

Classical Conditioning of Metallothionein Synthesis in Mice

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To clarify the role of metallothionein (MT), an anti-stress protein, induced by psychological stress, we carried out an experiment to determine whether hepatic MT synthesis induced by restraint stress can be classically conditioned by an olfactory odor cue of camphor. Mice were conditioned by exposure to the odor of camphor 1 hr before restraint stress and partly during restraint stress. Although exposure to the odor of camphor did not elevate hepatic MT levels in itself, the odor stimuli significantly elevated hepatic MT levels in mice conditioned for 3 days. The results indicate that MT synthesis is induced by an association of the central nervous system with psychological stress.

Key words — metallothionein, conditioning, restraint stress

INTRODUCTION

Stress is generally defined as a state of altered homeostasis caused by an external or internal stimulus, which has been shown to be restored to the control level by a variety of mechanisms for adaptation *via* the neuroendocrine system.¹⁾ In mammals, stress is known to induce an acute-phase response in the liver that is correlated with plasma glucocorticoid levels and with production of proinflammatory cytokines. The relationship between psychological stress and disease has become an important issue since the proposal of the stress theory by Selye.²⁾

Indeed, Adachi *et al.* reported that psychological stress induced oxidative DNA damage, which is a potent factor in development and progression of cancer,³⁾ and it has also been shown that oxidative DNA damage was conditioned to a novel taste.⁴⁾ However, anti-psychological stress factors have not been fully elucidated.

Metallothionein (MT), a low-molecular-weight and cysteine-rich protein, is induced by various stressful situations, including exposure to heavy metals, anticancer drugs, glucocorticoid, cytokines, fasting stress and restraint stress.^{5–7)} MT is known to play protective roles against various stresses mainly *via* scavenging of reactive oxygen species (ROS).^{7,8)} In fact, the ability of MT to capture hydroxyl radicals is more than 300-times greater than that of glutathione.⁹⁾ Various ROS generators, including cisplatin, antimycin A and paraquat, induced MT synthesis.^{9–11)} DNA damage was caused by hydroxyl radicals.¹²⁾ Treatment with 7,12-dimethylbenz[α]anthracene caused the development of skin tumors, and the frequency of occurrence of tumors in MT-null mice was higher than that in wild-type mice.¹³⁾ MT synthesis was induced by restraint stress, which is included in the definition of physiological and psychological stress, by wrapping of a metallic net or placing in a plastic tube.¹⁴⁾ MT synthesis was also induced by oxidative stress, but the activities of antioxidative enzymes such as glutathione-peroxidase and superoxide dismutase did not change.¹¹⁾ Taken together, the results of previous studies suggest that MT plays a protective role against psychological stress, which could cause DNA damage. However, direct evidence of the induction of MT by psychological stress has not yet been obtained.

In this study, we investigated whether hepatic MT synthesis can be conditioned by an olfactory odor cue of camphor, which can not induce MT synthesis in itself.

MATERIALS AND METHODS

Chemicals and Animals — All reagents are of research grade. Male ICR and female C57BL mice (5 week old) were purchased from Kansai SLC (Shizuoka, Japan) and maintained on standard rodent chow and water *ad libitum* in an environmentally controlled room ($23 \pm 1.5^\circ\text{C}$) with a 12-hr light, 12-hr dark cycle. The mice were allowed a period of at least 1 week to adapt and then they were placed in new cages daily for an additional 1 week in order

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Table 1. Conditioning Procedure

Groups	Days			
	0	1	2	4
CND	CS + Stress ^{a)}	CS + Stress ^{a)}	CS + Stress ^{a)}	none
CND/odor	CS + Stress ^{a)}	CS + Stress ^{a)}	CS + Stress ^{a)}	CS ^{b)}

^{a)} Mice were exposed to the odor of camphor followed by the restraint stress as described in materials and methods. ^{b)} Mice were exposed to the odor of camphor. CS, conditioned stimuli.

for them to adapt to handling by humans before being used in experiments. Camphor (Wako, Osaka, Japan) solution was prepared by dissolving 28.35 g of camphor in 150 ml of vaseline while stirring over low heat.

