Mechanisms of Protection by S-Alllylmercaptocysteine Against Acetaminophen-Induced Liver Injury in Mice

Isao Sumioka¹, Tatsuya Matsura², Shigeo Kasuga¹, Yoichi Itakura¹ and Kazuo Yamada², *¹

¹Institute for OTC Research, Wakunaga Pharmaceutical Co., Ltd., 1624 Shimokotachi, Koda-cho, Takata-gun, Hiroshima 739-1105, Japan
²Department of Biochemistry, Faculty of Medicine, Tottori University, 85 Nishi-cho, Yonago 683-8503, Japan

Received May 13, 1998 Accepted July 21, 1998

ABSTRACT—S-Alllylmercaptocysteine (SAMC), one of the water-soluble organosulfur compounds in ethanol extracts of garlic (Allium sativum L.), has been shown to protect mice against acetaminophen (APAP)-induced liver injury. In this study, we examined the mechanisms underlying this hepatoprotection. SAMC (100 mg/kg, p.o.) given 2 and 24 hr before APAP administration (500 mg/kg, p.o.) suppressed the plasma alanine aminotransferase activity increases 3 to 12 hr after APAP administration significantly. The hepatic reduced glutathione levels of vehicle-pretreated mice decreased 1 to 6 hr after APAP administration, but SAMC pretreatment suppressed the reductions 1 to 6 hr after APAP administration significantly. These inhibitory effects of SAMC were dose-dependent (50–200 mg/kg) 6 hr after APAP administration. As SAMC pretreatment (50–200 mg/kg) suppressed hepatic cytochrome P450 2E1-dependent N-nitrosodimethylamine dimethylase activity significantly in a dose-dependent manner, we suggest that one of its protective mechanisms is inhibition of cytochrome P450 2E1 activity. SAMC pretreatment also suppressed the increase in hepatic lipid peroxidation and the decrease in hepatic reduced coenzyme Q9 (CoQ9H2) levels 6 hr after APAP administration. The hepatic CoQ9H2 content of the SAMC pretreatment group was maintained at the normal level. Therefore, we suggest that another hepatoprotective mechanism of SAMC may be attributable to its antioxidant activity.

Keywords: Acetaminophen, Hepatoprotective agent, S-Alllylmercaptocysteine, Garlic, Cytochrome P450 2E1

Since ancient times, garlic (Allium sativum L.) has been used world-wide as a food and a folk medicine. In recent years, many studies have shown that garlic possesses antimicrobial (1), antithrombotic (2), antitumor (3), antihyperlipidemic (4, 5), antioxidative (6–8) and hepatoprotective (9–12) properties.

S-Alllylmercaptocysteine (SAMC) is one of the water-soluble organosulfur compounds in the aged garlic extract obtained by ethanol-extraction of sliced garlic bulbs. SAMC has been shown to protect the liver against a number of hepatotoxins, such as acetaminophen (APAP), carbon tetrachloride and D-galactosamine (9–11). In a previous study, we demonstrated that SAMC pretreatment protected mice against APAP-induced liver injury (11). However, the hepatoprotective mechanisms of SAMC are still poorly understood.

APAP is widely used as an analgesic and antipyretic drug. After therapeutic doses, it is biotransformed and eliminated as nontoxic glucuronic acid and sulfate conjugates. Only a small proportion of APAP is converted to N-acetyl-p-benzoquinoneimine (NAPQI) (13), a cytochrome P450 (P450)-mediated intermediate metabolite, which is normally detoxified by conjugation with reduced glutathione (GSH). However, after high doses of APAP, the capacity for its removal by hepatic conjugation with glucuronide and sulfate is exceeded, and more of the reactive metabolite NAPQI is formed. Consequently, more NAPQI is conjugated with GSH, and when hepatic GSH is depleted, more NAPQI will bind covalently to cellular macromolecules (14, 15). This is thought to lead to a loss of protein thiol groups (16, 17) and ultimately to cellular death.

