Free Radical Research, April 2007; 41(4): 489–497

informa
healthcare

S-allyl cysteine prevents CCl_4-induced acute liver injury in rats

SHINTARO KODAI[1], SHIGEKAZU TAKEMURA[1], YUKIKO MINAMIYAMA[1,2], SEIKAN HAI[1], SATOSHI YAMAMOTO[1], SHOJI KUBO[1], YASUKAZU YOSHIDA[3], ETSUO NIKI[3], SHIGERU OKADA[2], KAZUHIRO HIROHASHI[1], & SHIGEFUMI SUEHIRO[4]

[1]*Department of Hepato–Biliary–Pancreatic Surgery, Graduate School of Medicine, Osaka City University, Osaka, Japan,* [2]*Department of Anti-Aging Food Sciences, Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University, Okayama, Japan,* [3]*Human Stress Signal Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Osaka, Japan, and* [4]*Department of Cardiovascular Surgery, Graduate School of Medicine, Osaka City University, Osaka, Japan*

Accepted by Professor N. Taniguchi

(Received 23 October 2006)

Abstract
Aged garlic extract (AGE) possesses multiple biological activities. We evaluated the protective effect of *S*-allyl cysteine (SAC), one of the organosulfur compounds of AGE, against carbon tetrachloride (CCl_4)-induced acute liver injury in rats. SAC was administrated intraperitoneally (50–200 mg/kg). SAC significantly suppressed the increases of plasma ALT and LDH levels. SAC also attenuated histological liver damage. CCl_4 administration induced lipid peroxidation accompanied by increases in the plasma malondialdehyde and hepatic 4-hydroxy-2-nonenal levels, and SAC dose-dependently attenuated these increases. The hepatic total level of hydroxyoctadecadienoic acid (HODE), a new oxidative stress biomarker, was closely correlated with the amount of liver damage. These results suggest that SAC decreased CCl_4-induced liver injury by attenuation of oxidative stress, and may be a better therapeutic tool for chronic liver disease.

Keywords: *Aged garlic extract,* S-*allyl cysteine, reactive oxygen species, hydroxyoctadecadienoic acid (HODE)*

Abbreviations: *AGE, aged garlic extract; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCl_4, carbon tetrachloride; CCl_3, trichloromethyl radical; Cys,* L-*cysteine; GSH, glutathione; HNE, 4-hydroxy-2-nonenal; HODE, hydroxyoctadecadienoic acid; LDH, lactate dehydrogenase; LPO, lipid peroxide; MDA, malondialdehyde; NAC,* N-*acetyl cysteine; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SAC,* S-*allyl cysteine; SAMC,* S-*allylmercaptocysteine; SOD, superoxide dismutase; SSA, sulfosalicylic acid; 8-iso-$PGF_{2\alpha}$, 8-iso-prostaglandin $F_{2\alpha}$; 8-OHdG, 8-hydroxy-2-deoxyguanosine*

Introduction

There is no doubt that reactive oxygen species (ROS) play important roles in pathological changes of the liver, particularly in alcoholic and toxic liver diseases [1]. Furthermore, repeated acute inflammation is well known to lead to chronic hepatitis and liver cirrhosis. At the current time, clinical solutions are urgently required to address the high global prevalences of these hepatopathies, which are among the most serious diseases in humans.

Garlic has been used as a traditional medicine for centuries, and scientific studies have revealed that it can prevent thrombosis, prevent inflammation and inhibit cellular oxidative stress [2,3]. However, the effective molecules among garlic ingredients and their pharmacological actions have not been clearly elucidated. The composition of aged garlic extract (AGE) has

Correspondence: S. Kodai, Department of Hepato–Biliary–Pancreatic Surgery, Graduate School of Medicine, Osaka City University, 1-4-3 Asahimachi, Abeno, Osaka 545-8585, Japan. Tel: 81 6 6645 3841. Fax: 81 6 6646 6057. E-mail: s-kodai@med.osaka-cu.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760601118361

