INTRODUCTION

Formaldehyde (FA) is a colorless, highly flammable gas, and a very reactive compound. FA can react with different macromolecules, such as proteins, nucleic acids and low molecular weight substances such as amino acids (Cheng et al., 2003; Metz et al., 2004). It is widely used in the building, textile, paper product, resin, wood composite, insulating material, paint, plastic, fabric, adhesive and cosmetic industries, and is also found in photochemical smog and even in various fruits (Conaway et al., 1996; Trézl et al., 1998). In animal physiology, FA can be formed by the metabolism of L-methionine, histamine or methylamine, and can react with folic acid to contribute to biological methylation (Trézl et al., 1998).

In 1995, the International Agency for Research on Cancer (IARC) concluded that FA is a probable human carcinogen (Cogliano et al., 2005; IARC, 1995). Therefore, the toxicity of FA has attracted extensive studies in recent years. It is suspected that exposure to low levels of FA induces or aggravates airway inflammation (Fujimaki et al., 2004). Moreover, inhalation of FA has been shown to inhibit learning and memory performance in mice (Lu et al., 2008) and to cause increased oxidative stress (Zhou et al., 2006) in rat testis and the developing rat liver (Kum et al., 2007a). Conversely, other studies have suggested that FA inhalation does not influence systemic genotoxicity (Speit et al., 2009), renal oxidative stress (Kum et al., 2007b), asthmatic response (Ezratty et al., 2007) or cerebellum development (Songur et al., 2008).

In the present study, we examined the effect of FA inhalation on oxidative stress and inflammation in mice. Male adult ICR mice were exposed FA in gaseous form (0.1 ppm), and blood, urine, brain, lung and liver were obtained for 24 hr. Levels of 8-hydroxy-2'-deoxyguanosine (8OHdG) and NO3⁻ were then determined by HPLC. A second group of mice were injected with 5 mg/kg lipopolysaccharide (LPS) after 24 hr of FA (3 ppm) inhalation and blood and organs were assayed for NO3⁻ level and SOD activity. After exposure to a low dose of FA (0.1 ppm), the 8OHdG/dG ratio significantly increased in plasma. However, the ratio in urine and organs significantly decreased during 24 hr of FA exposure. The NO3⁻ levels mirrored the 8OHdG/dG ratio. After 24 hr exposure to a high dose of FA (3 ppm), NO3⁻ levels in plasma and liver were significantly lower than in control mice exposed to air only. The SOD activity of blood and urine were conversely increased in FA exposed animals. In the present study, we suggest that inhalation of FA at low doses influences the oxidative stress response in a tissue-specific manner. The FA may partially alleviate in some tissues like preconditioning in oxidative stress.

Key words: 8-hydroxy-2'-deoxyguanosine (8OHdG), Formaldehyde, Lipopolysaccharide (LPS), Nitric oxide (NO), Toxicity
contrast, inhalation of FA at low doses was shown to have a protective effect against allergic reactions and cell death induced by ovalbumin immunization (Fujimaki et al., 2004; Lino et al., 2009; Tsukahara et al., 2006).