Protection against APAP-induced liver injury can be achieved by various mechanisms. Cysteine prodrugs, including N-acetylcysteine, the most widely used antidote for APAP overdose, have been reported to protect the

* To whom correspondence should be addressed.
liver against APAP-induced injury. The mechanism responsible for this protection may be metabolism of these prodrugs to L-cysteine, which is incorporated into hepatic glutathione (18–22). Moreover, several P450 enzymes have been reported to play important roles in the bioactivation of APAP to NAPQI (23–26) and P450 inhibitors, such as dialyl sulfide, phenethyl isothiocyanate, and 2-(allylthio)pyrazine, have been shown to protect the liver against APAP-induced injury (12, 27, 28). Furthermore, recent studies suggest that APAP-induced liver injury is also caused by cellular oxidative stress, resulting in hepatic lipid peroxidation (29–31), and several antioxidants and antioxidative enzymes have been shown to prevent APAP-induced liver injury (31–34). Finally, enhancing the capacity for conjugation with glucuronic acid may protect against APAP-induced liver injury, as shown in an experiment using butylated hydroxyanisole (35).

However, it is still not known which mechanisms are responsible for the protective effect of SAMC against APAP-induced liver injury. Therefore, we examined whether SAMC affected hepatic glutathione, protein thiol, lipid peroxide and endogenous lipid-soluble antioxidant levels in APAP-treated mice and hepatic P450 2E1, uridine diphosphate glucuronyltransferase (UDP-GT) and sulfotransferase activities in normal mice.

MATERIALS AND METHODS

Chemicals

SAMC (CH$_2$=CH-CH$_2$-S-S-CH$_2$-CHNH$_2$-COOH) was synthesized as described previously (9). APAP, 2-vinylpyridine, trichloroacetic acid (TCA), sodium dodecyl sulfate (SDS) and 1,1,3,3-tetraethoxypropane were purchased from Wako Pure Chemical Industries (Osaka). GSH, glutathione reductase, isocitric acid, isocitric dehydrogenase, adenosine 3'-phosphate 5'-phosphosulfate, p-nitrophenyl sulfate and 2-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). NADPH, NADP, β-NADH, lactate dehydrogenase (from beef heart), phosphoenolpyruvate, pyruvate kinase and uridine-5'-diphospho-glucuronic were purchased from Boehringer Mannheim Biochemicals (Mannheim, Germany). 2-Thiobarbituric acid (TBA), phosphotungstic acid, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and ethylenediaminetetraacetic acid (EDTA) were purchased from Katayama Chemical Industries Co. (Osaka). N-Nitrosodimethylamine (NDMA), 3-methyl-2-nitrophenol and p-nitrophenol were purchased from Tokyo Kasei Industry (Tokyo). Chromatographically pure oxidized coenzyme Q$_8$ (CoQ$_8$), oxidized coenzyme Q$_{10}$ (CoQ$_{10}$) and α-tocopherol (α-Toc) were obtained from Eisai Co. (Tokyo), and reduced coenzyme Q$_8$ (CoQ$_{8}$H$_2$) and coenzyme Q$_{10}$ (CoQ$_{10}$H$_2$) were prepared by reducing CoQ$_8$ and CoQ$_{10}$, respectively, with sodium borohydride. All the other chemicals used were of analytical grade.

SAMC and APAP were suspended in 1% gum arabic and 1% carboxymethyl cellulose sodium salt solutions, respectively.

Animals and treatments

Male ddY mice were purchased when they were 5-week-old and housed, 5–6 per cage, in plastic cages under a 12-hr light/dark cycle for 1 week before experimental use. They were allowed free access to standard laboratory food and water, but fasted overnight before APAP administration. During food deprivation and experimentation, they were housed in cages with wire-mesh floors to prevent them from ingesting their bedding materials and excreta.

Mice were given SAMC (50, 100 or 200 mg/kg body weight, p.o.) 2 and 24 hr before APAP administration as described previously (11). A single dose of APAP (500 mg/kg, p.o.) was administered between 9 a.m. and 11 a.m. to avoid any circadian variation. The dose volumes of both compounds and vehicle were 10 ml/kg. Mice in the normal group were also fasted, but given neither SAMC nor APAP.

