the second DNP treatment for determination of TBARS and MT. Pretreatment with BSO did not affect the basal levels. (A) TBARS, (B) MT. Values are means $\pm$ S.E.M. of 4–12 mice ($n=12$ for DNP, $n=6$ for BSO/DNP treatment groups). Groups without superscript letter are significantly different at $p < 0.05$.

ROS production in mitochondria by DNP. In addition, antimycin A, a radical generator in mitochondria that has a different mechanism of action from that of DNP, also significantly increased hepatic TBARS and mitochondrial TBARS levels (data not shown). These data indicate that mitochondrial inhibitor such as DNP induces oxidative stress in the liver.

Enhancement of Lipid Peroxidation and MT Synthesis Induced by DNP To further study the involvement of oxidative stress in mitochondrial inhibitor-induced MT synthesis, we investigated the effects of BSO and vitamin E on hepatic MT levels. BSO is a specific inhibitor of γ -glutamyl-cysteine synthase and is therefore used as a specific inhibitor of the synthesis of GSH, a potent radical scavenger in physiological conditions. To confirm that production of oxidative

Fig. 3. Effects of Pretreatment with Vitamin E on the Concentrations of TBARS in the Liver Homogenates (A) and Mitochondria (B), and Liver MT (C) of DNP-Treated Mice

The mice received s.c. 35 mg/kg DNP 8 h before the second injection of DNP at 45 mg/kg. Vitamin E (0.56 mmol/kg, p.o.) was injected at 50 h, 25 h and 3 h prior to the second DNP injection. The livers were removed 12 h after the second DNP treatment for determination of TBARS and MT. Values are means $\pm$ S.E.M. of 3–12 mice ($n=4$ for saline, $n=3$ for vitamin E, $n=12$ for DNP, $n=6$ for vitamin E/DNP treatment groups). Groups without superscript letter are significantly different at $p < 0.05$.

stress was involved in MT synthesis by administration of mitochondrial inhibitors, we estimated the hepatic MT levels under GSH-depleted conditions due to administration of BSO. Treatment of mice with 6 mmol/kg of BSO reduced their hepatic GSH levels to 30% of the control level after 2 h (data not shown), and the BSO treatment did not affect the basal levels of TBARS and MT. Administration of DNP to BSO-pretreated mice greatly increased hepatic TBARS levels in comparison with levels in mice that received DNP without BSO-pretreatment (Fig. 2A). Compared with the control, an increase in MT concentration was observed in the livers of DNP-treated mice, and pretreatment with BSO caused a significant augmentation of MT levels induced by DNP (Fig. 2B). Administration of antimycin A to BSO-pretreated mice increased hepatic MT levels in comparison with the levels in mice that received antimycin A without BSO pretreatment (data not shown).

Figure 3 shows the effects of vitamin E on hepatic TBARS, mitochondrial TBARS and MT levels increased by DNP. The hepatic TBARS and mitochondrial TBARS levels increased by DNP were significantly decreased by pretreatment with vitamin E (Figs. 3A, B). The data suggest that vitamin E was distributed in mitochondria and inhibited DNP-induced ROS generation in mitochondria. Pretreatment with

Fig. 4. Expression of MT in DNP-Treated Fibroblast Cells

Fibroblast cells were incubated with DNP (400 μ M) for the durations indicated. (A) MT concentrations (% of control), and (B) MT-I, MT-II and GAPDH mRNA expression were determined. MT concentrations were examined by Cd-heme method and RT-PCR. These figures are representative of three independent experiments. Values are means $\pm$ S.E.M. ($n=6$, ** $p<0.01$ vs. control).

Fig. 5. Plasma ALT Levels in MT-Null and Wild-Type Mice after DNP Administration

129Sv mice (MT $^{+/+}$ and MT $^{-/-}$ mice) were injected with DNP (30 mg/kg) after DNP administration (45 mg/kg). ALT levels were determined 12 h later. Values are means $\pm$ S.E.M. of 4–12 mice. Groups without superscript letter are significantly different at $p<0.05$.

vitamin E prevented an increase in MT level by DNP administration to the control level (Fig. 3C).

Administration of DNP (Fig. 3) to mice increased the hepatic concentration of MT. It is possible that the injected chemical such as DNP, induces secretion of inflammatory cytokines such as interleukin 6 and tumor necrosis factor- α , which can induce MT synthesis.¹³⁾ Next, we examined induction of MT synthesis in cultured fibroblast cells treated with DNP without inflammation. Intracellular MT concentration in fibroblast cells was increased to 150% of that in untreated cells by treatment with DNP (400 μ M) for 12 h (Fig. 4A). The time course of MT-I and MT-II mRNA expression in fibroblast cells after DNP (400 μ M) exposure is shown in Fig. 4B. DNP induced expression of MT-I and MT-II mRNA in cells 6 h after the treatment, and the increase in MT-II mRNA expression was maintained at 12 h after the DNP treatment.

Effect of DNP on Liver Function in MT-Null Mice
The ALT level in plasma in MT-null mice was similar to that

Fig. 6. Production of H₂O₂ in DNP-Treated MT $^{+/+}$ or MT $^{-/-}$ Fibroblasts

MT $^{+/+}$ and MT $^{-/-}$ fibroblast cells were treated with DNP (200 μ M) for 60 min, and then DCFH-DA (5 μ M) was applied for 30 min. After DCFH-DA treatment, the amount of intracellular H₂O₂ was measured by flow cytometry. Results shown are from one experiment that is representative of three experiments.

in wild mice. Administration of DNP to mice caused an increase in ALT in the plasma (Fig. 5). The ALT level in plasma was greater in MT-null mice than in wild mice, suggesting that MT prevents liver damage caused by ROS.