been reported [4], and the unstable and highly odorous compounds found in fresh garlic are known to be converted into more stable, odorless and water-soluble compounds, such as *S*-allyl cysteine (SAC), *S*-allylmercaptocysteine (SAMC) and others [2]. Some of these compounds, such as SAC [5–7] and SAMC [8,9], have antioxidant properties. It has been postulated that SAC may be one of the active compounds responsible for the protective effects of AGE observed in several experimental models associated with oxidative stress [3,5,7,10,11]. Pharmacokinetic studies of SAC, the most abundant organosulfur compound in AGE (6.1 ± 2.7 mg/g dry extract) [4], in animals have revealed that SAC is easily absorbed from the gastrointestinal tract and becomes distributed into the plasma, liver, kidneys, lungs and heart with a bioavailability of 98% in rats [12]. Furthermore, SAC has the following useful characteristics: (i) it is water-soluble; (ii) it is stable for up to 2 years [4]; (iii) it is odorless; (iv) it is 30-fold less toxic than allicin and diallyl disulfide [2]; and (v) it is relatively inexpensive, since its synthesis is easy and cheap.

In vitro, SAC is able to scavenge $O_2^{\cdot-}$ and $HO^{\cdot}$ [5]. SAC also prevents H_2O_2-induced endothelial cell injury and lipid peroxidation [10], as well as low-density lipoprotein oxidation [13]. In addition, TNF-α and H_2O_2 can induce high levels of NF-κB activation in human T lymphocytes, and SAC can inhibit this NF-κB activation [14]. There are several reports regarding the biological effects of SAC in different organs. For example, SAC reduces ischemic brain edema in rats by inhibiting lipid peroxidation [7,11], and reduces histological damage to the heart and liver in mice treated with doxorubicin, an anticancer drug [15]. Recently, we reported that SAC attenuates carbon tetrachloride (CCl_4)-induced pulmonary fibrosis in rats (in press) [15].

Reduced glutathione (GSH) plays important roles in the protective effect against antioxidative injury, and both *N*-acetyl cysteine (NAC) and L-cysteine (Cys) have been reported to act as glutathione prodrugs. Although hepatoprotective properties of NAC have been reported in several previous studies [9,16,17], the effects of SAC in the rat liver have not yet been investigated.

The present study aimed to examine the effects of oral administration of SAC on CCl_4-induced liver injury in comparison with the effects of NAC and Cys.

Materials and methods

Chemicals

SAC ($CH_2{=}CH{-}CH_2{-}S{-}CH_2{-}CHNH_2{-}COOH$) was kindly provided by Wakunaga Pharmaceutical Co. (Osaka, Japan). CCl_4, L-012 and GSH were purchased from Wako Pure Chemical Co. (Osaka, Japan). NAC, Cys, phorbol 12-myristate 13-acetate (PMA) and Cu/Zn-superoxide dismutase (SOD) were purchased from Sigma Chemical Co. (St Louis, MO). All other reagents used were of analytical grade.

Animal treatment

Male Wistar rats (8W) weighing 180–200 g were purchased from SLC (Shizuoka, Japan) and maintained for 1 week before experimental use. All rats had free access to tap water and a commercial rodent diet (CE-2; CLEA Japan Inc., Tokyo, Japan), and were maintained on a 12-h light/dark cycle at a constant temperature of 25°C. All rats were taken care of according to the specifications outlined in the Guiding Principles for the Care and Use of Laboratory Animals approved by the local ethics committee on experimental animal research.

CCl_4 was applied to the rats *via* intraperitoneal (i.p.) injection as a 25% solution in corn oil (2 ml/kg). Intragastric (i.g.) administration of SAC, NAC or Cys was performed at 30 min before the CCl_4 injection. The animals were randomly divided into five groups as follows: (1) sham group, treated with i.g. water and i.p. corn oil; (2) vehicle group, treated with i.g. water and i.p. CCl_4; (3) SAC group, treated with i.g. SAC (25, 50, 100 or 200 mg/kg) and i.p. CCl_4; (4) NAC group, treated with i.g. NAC (200 or 600 mg/kg) and i.p. CCl_4; and (5) Cys group, treated with i.g. Cys (200 or 600 mg/kg) and i.g. CCl_4. The animals were sacrificed under urethane anesthesia (5 g/kg, i.p.) at 24 h after the CCl_4 treatment. Following collection of blood from the abdominal aorta, each rat was perfused with ice-cold saline and the liver was removed. Part of the left lobe was excised, fixed in 10% buffered-formaldehyde solution and embedded in paraffin blocks, while the remaining liver was immediately frozen under liquid nitrogen and stored until use.