The mice were anesthetized with diethyl ether, and then blood samples were taken from the right ventricle using a heparinized syringe and their livers were removed 0, 1, 3, 6 or 12 hr after APAP administration. The livers were frozen immediately and stored in liquid nitrogen until assayed.

Measurement of plasma alanine aminotransferase (ALT) activity

Plasma ALT activity was determined spectrophotometrically by means of a commercially available kit based on the method of Wróblewski and LaDue (36), which involves calculating the enzyme activity from the decrease in NADH absorbance at 340 nm. The results are expressed in IU/l.

Glutathione assays

The hepatic total glutathione content, the sum of the GSH and oxidized glutathione (GSSG) contents, was determined by the glutathione reductase-DTNB recycling assay using the method of Griffith (37). Frozen liver tissue was homogenized in 5% TCA / 5 mM EDTA under a stream of nitrogen gas, centrifuged at 20,000 × g for 10 min at 4°C and the TCA in the supernatant was removed by extracting 3 times with diethyl ether. The reaction mixture comprised 200 µM NADPH, 600 µM DTNB, 0.5 U glutathione reductase and 200 µl of the sample in a final volume of 1 ml. The reaction was initiated by adding glutathione reductase, and the rate of DTNB reduction
was determined by measuring the absorbance at 412 nm. The GSSG level was determined after GSH had been allowed to react with 2-vinylpyridine, and the GSH level was calculated by subtracting the GSSG level from the total glutathione level.

**Protein thiol assay**

The hepatic protein thiol content was determined by the method of Sedlak and Lindsay (38) using GSH as the standard. Frozen liver tissue was homogenized in 20 mM EDTA under a stream of nitrogen gas. Aliquots (250 μl) of the homogenates were mixed with 750 μl of 200 mM Tris-HCl (pH 8.2) and 50 μl of 10 mM DTNB. The mixture was made up to 5 ml with absolute methanol, allowed to stand with occasional shaking for 15 min and then centrifuged at 3,000 × g for 15 min at room temperature. The absorbance of the supernatant at 412 nm was measured to determine the total thiol concentration, and the nonprotein thiol content was determined after the protein had been precipitated with TCA. The protein thiol content was calculated by subtracting the non-protein thiol content from the total thiol content.

**Subcellular fractionation and enzyme assays**

Mice were given SAMC (50, 100 or 200 mg/kg, p.o.) 2 and 24 hr before decapitation, immediately after which their livers were perfused with chilled 154 mM KCl, removed and homogenized in a homogenizing buffer (50 mM Tris-HCl, 154 mM KCl, pH 7.4). The homogenates were centrifuged at 9,000 × g for 20 min at 4°C, and the microsomal and cytosolic fractions were separated from the supernatant by centrifugation at 105,000 × g for 90 min at 4°C. The resulting supernatant was retained and used as the cytosol, and the microsomal pellet was washed with homogenizing buffer, centrifuged again at 105,000 × g for 90 min at 4°C and then suspended in 250 mM sucrose. The microsomal and cytosolic fractions were stored at −80°C until assayed.

NDMA demethylase activity was determined by the method of Peng et al. (39). The assay mixture comprised 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 150 mM KCl, 4 mM NDMA, 10 mM isocitrate, 400 μM NADP, 0.3 U isocitrate dehydrogenase and 1 mg/ml microsomal protein in a final volume of 1 ml. The reaction was initiated with the NADPH-generating system, carried out with shaking for 30 min at 37°C and then terminated by adding 100 μl each of 25% ZnSO₄ and saturated Ba(OH)₂. This mixture was centrifuged at 2,000 × g for 10 min at room temperature, and 700 μl of supernatant was mixed with 300 μl of concentrated Nash reagent (5 g of ammonium acetate and 70 μl of acetylacetone in 6 ml of 3% acetic acid) for formaldehyde determination (39, 40). This mixture was incubated for 30 min at 50°C, the absorbance at 412 nm was measured, and the NDMA demethylase activity was expressed as nmol HCHO formed/min/mg protein.