MT Regulates Production of Oxidative Stress To investigate the possibility that MT induced under the condition of oxidative stress in mitochondria has a function as a radical scavenger, the effects of DNP on the production of ROS in MT-null cells and wild-type cells were studied using a cell-permeable probe, DCFH-DA. As shown in Fig. 6, the basal levels of intracellular ROS in wild-type cells and MT-null cells were similar. DNP-treated cells exhibited increases in ROS generation, and the intracellular ROS levels were significantly greater in DNP-treated MT-null cells than in DNP-treated wild-type cells.

DISCUSSION

It has been shown in numerous studies that antioxidant systems protect cells against ROS produced in different cell compartments. Mitochondria are a major source of endogenous ROS, and analysis of antioxidant systems against mitochondrial ROS is thought to be important. Therefore, we focused on antioxidant systems against mitochondrial ROS. Dryer *et al.*³⁵⁾ reported greatly enhanced activities of mitochondrial MnSOD in the livers of DNP-treated rats, indicating that MnSOD is important in the protection against mitochondrial ROS generated by DNP administration. We previously reported strong induction of MT and only slightly enhanced activities of MnSOD in the livers of DNP-treated mice.²⁵⁾ The difference in the activities of MnSOD might be species-specific. A mitochondrial inhibitor might induce the production of superoxide and convert it to hydrogen peroxide by MnSOD. The formed hydrogen peroxide may diffuse from mitochondria to the cytoplasm and nucleus, and induce MT and MnSOD synthesis. The data indicate that MT and MnSOD may cooperatively play a role as an antioxidant against mitochondrial ROS. It is interesting to note the cooperative contribution of MT and MnSOD to the antioxidant action for mitochondrial ROS. However, the involvement of mitochondrial oxidative stress in MT synthesis induced by a mitochondrial inhibitor such as DNP and the biological functions of induced MT remain obscure.

Administration of DNP to mice increased MT expression in protein (Fig. 2) and mRNA at 24 h after administration (data not shown). The induction was dose-dependent. However, whether MT synthesis is induced by DNP directly or indirectly is uncertain, because administration of some chemicals often increases secretion of inflammatory cytokines such as interleukin 6 and tumor necrosis factor- α which can induce MT synthesis.¹³⁾ In this study, therefore, we investigated whether MT synthesis is induced in cells. DNP induced expression of MT-I and MT-II mRNA and protein in fibroblast cells (Fig. 4), indicating that MT synthesis is induced by DNP directly. We also investigated the possible contribution of oxidative stress to MT synthesis induced by administration of DNP. Hepatic TBARS levels were increased in DNP-treated mice (Fig. 1A), and enhancement of ROS levels was observed in DNP-treated fibroblast cells (Fig. 6). These results might be due to the fact that inhibition of the normal electron transfer system by DNP incorporated into cells induced the generation of a superoxide anion from mitochondria. Indeed, DNP enhanced mitochondrial TBARS levels in the liver of mice (Fig. 1B).

Both TBARS level and MT synthesis induced by DNP administration were enhanced by BSO-mediated depletion of GSH, a ubiquitous and abundant radical scavenger (Fig. 2). In addition, pretreatment with vitamin E significantly suppressed the enhancement of both TBARS levels and MT synthesis by DNP (Fig. 3). These findings suggest that induction of MT synthesis by DNP administration is mediated through mitochondrial ROS. This possibility is also supported by the fact that increases in MT concentration and MT mRNA level after DNP exposure were observed. There is a possibility that superoxide in mitochondria generated by mitochondrial inhibitors or hydrogen peroxide reduced by Mn-SOD was released from mitochondria,³⁶⁾ which then activates MT transcription by increasing the pool of free Zn or by hydrogen peroxide diffused across the nuclear membrane.^{37,38)} An experimental model using DNP may be useful for studies on the role and effects of mitochondrial ROS. The production level of hydrogen peroxide in the presence of DNP in the culture medium was smaller in cells overexpressing the phospholipid hydrogen peroxide-GSH peroxidase, which is localized in mitochondria, than in the wild cells (data not shown), indicating that DNP induced oxidative stress in mitochondria. Further studies on the mechanism by which MT synthesis is induced by mitochondrial oxidative stress are needed.

Recent studies have indicated that the function of MT as an ROS scavenger is dependent on its intracellular localization,^{20,21)} and, moreover, the regulation of metabolism of metals such as zinc by MT was shown to be dependent on the intracellular localization of MT.²³⁾ Recently, Ye *et al.*²⁴⁾ demonstrated the localization of MT in mitochondria. It is interesting that MT is localized in the intermembrane space of sub-mitochondrial fractions, which is the main site of electron transfer *via* cytochrome *c* in mitochondrial respiration.²⁴⁾ Release of cytochrome *c* from mitochondria into the cytosol is a trigger signal in the mitochondria-dependent apoptosis pathway.³⁹⁾ The cytochrome *c* release induced by doxorubicin was inhibited by the scavenging of mitochondrial ROS generated by doxorubicin in MT-overexpressing cardiomyocytes.⁴⁰⁾ Taken together, these findings suggest that MT plays roles in the regulation of intracellular signaling *via* mito-

chondrial ROS production and in prevention of toxicity of ROS produced in mitochondria as shown in Fig. 6. This is the first report to indicate the involvement of MT in the regulation of mitochondrial ROS production.

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