Reactive oxygen species (ROS), including singlet oxygen, hydrogen peroxide, superoxide anion and hydroxyl radical can be produced by endogenous sources, as well as through cellular aerobic metabolism and inflammation, or through exposure to a variety of chemical and physical agents. ROS are important mediators of cellular injury, and play a putative causing in oxidative stress. ROS can contribute to a variety of diseases and may be present in situations of toxicity (Halliwell, 1997; Kadiiska and Mason, 2000). ROS-initiated oxidative stress can be regulated by cell defense mechanisms, including superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH). One major pathway of ROS-induced DNA damage involves a reaction at the C-8 position of 2'-deoxyguanosine to form 8-hydroxy-2'-deoxyguanosine (8OHdG). 8OHdG has been shown to cause mispairing during DNA replication, giving rise to G to T conversions, and consequently a G:C → T:A mutation occurs (Halliwell, 1997; Pilger and Rüdiger, 2006). Under normal conditions or less stressful conditions, oxidized DNA can be repaired in vivo by endonucleases, or by a basesspecific glycosylase. In such cases, 8OHdG is transported through the blood and excreted into the urine without further modification. However, in pathological conditions such as cancer, inflammation, diabetes and neurodegeneration, 8OHdG levels increase in tissues, blood or urine in a cellular environment of oxidative stress. As such, 8OHdG is considered to be a marker of DNA oxidative stress (Loft et al., 1993; Pilger and Rüdiger, 2006). Nitric oxide (NO) and the oxidative metabolite, peroxynitrite (ONOO-) are referred to as reactive nitrogen species (RNS). NO is synthesized from L-Arginine by three kinds of NO synthase (NOS) and can cause modification of neuronal function, dilution of blood vessels, disruption of host defenses, and inflammatory responses (Brüne et al., 1998; Gordge, 1998).

In the present study, we exposed ICR mice to low amounts of FA gas and investigated the resultant levels of oxidative stress in plasma, urine and tissues. Moreover, we tested the effects of FA inhalation on sensitivity to lipopolysaccharide (LPS) challenge.

**MATERIALS AND METHODS**

**FA exposure chamber**

The experimental chamber [38 (W) x 52 (L) x 32 (H) cm] was made from glass and consisted of two compartments: the experimental chamber and the FA vaporizing chamber, respectively (Fig. 1A). The two compartments were connected via a hole (5 cm diameter) with ventilator fan operating at 0.3 m$^3$/min to diffuse the FA into the experimental chamber. Another hole (5 cm diameter) was located in the experimental chamber for exhaust. A beaker containing an appropriate concentration of formalin (Wako, Osaka, Japan) was set over a stirrer in the FA vaporizing compartment to produce gaseous FA. The concentration of FA in the experimental chamber was measured before and during each experimental phase by a smell sensor (Smell sensor portable model XG100V, New Cosmos Electric, Osaka, Japan) set adjacent to the exhaust. In accordance with previous reports (Naya and Nakanishi, 2005), we used FA concentrations of 0.1 ppm (low dose) and 3 ppm (high dose).

**Animals**

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Showa University. Male ICR mice (8 weeks of age) weighing 30-40 g were obtained from Japan Crea (Tokyo, Japan). All mice were housed in a temperature- and humidity-controlled environment for at least 2 weeks prior to the experiment.

**Low dose FA exposure**

A total of 43 male ICR mice were divided into two experimental groups; a non-FA inhalation control (n = 7) and four FA inhalation groups (n = 9 in each group). After 0 (non-FA inhalation control), 2, 4, 8, or 24 hr FA exposure, body weight was measured, and then mice were sacrificed by cervical dislocation. Urine (50 µl) and heparinized blood from the right ventricle were collected and the animals were perfused with PBS through the left ventricle to remove the blood. The urine samples (50 µl) were immediately added to 350 µl of preserving solution (0.5 mM EDTA, 5% methanol, 20% glycerol) to avoid further oxidation. Tissue samples (lung, liver, and brain) of approximately 150 mg were collected and stored in 250 µl of tissue preserving solution (0.5 mM EDTA, 5% methanol, 40% glycerol). Plasma (200 µl) was mixed with an equal volume of preserving solution. All samples were stored at 4°C. An overview of the experiment is shown in Fig. 1B.

**High dose FA exposure**

We analyzed the effect of FA exposure on the inflammatory response induced by LPS (E.coli O111:B4, Seikagaku Corp., Tokyo, Japan). The mice were divided into two groups: treated with either only LPS injected intraperito-
neally at 5 mg/kg (n = 12) was defined “LPS” group or was it LPS injection followed by FA (3 ppm) exposure at 5 mg/kg (n = 12) was defined “FA + LPS” group. The mice in the latter group were exposed to high dose FA for 24 hr, injected intraperitoneally with LPS and further exposed to FA for 4 hr. At the end of the experiment, the mice were sacrificed as previously described. An overview of the experiment is shown in Fig. 1C.

