Site-Specific Induction of Metallothionein in N-Nitrosodimethylamine-Treated Rat Liver

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The effects of dietary protein and sulfur-containing amino acids on oxidative responses against N-nitrosodimethylamine (NDMA) treatment were investigated in rat liver, focusing on the mechanism of metallothionein (MT) induction. Rats were fed a 10% soybean protein isolate (SPI) diet or SPI supplemented with 0.3% L-methionine (SPI-Met) for 3 weeks after weaning. Rats fed on SPI showed lower glutathione (GSH) levels and higher MT levels in the liver than those fed on SPI-Met. After treatment with NDMA (20 mg/kg, i.p.), MT levels were elevated significantly in both groups, whereas GSH levels were only elevated in the SPI group. Pathologically, necrosis and hemorrhage were seen in the livers of NDMA-treated rats of both dietary groups, suggesting oxidative damage caused by NDMA treatment. Immunological histology using anti-MT antibody showed that most of the MT was distributed in the cytosol region of all the rat groups. The ratios of the MT-positive cells were correlated to MT levels that were chemically analyzed. It was notable that the localization of MT staining in the hepatic lobules was altered after NDMA injection. The MT staining was principally observed around the central vein before injection, whereas it was more markedly observed at the peripheral region after NDMA treatment. These results indicate that the synthesis of hepatic MT basically occurs around the central vein with oxygen stress caused by insufficient oxygen supplement and/or stimulated oxygen consumption. NDMA could be oxidatively metabolized at the peripheral region with sufficient supplemental oxygen from the artery, resulting in oxidative stress and stimulation of MT synthesis there. Thus, distribution of tissue MT might vary depending on the site of oxidative stress.

Key words — metallothionein, N-nitrosodimethylamine, oxidative response, liver, rats

INTRODUCTION

N-Nitrosodimethylamine (NDMA), a potent carcinogenic and cytotoxic agent,1–3 was oxidized during metabolic activation with cytochrome P450,4–6 resulting in stimulated production of active oxygen species,7–10) Glutathione (GSH), the most abundant thiol compound in the cytosol fraction, protects organisms against NDMA toxicity.11) Metallothionein (MT), another cytosolic thiol-containing compound, is considered to function as a free-radical scavenger,12–15) as well as playing essential roles in the metabolism and detoxification of heavy metals.16) Cadmium toxicity in GSH depletion was overcome by induction of MT,17) suggesting a compensatory mechanism of MT for GSH.

GSH is known as a cysteine reservoir in the liver, and hepatic GSH levels declined when rats received diets deficient in sulfur-containing amino acids.18) In contrast to GSH, MT levels were increased in rats maintained on a sulfhydryl-deficient diet,19) or on a diet restricted by sulfur-containing amino acids.20) After administration of NDMA, MT levels were elevated in rat liver in addition to GSH, and one role of MT was thought to be the protection of organisms against toxicity of NDMA.21)

In rats fed on a diet consisting of low sulfur-amino acids, NDMA-induced oxidative toxicity in the liver was increased, as indicated by a rise in GSH and MT levels, the elevation of r-glutamyltransferase activity, and a reduction in anti-oxidative enzyme activities in the tissues.22) Here, the effects of dietary sulfur amino acids on NDMA-induced oxidative responses were investigated in rat liver, focusing on the changes in MT levels and its regional distribution to clarify the mechanism of MT induction.

MATERIALS AND METHODS

Chemicals —— N-Nitrosodimethylamine (NDMA, for gas chromatograph), glutathione (reduced form),
diethylmalate, CdCl₂, 5 mM (1000 ppm) HgCl₂ standard solution, and 100%(w/v) trichloroacetic acid (TCA) aqueous solution were purchased from Wako Pure Chemical Ind., Ltd., (Osaka, Japan). Ovalbumin was obtained from Sigma Co. (St. Louis, MO, U.S.A.).

Glutathione reductase (EC 1.6.4.2; from yeast) and NADPH were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other chemicals were of reagent grade.

**Animals and Treatment**

Weaning rats (male, Wistar; Charles River, Shizuoka, aged 21 days; body weight : 52 ± 3 g) were maintained for 3 weeks on semi-synthetic diets, soybean protein isolate (SPI) and SPI supplemented with 0.3% l-methionine (SPI-Met), which were made principally according to AIN-93 recommendations,²⁰ with the compositions as shown in Table 1. SPI was prepared using soybean protein isolate (Fujipro R; Fuji Seiyu Co., Osaka, Japan) as a protein source (10% of total content). The crude protein content of the soybean protein isolate was 91.1 g/100 g, and the methionine content of this crude protein was 1.4 g/100 g, as determined by the Fuji Seiyu Research Institute (Osaka, Japan). When the SPI-Met, 0.3 g/100 g was added to raise the amino acid to levels similar to those of a commercially available laboratory chow CE-2 (CLEA Japan, Osaka, Japan). Rats were given drinking water ad libitum and housed in individual cages in a temperature-controlled room at 20–22°C with a controlled 12-hr cycle of light and dark.