UDP-GT activity was evaluated by the method of Mulder and Van Doorn (41), which is based on the determination of UDP production from UDP-glucuronate during the reaction. UDP production was measured continuously by coupling it to the conversion of NADH into NAD⁺ through pyruvate kinase and lactate dehydrogenase. The assay mixture comprised 75 mM Tris-HCl (pH 7.3), 5 mM MgCl₂, 200 μM phosphoenol pyruvate, 200 μM NADH, 5 U/ml pyruvate kinase, 0.625 U/ml lactate dehydrogenase, 1.5 mM UDP-glucuronate, 300 μM 3-methyl-2-nitrophenol and 150 μg/ml microsomal protein in a final volume of 500 μl. Triton-X, to produce a concentration of 0.25% (v/v), was added to the microsomal suspension (10 mg microsomal protein/ml) prior to adding it to the assay mixture. The reaction was initiated by adding the microsomal protein and allowed to proceed for 4 min at 29°C. The enzyme activity was calculated from the decrease in NADH absorbance at 340 nm.

Sulphotransferase activity was determined by the method of Ramaswamy and Jakoby (42) with a slight modification. The assay mixture comprised 375 mM phosphate buffer (pH 7.4), 7.5 mM 2-mercaptoethanol, 250 μM p-nitrophenol, 200 μM adenosine 3'-phosphate 5'-phosphosulphate and 500 μg of cytosolic protein in a final volume of 400 μl. The reaction was initiated by adding p-nitrophenol and allowed to proceed for 20 min at 37°C. The reaction was terminated by adding 500 μl of methylene blue solution (25 mg of methylene blue, 5 g of anhydrous sodium sulfate and 1 ml of H₂SO₄ dissolved in distilled water to produce a final volume of 100 ml), followed by 2 ml of chloroform. The two phases were agitated with a vortex mixer for 20 sec and separated by centrifugation at 2,000 × g for 10 min at room temperature. The chloroform layer was transferred to a tube containing about 100 mg of anhydrous sodium sulfate and the absorbance at 651 nm was determined.

**Lipid peroxidation assay**

Frozen liver tissue was homogenized in 50 mM phosphate buffer / 20 mM EDTA (pH 7.4) under a stream of nitrogen gas. Lipid peroxidation was evaluated by the method of Masugi and Nakamura (43) with some modifications. Briefly, 200 μl of 7% SDS, 2 ml of 0.1 N HCl, 300 μl of 10% phosphotungstic acid and 1 ml of 0.5% TBA were added to 100 μl of tissue homogenate. The mixture was heated in boiling water for 45 min, cooled for 5 min, and then 5 ml of n-butanol was added and mixed vigorously. After centrifugation, the fluorescence of the n-butanol layer was measured (excitation, 515 nm;
emission, 553 nm). The TBA-reactive substance (TBARS) levels are expressed as malondialdehyde equivalents using 1,1,3,3-tetraethoxypropane as the standard.

**Assay of CoQ9H2, CoQ10H2 and α-Toc**

Simultaneous determination of the hepatic CoQ9H2, CoQ10H2 and α-Toc levels was carried out by the method of Ikenoya et al. (44). Frozen liver tissue was homogenized in ice-cold water under a stream of nitrogen gas, and CoQ9H2, CoQ10H2 and α-Toc in the homogenate (1 vol.) were extracted 3 times with a 2:5, by volume, mixture of ethanol and n-hexane. The n-hexane layer was collected and evaporated under a stream of nitrogen gas, and the residue was redissolved in ethanol and subjected to high-performance liquid chromatography using a system consisting of a Jasco 880-PU pump (Jasco, Tokyo), a Rheodyne injection valve with a 100 µl loop, a Chemosorb ODS-H column (4.6 × 250 mm, 7-μm particle size) and a Jasco 840 EC detector. The mobile phase consisted of ethanol/methanol/70% HClO4 (700:300:1, v/v) and 0.7% (w/v) NaClO4. The eluate was passed through an electrochemical detector (applied potential 0.6 V vs Ag+/AgCl) to measure the CoQ9H2, CoQ10H2 and α-Toc levels.

**Protein assay**

Protein contents were determined by the method of Lowry et al. (45) using bovine serum albumin as the standard.