Another set of animals (n = 12) were examined SOD activity. After 24 hr high dose FA or room air exposure,
plasma, urine and liver were collected as described above and were measured SOD activity.

Measurement of 8OHdG and dG
Tissue samples were prepared with a homogenizer (FastPrep FP120, BIO 101, La Jolla, CA, USA) and were centrifuged at 15,000 rpm for 10 min at 4°C. The supernatants were then treated on ice by a micro-dialysis system with a molecular weight cut-off of 50 kDa (Eicom, Kyoto, Japan). Efficiency of recovery of dG and 8OHdG were measured using the standard solutions to enable accurate correction for differences in individual membranes. dG and 8OHdG in the samples were then separated out with a column (CA5-ODS, Eicom) maintained at 2°C and quantified using an electrochemical detector (ECD) system (HITEC, Eicom). The electrode potential was set to 550 mV and flow rate at 230 µl/min with a mobile phase consisting of 0.1 M sodium phosphate buffer (pH 7.4), 2.5% methanol, and 90 mg/l sodium 1-octanesulfate. 8OHdG was detected at 3.32 nA and dG was detected by UV at 254 nm.

Measurement of NO metabolites
NO is highly unstable in solution and cannot be readily assayed. However, NO is metabolized to stable nitrite (NO₂⁻) and nitrate (NO₃⁻) ions and were thus measured as NO₃⁻ after conversion of NO₂⁻ to NO₃⁻ using nitrate reductase with HPLC (Yamada and Nabeshima, 1997). In brief, NO₂⁻ and NO₃⁻ concentrations in plasma, urine or supernatant of tissue extractions were measured using the HPLC NO detector system (ENO-10, Eicom). The injected sample was mixed with an equivalent volume of methanol and was subsequently separated using a reverse-phase separation column packed with polystyrene polymer (NO-PAK, 4.6 × 50 mm, Eicom). NO₂⁻ was reduced to NO₃⁻ in a reduction column packed with copper-plated cadmium filings (NO-RED, Eicom). The NO₂⁻ concentration was measured by addition of Griess reagent (Archer, 1993; Guevera et al., 1998) to form a purple azo dye, and the resulting peak at 540 nm was quantified. In the present study, we measured the peak of NO₃⁻ because the majority of NO metabolite was NO₃⁻ and the peak of NO₂⁻ was not suitable for quantitative analysis.

Measurement of plasma IL-6 levels
Plasma IL-6 levels, which are an early inflammatory marker, were measured in a bioassay using an IL-6 dependent murine hybridoma cell line, B9. B9 cells were maintained in RPMI 1640 with 25 mM HEPES containing 10% heat inactivated (56°C, 30 min.) fetal bovine serum and a 1% antibiotic-antimycotic solution in the presence of hybridoma growth factor (20 units/ml) and recombinant murine IL-6 (Boehringer Mannheim Biochemica, Mannheim, Germany). In 96-well plates, plasma samples (2 µl) were added to 198 µl of culture medium without IL-6. The 200 µl samples were then serially diluted 8-fold. Each plate included a standard row containing a dilution series of the recombinant murine IL-6, in which the concentration of the first well was 5 pg/ml. B9 cells (100 µl) (2 × 10⁴/ml washed in culture medium were then added to each well and incubated in 5% CO₂ at 37°C for 72 hr. Bioactivity of IL-6 in the samples was measured fluorometrically following addition of Alamar Blue (20 µl), a fluorometric growth indicator (Alamar Biosciences Inc., Sacramento, CA, USA), for the final 8 hr of the incubation process. Fluorescence was measured using a Fluoroskan Ascent FL (LABOSYSTEMOY, Helsinki, Finland). The specificity of the B9 cell bioassay for measuring the bioactivity of IL-6 is well-established for in vitro and in vivo experiments (Yagi et al., 2005).