Biochemical analysis of liver enzymes

Blood was centrifuged at 12,000*g* for 15 min at 4°C, and the plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were determined by an automated analysis system.

Histological analysis

Liver tissue was processed using a paraffin slice technique and 4-μm liver sections were stained with hematoxylin and eosin.

Measurement of lipid peroxide (LPO) levels

The LPO levels in the liver homogenates were measured using an ELISA kit (LPO-586™; OXIS International Inc., Portland, OR). In general, frozen liver tissue (about 200 mg) was homogenized in 0.4 ml

of 20 mM phosphate buffer, pH 7.4. However, when LPO was investigated as a marker of cellular injury, the same amount of frozen liver tissue was homogenized in 20 mM phosphate buffer (pH 7.4) containing 0.5 M butylated hydroxytoluene. Each homogenate was centrifuged at 3000*g* for 10 min at 4°C to remove any large particles. An aliquot of each sample was removed for protein determination, while the remainder of the sample was used for measurement of the malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) levels with the BIOXYTECH® LPO-586™ assay kit (OXIS International Inc.) according to the manufacturer's instructions. This assay is based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole, with MDA and 4-hydroxyalkenals at 45°C, resulting in a stable chromophore with a maximal absorbance at 586 nm.

Western blot analysis of liver tissues

Frozen liver tissue (about 200 mg) was homogenized in 0.4 ml of sample buffer (50 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate, 10 mM dithiothreitol, 10% glycerol and 1 mM phenylmethylsulfonyl fluoride). Proteins (20 μg) of the soluble and particulate fractions were separated by 12.5% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (MILLIPORE Co., Billerica, MA). Next, the membrane was blocked with 3% BSA and incubated with a mouse anti-HNEJ-2 monoclonal antibody (3 μg/ml; Japan Institute for The Control of Aging, Shizuoka, Japan) at 4°C overnight. Following three washes in Tris–buffered saline (pH 7.4) containing 3% skim milk, the membrane was incubated with a secondary anti-mouse IgG (1:2000; Dako Cytomation, Kyoto, Japan) for 40 min at room temperature, and then washed three times in Tris–buffered saline (pH 7.4) containing 0.1% Tween. Reactive bands were identified by enhanced chemiluminescence (Amersham, Bucks, UK) and autoradiography following exposure to X-ray film, according to the manufacturer's instructions. Finally, the western blots were quantified using Scion Image software (version 1.63; Scion Corp., Frederick, MD)

Analyses of total hydroxyoctadecadienoic acid (tHODE) and 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) in the plasma and liver

The levels of tHODE and 8-iso-$PGF_{2\alpha}$ were measured as described previously [18], with slight modifications. Briefly, internal standards of 8-iso-$PGF_{2\alpha}$-d_4 (100 ng) and 9-HODE-d_4 (100 ng) and 1 ml of methanol were added to the plasma samples (2.0 ml), followed by reduction with an excess amount of sodium borohydride at room temperature for 5 min under nitrogen. The liver was homogenized in saline (liver:saline = 1:3, w/w) using a Polytron PT-3100 (KINEMATICA AG, luzern, Switzerland), and an aliquot (300 μl) was further diluted with saline (1700 μl). The internal standards and 1 ml of methanol were added to this solution followed by reduction as described above. Subsequently, the reduced sample was mixed with 1 M KOH in methanol (1 ml) under nitrogen and incubated in a shaker at 40°C for 30 min in the dark. Following centrifugation at 3000*g* at 4°C for 10 min, the supernatant was diluted with a 4-fold volume of water (pH 3) and acidified (pH 3) using 2 N HCl. The acidified sample was centrifuged at 3000*g* at 4°C for 10 min, and the supernatant was subjected to solid-phase extraction [18]. After evaporation of the eluted solution with nitrogen gas, 30 μl of a silylating agent (*N*,*O bis*), trimethylsilyl trifluoroacetamide (BSTFA), was added to the dried residue, vigorously mixed using a vortex mixer for 1 min and incubated at 60°C for 60 min to obtain trimethylsilyl esters and ethers. Next, the solution was diluted with 70 μl of acetone and an aliquot of this sample was injected into a gas chromatography system (GC 6890 N; Agilent Technologies Co. Ltd, Palo Alto, CA) equipped with a quadrupole mass spectrometer (5973 Network; Agilent Technologies Co. Ltd). A fused-silica capillary column (HP-5MS, 5% phenyl methyl siloxane, 30 m × 0.25 mm; Agilent Technologies Co. Ltd) was used. Helium was used as the carrier gas at a flow rate of 1.2 ml/min, and the temperature was programmed to increase from 60 to 280°C at 10°C/min. The injector temperature was set at 250°C and the temperatures of the transfer lines to the mass detector and ion source were 250 and 230°C, respectively. The electron energy was set at 70 eV. The amounts of 8-iso-$PGF_{2\alpha}$ and tHODE were determined using fragment ions of m/z 481 and 440, respectively. The internal standards used for the quantification of 8-iso-$PGF_{2\alpha}$ and tHODE were 8-iso-$PGF_{2\alpha}$-d_4 ($m/z = 485$) and 9-HODE-d_4 ($m/z = 444$), respectively.