**Statistical analyses**

The results are expressed as means ± S.E. Differences between means were determined using one-way analysis of variance or the Kruskal-Wallis method for multiple comparisons and Student’s t-test or the Aspin-Welch method for single comparisons. Differences at P < 0.05 were considered to be significant.

**RESULTS**

**Effect of SAMC pretreatment on plasma ALT activity**

APAP (500 mg/kg, p.o.) was administered, and the plasma ALT activity was measured as an index of liver damage. The time courses of the ALT activity changes are shown in Fig. 1. The plasma ALT activity started to increase 3 hr after APAP administration and reached its maximum 6 hr after APAP administration. SAMC pretreatment suppressed the plasma ALT activity increases 3, 6, and 12 hr after APAP administration significantly. The dose-dependency of this protective effect was investigated 6 hr after APAP administration. As shown in Fig. 2, the SAMC pretreatment groups showed dose-dependent plasma ALT activity reductions, and the effects of 100 and 200 mg/kg were significant. The percentages of inhibition of plasma ALT activities were calculated using the following equation:

\[ 100 \times \frac{(\text{ALT in vehicle group} - \text{ALT in SAMC group})}{(\text{ALT in vehicle group} - \text{normal ALT level})} \]

For the groups pretreated with 50, 100 and 200 mg/kg...
SAMC, the percentages of inhibition were 79%, 97% and 100%, respectively. Pretreatment of mice with 200 mg/kg SAMC afforded complete protection against APAP-induced liver injury.

**Effect of SAMC pretreatment on hepatic glutathione content**

As GSH plays an important role in the detoxification of APAP, the hepatic GSH content was examined. The time courses of the changes in the hepatic GSH contents are shown in Fig. 3. The hepatic GSH content of the vehicle-treated group decreased rapidly to 9% of the initial (0 hr) level 1 hr after APAP administration with no reciprocal increase in the GSSG content, suggesting that GSH loss may have resulted from the detoxification of NAPQI by GSH conjugation, rather than from the consumption of GSH by glutathione peroxidase. However, 12 hr after APAP administration, the GSH content was higher than the initial level. SAMC pretreatment suppressed the hepatic GSH content reductions 1, 3 and 6 hr after APAP administration significantly. The dose-dependency of this effect was investigated 6 hr after APAP administration. As shown in Fig. 4, dose-dependent increases in the hepatic GSH contents were observed and the effects of 100 and 200 mg/kg were significant.

**Effect of SAMC pretreatment on hepatic protein thiol content**

Because NAPQI reacts not only with GSH, but also with protein thiol, the hepatic protein thiol contents were examined 0 and 6 hr after APAP administration. As shown in Table 1, the hepatic protein thiol content was unaffected by pretreatment with SAMC (100 mg/kg) in comparison with that of vehicle-pretreated mice. However, the hepatic protein thiol content in the vehicle group decreased to 81% of that in the normal group 6 hr after APAP administration. Pretreatment with SAMC might suppress the reduction of the hepatic protein thiol content, maintaining it at the normal level.

---

**Fig. 3.** Effects of SAMC pretreatment on the hepatic GSH and GSSG contents of mice at various times after APAP administration (500 mg/kg, p.o. at 0 hr). SAMC (100 mg/kg, p.o.) was given 2 and 24 hr before APAP. The vehicle-treated group received 1% gum arabic solution (10 ml/kg). The data are expressed as means±S.E. of at least 5 animals. *, **Significantly different from the vehicle-treated group (P<0.05, P<0.01, respectively). ○: Vehicle, ●: SAMC.

**Fig. 4.** Effects of SAMC pretreatment on the hepatic GSH and GSSG contents of mice 6 hr after APAP administration (500 mg/kg, p.o.). SAMC (30, 100 or 200 mg/kg, p.o.) was given 2 and 24 hr before APAP. Mice in the normal group were given neither SAMC nor APAP. The data are expressed as means±S.E. of at least 5 animals. The normal levels of hepatic GSH and GSSG contents were 22.4±2.3 (n=6) and 1.9±0.3 nmol/mg protein (n=6), respectively. *Significantly different from the vehicle-treated group (P<0.05).
Table 1. Effects of SAMC pretreatment on the hepatic protein thiol contents of mice 0 and 6 hr after APAP administration