Measurement of SOD activity levels
SOD activity in plasma, urine and supernatant of liver homogenates were measured by nitrite methods (Oyanagui, 1984) according to instruction manual (SRL, Tokyo, Japan). In brief, hydroxylamine, xanthine oxidase, hypoxanthine, EDTA-2Na, and the sample were incubated with or without KCN at pH 8.2, 37°C for 30 min. Then, Diazo dye-forming reagent formed by sulfanilic acid, N-1-naphthylethylene diamine and acetic acid was added and the absorption was measured at 550 nm.

Statistics
Data were analyzed using the Kruskal Wallis non-parametric analysis of variance test followed by Dunnnett’s multiple comparison test using JMP® software (Cary, NC, USA). A p < 0.05 was considered statistically significant.

RESULTS
To determine effects of FA inhalation on ROS, the 8OHdG/dG ratios were measured in urine, plasma, and tissues (lung, liver, and brain) for prior exposure to FA of up to 24 hr (Figs. 2 and 3). The urine dG level was 1.6 ± 0.2 µg/ml at 0 hr and increased significantly to 2.5 ± 0.2 µg/ml by 24 hr (p < 0.05). In contrast, plasma dG decreased significantly with time. There was no significant change over time in 8OHdG concentration in urine or plasma. Accordingly, due to the changes in dG concentration, the 8OHdG/dG ratio significantly decreased in urine over 24 hr (from 3.0 ± 0.4; p < 0.05 to 1.2 ± 0.2), and increased in plasma (from 9.0 ± 1.4; p < 0.05 to 25.8 ±
Tissue levels of dG, 8OHdG and the corresponding ratios are shown in Fig. 3. Levels of dG in lung and liver increased after low dose FA exposure, whilst levels of 8OHdG did not change significantly. Thus, the 8OHdG/dG ratios in lung and liver significantly decreased after 24 hr (lung, from 12.1 ± 1.4/10^3 to 5.0 ± 1.6/10^3 dG, p < 0.05; liver, from 4.5 ± 1.1/10^3 to 1.1 ± 0.2/10^3 dG, p < 0.05). However, levels of dG and 8OHdG in the brain did not show any significant changes over time. These results suggest that FA exposure influences oxidative stress even at low doses.

NO₃⁻ levels were also affected by FA exposure (Fig. 4). NO₃⁻ levels in the urine, liver and brain decreased by approximately 50% after 24 hr of FA exposure (urine, from 1.4 ± 0.2 to 0.8 ± 0.1 mM, p < 0.05; liver, from 19.2 ± 2.1 to 8.0 ± 1.2 μM, p < 0.05; brain, from 17.9 ± 3.1 to 5.8 ± 0.7 μM, p < 0.05). However, NO₃⁻ levels in plasma increased approximately 2-fold after 24 hr. No significant changes in NO₃⁻ levels were observed in the lung during this experiment. These results confirmed the 8OHdG/dG data in showing that FA exposure impacts oxidative stress levels, with increasing stress in plasma and decreasing stress in urine and tissues.

Levels of IL-6 were determined in plasma to analyze...
Fig. 3. Effect of low dose FA exposure on levels of dG (A, D, G), 8OHdG (B, E, H) and ratio of 8OHdG/dG (C, F, I) in lung (A, B, C), liver (D, E, F) and brain (G, H, I) of mice. Data was shown as mean ± S.E. (n = 7-9). * : p < 0.05 compared with control.
the effect of FA exposure on the inflammatory response (Fig. 5). In plasma of mice exposed to low dose of FA, IL-6 levels did not show any significant changes during these experimental periods. We then tried to clarify whether FA pre-exposure would affect NO3⁻ levels following LPS injection. After 24 hr high dose FA exposure (3 ppm), mice were injected with LPS (5 mg/kg) and blood, urine and tissues were examined after another 4 hr. NO3⁻ levels in the plasma and liver were significantly lower than in that of the room air-exposed control mice. On the other hand, there was no difference in NO3⁻ levels in lung and brain (Fig. 6).