Total thiol assay

Excised liver tissues were homogenized immediately in 4 volumes of ice-cold 5% sulfosalicylic acid (SSA) solution, while plasma samples were mixed with 0.5 volumes of ice-cold 10% SSA solution. All the samples were centrifuged at 12,000*g* for 15 min, and the total thiol concentrations in the acid-soluble fractions were determined using Ellman's reagent [19].

Analyses of lipid peroxidation induced by enzymatic reduction of CCl_4

Hepatic microsomes were prepared as described previously [20]. Briefly, tissues (about 4 g) were placed in 1.15% KCl and homogenized at 4°C in a Polytron homogenizer for 10 s, and then in a Teflon

homogenizer for 12 strokes. The homogenate was centrifuged at 9000*g* for 20 min. The supernatant fraction was centrifuged further at 105,000*g* for 40 min. The microsomal fraction was resuspended in a 50 mM sodium phosphate buffer solution (pH 7.4) containing 1 mM EDTA and 20% glycerol. All centrifugation was performed at 4°C. Microsomal incubations (1 mg protein/ml) were performed in 1 ml of 0.1 M potassium phosphate buffer, pH: 7.4, in the presence of 0.1 mM EDTA and a NADPH generating system containing 1 mM NADP, 10 mM glucose-6-phosphate and 2 units of glucose-6-phosphate dehydrogenase. Next, the microsomes pre-incubated with SAC (0.1, 0.5, or 1 mM), NAC (0.1, 0.5, or 1 mM), or Cys (0.1, 0.5, or 1 mM) for 5 min at 37°C, before the reaction was started by adding CCl_4. Peroxidation was started by addition of 5 μl of CCl_4 1 M in ethanol [21]. After 10 min incubation at 37°C MDA and HNE levels were measured as described above with the BIOXYTECH® LPO-586™ assay kit (OXIS International Inc.).

Detection of ROS generation by neutrophils isolated from human blood

Heparinized venous blood was obtained from normal healthy subjects. The anti-coagulated blood was purified to only contain neutrophils by using Mono-Poly Resolving Medium (Dainippon Pharmaceutical Co., Osaka, Japan). Next, the isolated neutrophils (5 μl; 1×10^8 cells/ml) were incubated in 980 μl of Krebs–Ringer phosphate buffer in the presence of 100 μM L-012, and then pre-incubated with NAC (10^{-6}–10^{-3} mol/l), SAC (10^{-6}–10^{-3} mol/l) or Cys (10^{-6}–10^{-3} mol/l) for 5 min [8] at 37°C, before the reaction was started by adding 5 nM PMA. The chemiluminescence intensity was recorded continuously for 1–10 min using a Luminescence Reader (BLR-201; Aloka, Tokyo, Japan). The intensity was completely abolished following the addition of SOD (5 μl; 10,000 U/ml).

Statistical analysis

Data are expressed as means ± SEM. Statistical analyses were performed by analysis of variance (ANOVA) followed by appropriate *post hoc* tests including Bonferroni correction with p-value < 0.05 being considered as statistically significant.

Results

Biochemical analyses of CCl_4-induced liver injury

The plasma ALT and LDH levels are shown in Figure 1A,B, respectively. The ALT and LDH levels were both significantly higher in the CCl_4-treated group than in the sham group. The increased levels were significantly attenuated by SAC or NAC in a dose-dependent manner, whereas Cys treatment had little effect, even at a high dose. The changes in the AST level showed the same pattern as those in the LDH level (data not shown). SAC showed the most potent attenuation of CCl_4-induced liver injury.