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein thiol content (nmol GSH equivalents/mg protein)</th>
<th>0 hr</th>
<th>6 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>74.7 ± 2.2</td>
<td>79.5 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>APAP + Vehicle</td>
<td>77.9 ± 1.2</td>
<td>64.5 ± 8.1</td>
<td></td>
</tr>
<tr>
<td>+ SAMC</td>
<td>77.6 ± 1.7</td>
<td>81.7 ± 1.8</td>
<td></td>
</tr>
</tbody>
</table>

SAMC (100 mg/kg, p.o.) was given 2 and 24 hr before APAP (500 mg/kg, p.o.). The vehicle group received 1% gum arabic solution (10 ml/kg). Mice in the normal group were given neither SAMC nor APAP. The data are expressed as means ± S.E. of at least 5 animals.

Effects of SAMC pretreatment on hepatic P450 2E1, UDP-GT and sulfotransferase activities

Since the hepatotoxicity of APAP results from the formation of NAPQI, the activity of P450 2E1, which is a major enzyme responsible for APAP bioactivation, was examined. As shown in Table 2, SAMC reduced the activity of P450 2E1-dependent NDMA demethylase in a dose-dependent manner, and the effect of each dose used was significant. The P450 2E1 activities of the groups given 50, 100 and 200 mg/kg SAMC were 74%, 69% and 45%, respectively, of those treated with vehicle.

UDP-GT and sulfotransferase activities were also examined because APAP is eliminated by glucuronic acid and sulfate conjugation. As shown in Table 2, SAMC pretreatment affected neither hepatic UDP-GT nor sulfotransferase activities.

Effect of SAMC pretreatment on hepatic lipid peroxidation

Hepatic lipid peroxidation was evaluated by measuring the TBARS levels 6 and 12 hr after APAP administration. The results are shown in Table 3. Six and 12 hr after APAP administration, the TBARS levels of the group treated with vehicle increased compared with the normal levels, and SAMC pretreatment suppressed the increases in the hepatic TBARS levels significantly.

Effects of SAMC pretreatment on the hepatic CoQ9H2, CoQ10H2 and α-Toc contents

The hepatic CoQ9H2, CoQ10H2 and α-Toc contents were measured 6 hr after APAP administration. The results are shown in Table 4. The hepatic CoQ9H2 levels decreased to 68% of the normal level 6 hr after APAP administration, and SAMC pretreatment suppressed this reduction significantly. The hepatic CoQ10H2 and α-Toc contents did not change significantly in any group.

DISCUSSION

This study has demonstrated that SAMC pretreatment suppresses the plasma ALT activity increases caused by APAP administration in a dose-dependent manner (Figs. 1 and 2). The inhibitory effect of SAMC observed in this study is consistent with that reported previously (11). We suggest that SAMC pretreatment has a strong protective effect against APAP-induced liver injury in mice. The aim of our study was to examine the mechanisms underlying this protective effect.

Many studies on the mechanisms of APAP-induced liver injury have demonstrated that GSH plays an important role in the detoxification of NAPQI, a reactive and toxic metabolite of APAP, and that liver necrosis begins when the GSH stores are almost exhausted (46). Cysteine prodrugs, such as N-acetylcysteine, have been reported to protect the liver against APAP-induced injury. With respect to the mechanism responsible for this protection, these prodrugs are thought to be metabolized to L-cysteine, thereby supplying a component for hepatic glutathione synthesis (18–22). As SAMC possesses part of the cysteine structure, we assumed that GSH synthesis would also be increased by SAMC pretreatment. We found that SAMC pretreatment suppressed the hepatic GSH content reductions that occurred 1, 3 and 6 hr after

Table 2. Effects of SAMC pretreatment on the hepatic NDMA demethylase, UDP-GT and sulfotransferase activities of mice