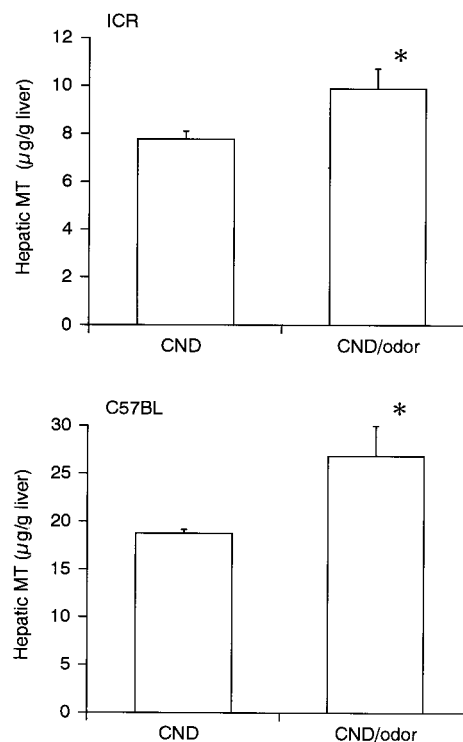
Conditioning Procedure — On the conditioning days, mice to be exposed to camphor were transferred into a new cage without water and chow, and transported to a room outside the vivarium starting at 10:00 a.m. 20 ml of camphor in vaseline was heated in a microwave oven for 1 min and then placed on the top of the mouse cage. A second cage was inverted over this to contain the camphor odor, and mice were exposed to camphor in this way for 1 hr. Mice were restrained by a metal net for 2 hr under the camphor odor, and mice were kept restrained for an additional 4 hr in the vivarium without the camphor odor. The mice were seeded under standard conditions. On the assay day, the control and conditioned groups were transferred into a new cage without water and chow. They were then transported to a room outside the vivarium, and only the conditioned group were exposed to camphor for 3 hr starting at 10:00 a.m. Then both groups were transported to the vivarium and were maintained under standard seeding conditions. After 4 hr, mice were killed under pentobarbital anesthesia, and the livers were removed and stored at -80°C until use.

MT Assay — Hepatic MT levels were determined by Cd-hem methods as described previously.¹⁵⁾

Statistical Analysis — Statistical analysis was performed by analysis of variance with Student's *t*-test.

RESULTS

MT synthesis is induced by various stresses, including fasting stress and restraint stress.^{5,14)} MT levels induced by stress are different between strains of animals.^{16,17)} To elucidate the MT synthesis in-

**Fig. 1.** Hepatic MT Synthesis Induced by Conditioned Stimuli

Mice were conditioned on days 0, 1 and 2 as described in MATERIALS AND METHODS. On day 4, the conditioned mice were stimulated with conditioned stimuli (CND/odor group) or not stimulated (CND group), and then the hepatic MT levels were determined. Data are means \pm SEM ($n = 4$). *Significant difference from the control group ($p < 0.05$).

duced by psychological stress, we examined whether MT synthesis in ICR and C57BL mice can be conditioned by exposure of the mice to the odor of camphor according to the procedure described in Table 1. Only exposure to the odor of camphor did not induce MT synthesis in this experimental conditions (data not shown). However, as shown in Fig. 1, MT synthesis induced by the conditioned stimuli was significantly elevated in the conditioned group compared to that in the non-conditioned group (7.81 ± 0.35 to $9.94 \pm 0.78 \mu\text{g MT/g liver}$ in ICR mice; 18.74 ± 0.34 to $26.76 \pm 3.23 \mu\text{g MT/g liver}$ in C57BL mice).

DISCUSSION

The classical conditioning phenomenon has been considered to be a part of stress responses.¹⁸⁾ Although MT was estimated to be an important protective factor in the adaptation to a variety of stresses, including chemical and physiological stresses,⁷⁾ conditioning of MT synthesis has never been reported. In this study, we investigated the possibility of conditioning of MT synthesis using camphor odor, which is a classical conditioning stimulant.¹⁹⁾ Although the odor of camphor in itself did not elevate hepatic MT levels in the experimental conditions (data not shown), MT synthesis was conditioned by the odor stimuli. These findings indicate that mice that are exposed to a camphor odor cue under a condition of restraint can associate the stimuli with the response of MT synthesis induced by restraint stress. It has been proposed that ROS play a significant role in the pathogenesis of cancer through several kinds of nuclear DNA injury.²⁰⁾ MT is a potent scavenger of ROS (Sato and Bremner, 1993), and MT deficiency promoted carcinogenesis in mice treated with 7,12-dimethylbenz[α]anthracene, by which oxidative damage of DNA was caused.^{13,21)} Irie *et al.* indicated the possibility of involvement of the central nervous system and psychological stress in the pathogenesis of cancer because of classical conditioning of oxidative DNA damage.⁴⁾ These results suggest that MT might play a role in prevention of carcinogenesis induced by psychological stress by its ability to protect against DNA damage induced by generation of ROS.

Further future study is needed to determine the involvement of neuroendocrine system in MT synthesis induced by stress. This is the first report to indicate the interaction between the central nervous system and MT synthesis.

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