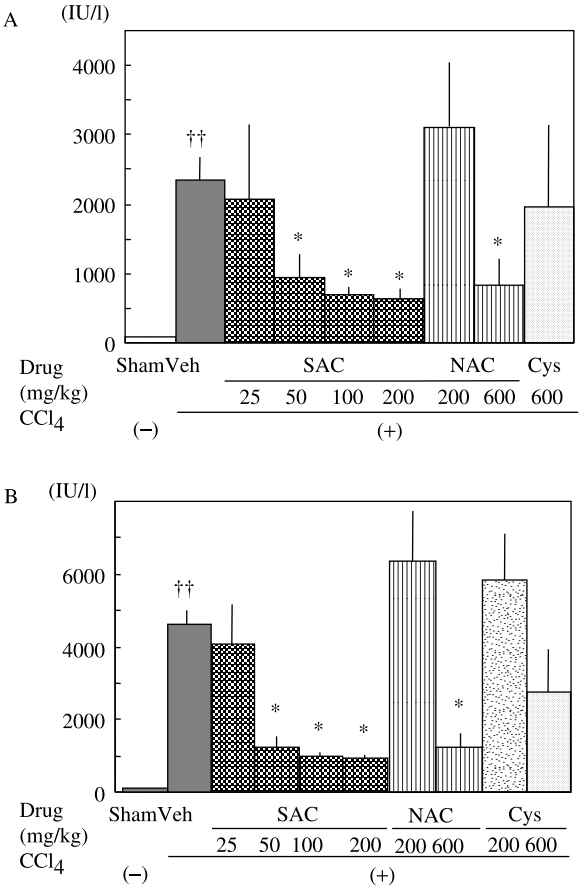

Figure 1. Effects of cysteine compounds on the plasma ALT and LDH levels following CCl_4 administration. At 30 min after treatment with SAC or the indicated compounds, the rats received an i.p. injection of 25% CCl_4 solution in corn oil (2 ml/kg). At 24 h after the CCl_4 treatment, the animals were sacrificed, and assays were performed as described in the Materials and Methods. (A) Plasma ALT levels. (B) Plasma LDH levels. Data are presented as the means ± SEM of at least four animals. ††, $p < 0.01$ versus the sham group. ⋆, $p < 0.05$ versus the vehicle group.

Histological findings in the liver

CCl_4 administration induced centrilobular cytoplasmic vacuolation, hepatic cell necrosis and inflammatory cell inflammation (Figure 2A). Both SAC (200 mg/kg; Figure 2B) and NAC (600 mg/kg; Figure 2C) attenuated the development of hepatic injury. Cys (600 mg/kg; Figure 2D) slightly inhibited the CCl_4-induced hepatic cell necrosis.