NDMA dissolved in saline solution (10 mg/ml) was injected intraperitoneally prior to the experiment according to Ahotupa *et al.*⁷ Since we found that 30 mg NDMA/kg body weight was hazardous enough to increase the hepatic TBARS levels in rats,⁹ a dose of 20 mg NDMA/kg was employed throughout the present experiment. Twenty-four hours after injection of NDMA, the rats were sacrificed under anesthesia by inhalation of diethylether at 9:00–10:00, and portions of the liver were removed for histochemistry and for GSH and MT assays.

**Assays of GSH and MT**

For a determination of GSH, a portion of the liver (0.3–0.5 g) was immediately homogenized in ice cold 5% perchloric acid containing 1 mM EDTA. Using the supernatant after centrifugation at 2500 × g for 10 min, glutathione levels were determined by the enzymatic recycling method.²¹ For MT analysis, a portion of the liver (0.3–0.5 g) samples was homogenized in ice-cold 1.15% KCl (5%, w/v) under N₂ atmosphere, and MT levels were assayed using the Hg²⁺ saturation method essentially according to Naganuma *et al.*²² with a slight modification using non-radioactive HgCl₂.²³ Briefly, the homogenate (1 ml) was treated successively with diethylmalate (5 µl) and 10 mM CdCl₂ (25 µl), and heated at 95°C for 5 min to precipitate high-molecular weight proteins. Following cooling and centrifugation, 0.5 ml of the supernatant was successively treated with 5 mM HgCl₂ (25 µl), 1 mM ovalbumin (225 µl), and 12.5% TCA (250 µl). After centrifugation, the supernatant was filtered through a membrane of 0.22 µm pore diameter (Ultrafree C3, Millipore). The total MT levels were determined by analyzing Hg concentration in the final filtrate. Hg levels in the final preparations were determined by the oxygen combustion-gold amalgamation method,²⁴ using an atomic absorption mercury detector MD-A (Nippon Instruments, Ltd., Osaka, Japan). MT levels were expressed as the amount of Hg bound to thionein molecules.

**Histochemical Staining**

An aliquot of liver (0.3 g) was fixed in phosphate-buffered 10% formaldehyde (pH 7.4), embedded in paraffin, and sectioned at 5-µm thickness. The sections were stained with hematoxylin and eosin (HE) to determine pathological changes. Distribution of MT was examined by staining with anti-mouse MT antibody (clone E9, Zymed Lab. Inc., San Francisco, U.S.A.). After blocking endogenous peroxidase activity with 3% H₂O₂, sections were treated with 10% normal bovine serum, incubated with primary antibody (1 : 400) overnight at 4°C, and then sequentially incubated with biotinylated goat anti-mouse IgG.

<table>
<thead>
<tr>
<th>Component</th>
<th>SPI</th>
<th>SPI-Met</th>
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<tbody>
<tr>
<td>Soybean protein isolate</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>l-Methionine</td>
<td>—</td>
<td>0.3</td>
</tr>
<tr>
<td>Mineral mixturea</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Vitamin mixturea</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Dextrin</td>
<td>15.5</td>
<td>15.5</td>
</tr>
<tr>
<td>Starch</td>
<td>49.75</td>
<td>49.45</td>
</tr>
</tbody>
</table>

* Table 1. Composition of SPI and SPI-Met Diets (g/100 g)

a) Composition of mineral and vitamin mixtures was that of the AIN-93 diet (Reeves *et al.* 1993) for growing rats; purchased from Oriental Yeast Co. (Tokyo, Japan).
After the first and second incubations, the sections were given three 5-min washes in phosphate buffered saline and then developed with 3,3-diaminobenzidine tetrahydrochloride and H2O2. The sections were counterstained with methyl green.

Statistical Analyses —— Each experiment was performed with 6 rats, and the results are presented as mean ± standard deviation (S.D.). Statistical analyses were performed with variance (ANOVA). Post hoc comparisons of means were performed with Fisher’s PLSD test. A probability of < 0.05 was considered significant.

RESULTS AND DISCUSSION

GSH and MT Levels

SPI was prepared using soybean protein isolate as a protein source (10% of total content) which was somewhat lower than recommended by Reeves et al.20) Since the methionine content of SPI was as low as 0.14%, 0.3% of methionine was supplied in SPI-Met to a level similar to that in commercially available laboratory chow.