<table>
<thead>
<tr>
<th>Group</th>
<th>NDMA demethylase&lt;sup&gt;a&lt;/sup&gt;</th>
<th>UDP-GT&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sulfotransferase&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>3.04 ± 0.24</td>
<td>33.7 ± 1.8</td>
<td>0.162 ± 0.021</td>
</tr>
<tr>
<td>SAMC 50 mg/kg</td>
<td>2.24 ± 0.17**</td>
<td>35.9 ± 1.4</td>
<td>0.150 ± 0.017</td>
</tr>
<tr>
<td>SAMC 100 mg/kg</td>
<td>2.10 ± 0.11**</td>
<td>36.6 ± 0.9</td>
<td>0.160 ± 0.013</td>
</tr>
<tr>
<td>SAMC 200 mg/kg</td>
<td>1.37 ± 0.16**</td>
<td>33.8 ± 1.0</td>
<td>0.167 ± 0.016</td>
</tr>
</tbody>
</table>

SAMC was given p.o. 2 and 24 hr before decapitation. The vehicle-treated group received 1% gum arabic solution (10 ml/kg). The data are expressed as means ± S.E. of 9 animals. <sup>a</sup>The values are expressed as nmol HCHO formed/min/mg protein. <sup>b</sup>The values are expressed as nmol/min/mg protein. **Significantly different from the vehicle-treated group (P < 0.01).
Table 3. Effects of SAMC pretreatment on hepatic lipid peroxidation in mice 6 and 12 hr after APAP administration

<table>
<thead>
<tr>
<th>Group</th>
<th>TBARS (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 hr</td>
</tr>
<tr>
<td>Normal</td>
<td>0.89±0.10</td>
</tr>
<tr>
<td>APAP + Vehicle</td>
<td>1.55±0.18</td>
</tr>
<tr>
<td>+ SAMC</td>
<td>1.03±0.08*</td>
</tr>
</tbody>
</table>

SAMC (100 mg/kg, p.o.) was given 2 and 24 hr before APAP (500 mg/kg, p.o.). The vehicle group received 1% gum arabic solution (10 ml/kg). Mice in the normal group were given neither SAMC nor APAP. The data are expressed as means±S.E. of at least 5 animals. *Significantly different from the vehicle-treated group (P<0.05).

APAP administration (Fig. 3), and this suppression was dose-dependent 6 hr after APAP administration (Fig. 4). However, the time course experiment revealed that the hepatic GSH content of SAMC-pretreated mice did not differ from that of vehicle-pretreated mice at the time (0 hr) of APAP administration (Fig. 3), suggesting that SAMC pretreatment did not affect hepatic GSH synthesis.

As inhibition of P450 activities, resulting in reduced generation of NAPQI, also suppresses GSH depletion, we investigated whether SAMC pretreatment affected P450 activities. P450 2E1, 3A4 and 1A2 have been shown to be the major enzymes responsible for APAP bioactivation in the human and rat liver (23–25). Similar P450 enzymes, particularly P450 2E1, are thought to be involved in APAP bioactivation in mice, because P450 2E1 inhibitors, such as diallyl sulfide, phenethyl isothiocyanate and 2-(allylthio)pyridine, have been shown to protect mice against APAP-induced liver injury (12, 27, 28). We found that SAMC pretreatment suppressed P450 2E1 activity in a dose-dependent manner (Table 3). Therefore, we suggest that SAMC protects mice against APAP-induced liver injury, at least in part, by inhibiting P450 2E1 activity. A previous study demonstrated that S-allylcysteine (CH\textsubscript{2}=CH-CH\textsubscript{2}-S-CH\textsubscript{2}-CHNH\textsubscript{2}-COOH) did not inhibit P450 2E1 activity (12), suggesting that the disulfide bond in the SAMC molecule is very important for P450 2E1 activity inhibition. The most significant physiological role of P450 2E1 is its adaptive response to high blood ethanol levels with a corresponding acceleration of ethanol metabolism. Moreover, P450 2E1 is also associated physiologically with the metabolism of lipids and ketones in starvation, obesity and diabetes (47). Although, we performed a 2-week repeated dose toxicity study to examine the effect of SAMC in normal mice, at the doses tested in this study, SAMC was free of toxic effects (S. Kasuga et al., unpublished data).