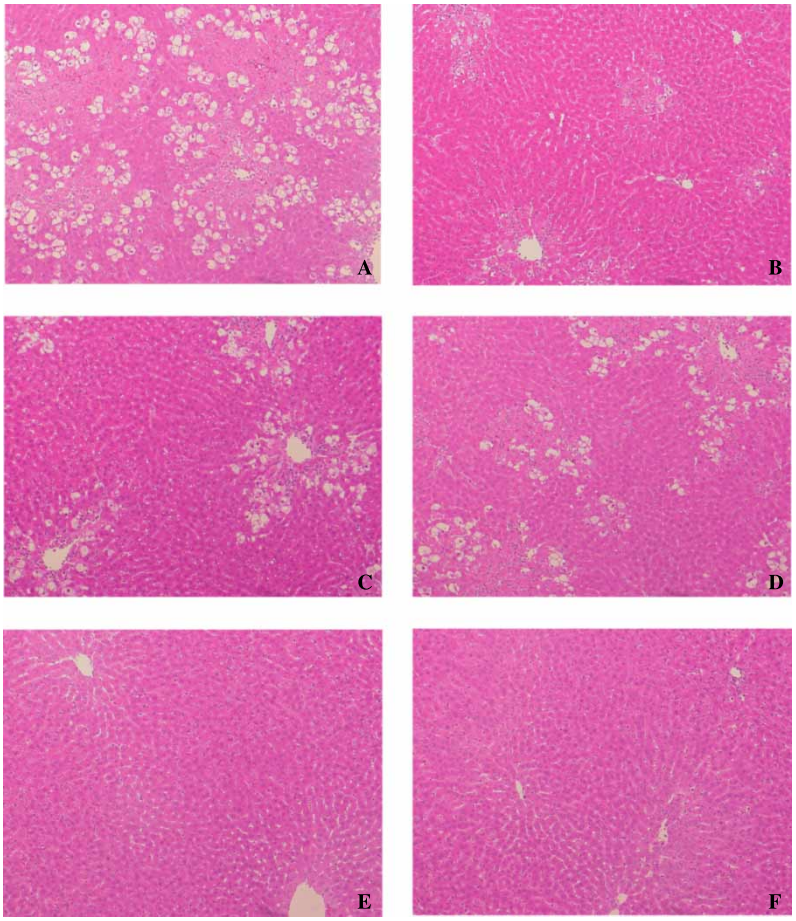

Figure 2. Representative pictures of hematoxylin and eosin staining of the liver at 24 h after CCl_4 administration. The animals were treated as described in the legend for Figure 1. At 24 h after the CCl_4 treatment, the animals were sacrificed and the right lobe was fixed and embedded in paraffin. (A) Vehicle. (B) SAC (200 mg/kg). (C) NAC (600 mg/kg). (D) Cys (600 mg/kg). (E) Vehicle treated without CCl_4. (F) untreated control.

Evaluation of oxidative stress induced by CCl_4

The hepatic LPO level was significantly higher in the vehicle group than in the sham group (Figure 3). SAC (200 mg/kg), NAC (600 mg/kg) and Cys (600 mg/kg) significantly suppressed the increased level.

CCl_4 administration significantly increased the expression of many HNE-modified proteins (28, 46, 50 kDa etc.) in the liver. The expression levels of representative HNE-modified protein (28 kDa) are shown in Figure 4. SAC (200 mg/kg) and Cys (600 mg/kg) significantly reduced the increased level, whereas NAC (600 mg/kg) had no effect.

The CCl_4-induced increases in the tHODE level in the liver are shown in Figure 5. SAC (200 mg/kg) and NAC (600 mg/kg) attenuated these increases that were well correlated with the extent of the liver injury.

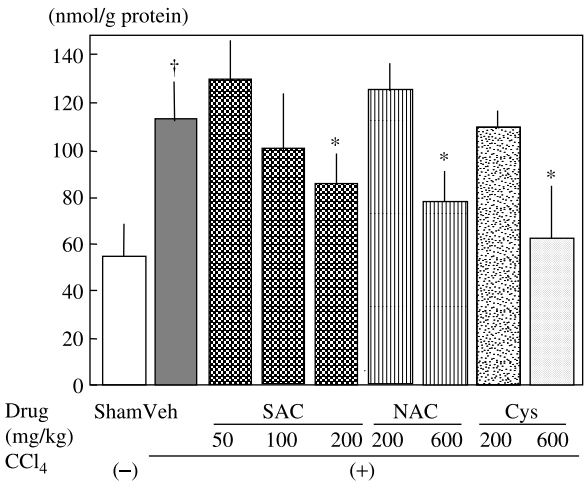

Figure 3. Effects of cysteine compounds on hepatic LPO production following CCl_4 administration. The hepatic LPO levels were measured as described in the Materials and Methods. Data are presented as the means ± SEM of 5–6 animals. †, $p < 0.05$ versus the sham group. ⋆, $p < 0.05$ versus the vehicle group.

Effects of cysteine compounds on hepatic and plasma thiol levels

The hepatic and plasma thiol levels at 30 min after administration of each drug are shown in Table I. SAC