The hepatic GSH and MT levels differ greatly between the groups fed on SPI and those on SPI-Met diet for 3 weeks after weaning (Table 2). The GSH levels in the SPI group were as low as 1.17 μmol/g tissue, which was 6.5 times lower than in the SPI-Met-fed rats. On the other hand, the SPI group showed 5.3 times higher hepatic MT levels than the SPI-Met group. Both levels of the SPI-Met group were nearly the same as those observed in laboratory chow-fed rats.9) Since the sulfur amino acid content in SPI was about one third of SPI-Met, the hepatic GSH levels were lowered corresponding to the sulfur amino acid contents. In contrast to GSH, MT levels were negatively correlated with the sulfur amino acid contents of the diet. Since the hepatic MT levels in the SPI-Met group were very close to the basal level found in laboratory chow-fed rats,10) SPI feeding would induce hepatic MT to compensate for the heightened oxidative status caused by the lowered GSH levels. As a result, a reverse relationship was shown between these two sulfhydryl compounds. Since body weight gains of SPI-fed rats were somewhat lower compared to SPI-Met rats,10) the SPI diet was considered to be inadequate for growth. However, since rats of the SPI group survived for at least several weeks with low hepatic GSH levels, those levels might be barely enough to survive. Elevation of MT levels in the liver of the SPI group could be explained as the result of oxidative stress enhanced by a shortage of GSH, which could have maintained the hepatocytes in a reduced state.

Intraperitoneal injections of NDMA (20 mg/kg) could cause mild damage to rat liver as indicated by alterations in lipid peroxidation and anti-oxidative enzyme activities.10) In the present study, NDMA treatment caused an increase in the hepatic GSH levels of the SPI group, whereas the levels in the SPI-Met group were not affected at all. On the other hand, MT levels increased in both dietary groups after NDMA treatment. NDMA at the present dose level would have caused enough oxidative stress to induce a protective response such as a novel synthesis of GSH and/or MT in the rat liver. In the SPI-Met group, however, the hepatic GSH levels might be high enough to inhibit GSH synthetase activity by a feed-back inhibition.

Pathological Findings

The pathological effects of intraperitoneal injections of NDMA (20 mg/kg) on rat liver were significant. NDMA was oxidized during metabolic activation by cytochrome P450,4–6 causing oxidative stress.7–10) The liver of the NDMA-treated rats of both dietary groups showed necrosis and hemorrhage 24 hr after the injection (Figs. 1b, 1d). In a previous paper, we found that SPI-fed rats showed a higher

<table>
<thead>
<tr>
<th>Diet</th>
<th>NDMA (20 mg/kg, i.p.)</th>
<th>GSH (mmol/g)</th>
<th>MT (nmol Hg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPI</td>
<td>—</td>
<td>1.15 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>165.2 ± 28.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.51 ± 0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>211.9 ± 51.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SPI-Met</td>
<td>—</td>
<td>7.77 ± 0.70&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.3 ± 21.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>7.60 ± 1.32&lt;sup&gt;c&lt;/sup&gt;</td>
<td>93.2 ± 43.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a–d</sup> Mean values within a column with unlike superscript letters were significantly different: p < 0.05.
susceptibility to NDMA hepatotoxicity than SPI-Met fed rats indicated by enhanced lipid peroxide levels and decreased antioxidative enzyme activities.\(^{10}\) In the present study, however, no pathological difference in NDMA-induced damaged cells could be found between the two dietary groups. If both GSH and MT were assumed to function in considerable part as a prevention against NDMA cytotoxicity, MT and GSH would be the dominant contributors in the SPI and SPI-Met groups, respectively. Since a detailed examination using a higher magnification showed that the SPI-Met group had a slightly higher rate of necrotic cells at the central region of the hepatic lobules than the SPI group (data not shown), the higher MT in the SPI group might function effectively against NDMA toxicity. Thus, alterations in biochemical markers such as lipid peroxide and antioxidative enzyme activities\(^{10}\) would not necessarily be consistent with pathological changes.

Tohyama et al.\(^{25}\) reported that the hepatic MT principally distributed in the cytosol fraction, though it transiently distributed in the nucleus after partial heptectomy. In the present study immunological staining of the liver with anti-MT antibody showed that MT was distributed in the cytosol fractions of the liver in both dietary groups. The staining density was higher in the SPI group (Fig. 2c), in agreement with the tissue MT levels determined above. The staining distribution was localized around the central vein in the hepatic lobules of both dietary groups before NDMA injection (Figs. 2a, 2c). It should be noted that 24 hr after NDMA treatment, strong MT staining appeared in the peripheral regions of the lobules (Figs. 2b, 2d). Since those regions are close to the interlobular arteries, the oxygen supply is abundant. These results indicate that the synthesis of hepatic MT basically occurs around the central vein. Since the cells need a stable oxygen supply to maintain homeostasis, an insufficient oxygen supply and/or stimulated oxygen consumption around the central vein may cause oxygen stress, which would lead to MT synthesis. After NDMA injection, the oxygen-consuming metabolism of NDMA was actively induced in the peripheral regions by the action of cytochrome P-450 using abundant oxygen, producing a reactive oxygen species\(^{7,8}\) which probably stimulated a novel MT induction. Thus, MT induction would possibly depend on the reactive oxygen species, and be increased by the oxidative metabolism of xenobiotics.

**Acknowledgements** The experimental protocol was approved by the Ethics Committee for Research on Animals of Nakamura Gakuen University.
REFERENCES


25) Tohyama, C., Suzuki, J. S., Hemelraad, J.,