We hypothesized that FA exposure might have induce an anti-stress as a mechanism for preconditioning. Then we measured an anti-oxidative stress marker SOD activity in plasma, urine and liver homogenate 24 hr after FA (3 ppm) exposure. SOD activity levels in the plasma and urine increased significantly 24 hr after FA exposure (plasma, from 10.6 ± 0.9 to 14.5 ± 0.7 U/ml, \( p < 0.05 \); urine, from 19.3 ± 1.2 to 31.7 ± 2.7 U/ml, \( p < 0.05 \)), but not in liver homogenate (Fig. 7).

**DISCUSSION**

In this study, we have assessed the effect of FA inhalation on oxidative stress in mice using an HPLC system.
Fig. 5. Concentration of IL-6 in plasma following low dose FA exposure. IL-6 concentration (pg/ml) was detected using a B9 bioassay. Data was shown as mean ± S.E. (n = 8).

Fig. 6. Levels of NO$_3^-$ 4 hr after LPS injection with or without FA pre-exposure for 24 hr. NO$_3^-$ levels in the plasma and liver significantly decreased in mice pretreated with FA. Data was shown as mean ± S.E. (n = 7). *: p < 0.05 compared with control (room air exposure mice).

Fig. 7. SOD activity in plasma and urine and liver following 24 hr FA exposure. Data was shown as mean ± S.E. (n = 10-12).
that is approximately 100 times more sensitive than ELISA or colorimetric methods (Kasai, 2003; Shimoi et al., 2002). We measured the levels of oxidative stress markers 8OHdG and NO$_3^-$ in blood, urine and other tissues for FA exposures up to 24 hr. Finally, we examined synergetic effects and changes in inflammatory response caused by LPS injections in mice exposed to FA. We also measured SOD activity to detect anti-stress system with or without FA inhalation.

For the initial experiments, we used a gaseous concentration of 0.1 ppm FA, a very low concentration that is near the criterion value of the Ministry of Health, Labour and Welfare in Japan (http://www.mhlw.go.jp/bunya/roudoukijun/anzenesai17/index.html). Our results showed that over the time period of 24 hr, the NO$_3^-$ levels and 8OHdG/dG ratios in tissues and urine significantly decreased compared to control. Conversely, the levels and 8OHdG/dG ratios in tissues and urine showed that over the time period of 24 hr, the NO$_3^-$ concentration of 0.1 ppm FA, a very low concentration that FA inhalation.

We then measured IL-6, a marker for inflammatory response in plasma, to test the hypothesis that inflammation in mice would increase following exposure to FA. However, IL-6 levels in plasma did not change significantly over the course of the experiment. Morphological changes also did not show any critical differences between the experimental groups (data not shown). These results suggest that while mice were affected by FA exposure, the inflammatory response or tissue damages was not induced. The phenomenon where some people who are sensitive to FA subsequently develop other allergies or immune diseases is known as sick-building syndrome. We then tested whether FA inhalation changes the sensitivity of immune and inflammatory responses in mice NO$_3^-$ levels in plasma and were lower in mice exposed to FA than in mice not exposed to FA. This result suggests that FA inhalation either decreased NO production or increased NO scavenging, which may have induced an anti-stress system as a mechanism for preconditioning. Preconditioning is a phenomenon in which non-lethal stresses such as short periods of ischemia or use of some anesthetics or chemicals induce a stress-responsive cascade in the brain, heart and other organs. Other studies have shown that increased stress responses result in the protection of host organs and tissues from lethal stress-

Oxidative stress by formaldehyde exposure

In conclusion, our results show that exposure to a low dose of gaseous FA influences oxidative stress, and that the effects vary in different tissues. It may be that inhalation of FA has resulted in triggering of a low level protective cascade.

REFERENCES


Vol. 35 No. 5