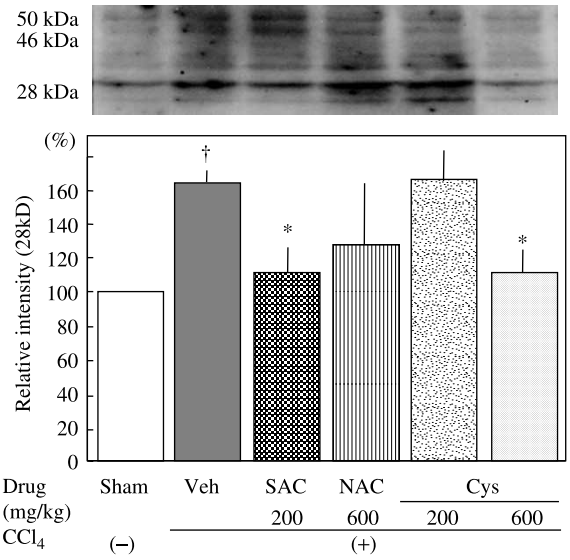

Figure 4. Effects of cysteine compounds on HNE-modified protein adducts following CCl_4 administration. The expression levels of HNE-modified protein in the liver after CCl_4 administration were analyzed by western blotting. The intensities of the 28 kDa protein bands were then plotted relative to the corresponding band intensity in the sham group. Data are presented as the means ± SEM of 5–6 animals. †, $p < 0.05$ versus the sham group. ⋆, $p < 0.05$ versus the vehicle group.

had no effect on either the hepatic or plasma thiol level. In contrast, NAC significantly increased both the plasma and hepatic thiol levels and Cys significantly increased the plasma thiol level. These increased levels returned to their normal levels after 24 h. As shown in Figure 6, the hepatic thiol levels slightly increased in SAC, NAC and Cys treatment.

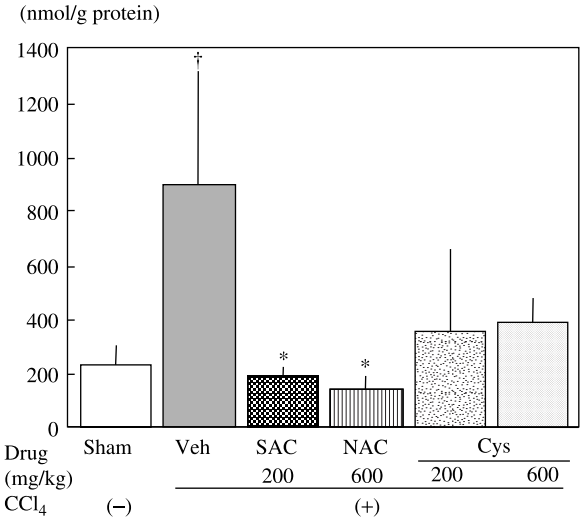

Figure 5. Effects of cysteine compounds on tHODE levels following CCl_4 administration. The hepatic tHODE levels were measured as described in the Materials and Methods. Data are presented as the means ± SEM of 5–6 animals. †, $p < 0.05$ versus the sham group. ⋆, $p < 0.05$ versus the vehicle group.

These levels were slightly decreased at 24 h after CCl_4 administration in all groups. Cys compounds did not affect these decreases induced by CCl_4. The plasma thiol level was decreased in the control group, and did not affect following administration of the Cys compounds.

Effects of cysteine compounds on $\cdot CCl_3$ radical-induced lipid peroxidation of hepatic microsomes

To examine the direct detoxifications of CCl_4-generated radicals or/and alters the metabolic activation of CCl_4 *in vivo*, we measured ?CCl_3 radical-induced lipid peroxidation of hepatic microsomes. As shown in Figure 7, addition of all Cys compounds had no effect on time course of lipid peroxidation induced by CCl_4.

ROS generation from isolated human neutrophils stimulated by PMA

To examine the direct inhibition by each drug *in vitro*, we measured PMA-stimulated ROS production by isolated human neutrophils using L-012 chemiluminescence. NAC and SAC each inhibited ROS production, whereas Cys had no effect. The IC_{50} (concentration for 50% inhibition) values for NAC and SAC were 2×10^{-4} and 2×10^{-3} M, respectively. The chemiluminescence intensity was completely abolished by SOD addition (Figure 8).

Discussion

The results of the present study have demonstrated that SAC is more effective than other Cys compounds for attenuating CCl_4-induced liver damage. Three possible mechanisms for this phenomenon may be considered as follows: (1) SAC directly detoxifies CCl_4-generated radicals or/and alters the metabolic activation of CCl_4; (2) SAC scavenges ROS produced by secondary infiltrating inflammatory cells; and (3) SAC acts as a glutathione prodrug. These three possible mechanisms are discussed in more detail below.

Regarding the first possible mechanism, CCl_4 is known to induce hepatic injury *via* reactive metabolites, such as the trichloromethyl radical ($\cdot CCl_3$), in the liver microsomal P-450 system [22]. The effect of the pre-treatment with these compounds on the generation of $\cdot CCl_3$ from CCl_4 *in vivo* has been unclear. We clarified that none of our Cys compounds (~1 mM) inhibited $\cdot CCl_3$ radical-induced lipid peroxidation of hepatic microsomes. However, the production of ROS derived from $\cdot CCl_3$ must be taken into consideration *in vivo*.