We also investigated whether SAMC pretreatment affected the activities of UDP-GT and sulfotransferase, because conjugation with glucuronic acid and sulfate are other pathways that metabolize APAP. Our results showed that SAMC pretreatment did not affect the activity of either enzyme (Table 2). Therefore, it is unlikely that enhanced glucuronidation or sulfation of APAP is the mechanism underlying the hepatoprotective effect of SAMC.

Several studies have shown that NAPQI can also react with the protein thiol groups of cellular macromolecules (16, 17), reducing the protein thiol content and disturbing cellular Ca\textsuperscript{2+} homeostasis, leading to cell death (17). In an attempt to establish whether another mechanism was involved in the sparing action of SAMC on the hepatic GSH content, we investigated whether SAMC pretreatment affected the hepatic protein thiol content. Our results showed that the hepatic protein thiol content of mice pretreated with SAMC did not differ from that of vehicle-pretreated mice at the time (0 hr) of APAP administration (Table 1), suggesting that SAMC pretreatment did not affect the hepatic protein thiol content.

Several studies, including those of our group, have

Table 4. Effects of SAMC pretreatment on the hepatic CoQ\textsubscript{H}\textsubscript{2}, CoQ\textsubscript{10}H\textsubscript{2} and \(\alpha\)-Toc contents of mice 6 hr after APAP administration

<table>
<thead>
<tr>
<th>Group</th>
<th>CoQ\textsubscript{H}\textsubscript{2} (pmol/mg protein)</th>
<th>CoQ\textsubscript{10}H\textsubscript{2} (pmol/mg protein)</th>
<th>(\alpha)-Toc (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>213.8±22.2</td>
<td>8.5±0.9</td>
<td>78.3±7.8</td>
</tr>
<tr>
<td>APAP + Vehicle</td>
<td>144.8±25.3</td>
<td>7.7±2.0</td>
<td>69.8±4.0</td>
</tr>
<tr>
<td>+ SAMC</td>
<td>247.1±21.8*</td>
<td>8.7±0.4</td>
<td>73.6±3.4</td>
</tr>
</tbody>
</table>

SAMC (100 mg/kg, p.o.) was given 2 and 24 hr before APAP (500 mg/kg, p.o.). The vehicle group received 1% gum arabic solution (10 ml/kg). Mice in the normal group were given neither SAMC nor APAP. The data are expressed as means±S.E. of at least 6 animals. *Significantly different from the vehicle-treated group (P<0.05).
shown that oxidative stress followed by lipid peroxidation plays an important role in the pathogenesis of APAP-induced liver injury (29–31) and that pretreatment with the antioxidants, CoQ10 and α-Toc (31), and the antioxidant enzymes, superoxide dismutase and catalase (34), prevents APAP-induced liver injury. We found that SAMC suppressed hepatic lipid peroxidation (Table 3) and maintained the hepatic CoQ9H2 content at normal levels, even 6 hr after APAP administration (Table 4). In a previous study, we found that APAP-induced liver injury reduced the hepatic CoQ8H2 and CoQ10H2 contents, as a result of their function as antioxidants, scavenging the lipid peroxyl radicals generated by APAP administration (31). As SAMC is also known to exhibit antioxidant activity (7, 8), it may also function as an antioxidant in the livers of APAP-treated mice. Therefore, the antioxidant activity of SAMC may be another mechanism through which it protects the liver against APAP-induced injury.

In conclusion, we have shown, using the plasma ALT activity as an index of liver damage, that SAMC pretreatment can protect mice against APAP-induced liver injury in a dose-dependent manner. The mechanisms responsible for this hepatoprotection appear to be inhibition of P450 2E1 activity by SAMC and the antioxidant activity of SAMC.

REFERENCES

25 Harvison PJ, Guengerich FP, Rashed MS and Nelson SD: Cytochrome P-450 isozyme selectivity in the oxidation of
35 Hazelton GA, Hjelde JJ and Klaassen CD: Effects of butylated hydroxyanisole on acetaminophen hepatotoxicity and glu-
41 Mulder GJ and Van Doorn ABD: A rapid NAD⁺-linked assay for microsomal uridine diphosphate glucurononyltransferase of rat liver and some observations on substrate specificity of the enzyme. Biochem J 151, 131–140 (1975)