Regarding the second possible mechanism, SAC is known to be capable of scavenging O_2^- [5], $HO\cdot$ [5] and H_2O_2 [3,10]. SAC was able to scavenge ROS

Table I. Hepatic and plasma thiol levels at 30 min after each drug administration.

Group	Sham	SAC 200 mg/kg	NAC 600 mg/kg	Cys 600 mg/kg
Liver (mmol/g)	7.15 ± 0.22	6.39 ± 0.77	8.47 ± 0.44*	7.70 ± 0.33
Plasma (mM)	19.5 ± 2.46	22.0 ± 2.31	111.1 ± 32.8*	33.6 ± 1.3*

At 30 min after oral administration of the cysteine compounds, the animals were sacrificed, and assays were performed as described in the Materials and Methods. Data represent the means ± SEM of at least four animals. *$p < 0.05$ versus the sham group.

generated from PMA-stimulated polymorphonuclear cell with one-tenth the potency of NAC, suggesting that the inhibitory effect of NAC on neutrophil-generated ROS production may be stronger than that of SAC *in vitro*. However, in the present study, SAC was more effective than NAC for attenuating liver injury (evaluated by histology, as well as the AST, ALT and LDH levels) and lipid peroxidation (evaluated by the LPO, HNE and tHODE levels) *in vivo*. Therefore, SAC may act *via* other mechanisms in addition to ROS inhibition. Additionally, SAC/SAC metabolites *in vivo* may work as antioxidant.

Regarding the third possible mechanism, it has been demonstrated that GSH plays important roles in the detoxification of reactive metabolites of CCl_4, and that liver necrosis begins when the GSH stores are almost exhausted [23]. Both NAC and Cys have been reported to act as Cys prodrugs and to protect against acetaminophen-induced liver injury [16,17,24,25]. In fact, NAC and Cys was found to significantly increase

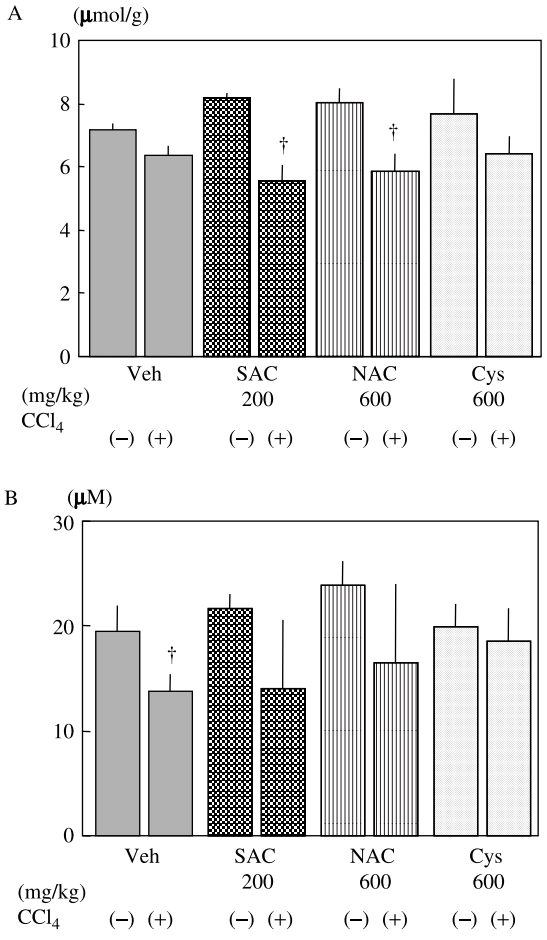

Figure 6. Effects of cysteine compounds on the hepatic and plasma thiol status. Cysteine compounds were orally administered at 30 min before treatment with CCl_4 (2 ml/kg, i.p.). At 24 h after the CCl_4 administration, the animals were sacrificed, and assays were performed as described in the Materials and Methods. (A) Hepatic thiol status. (B) Plasma thiol status. Data represent the means ± SEM of at least four animals. †, $p < 0.05$ versus each compound without CCl_4 treatment.

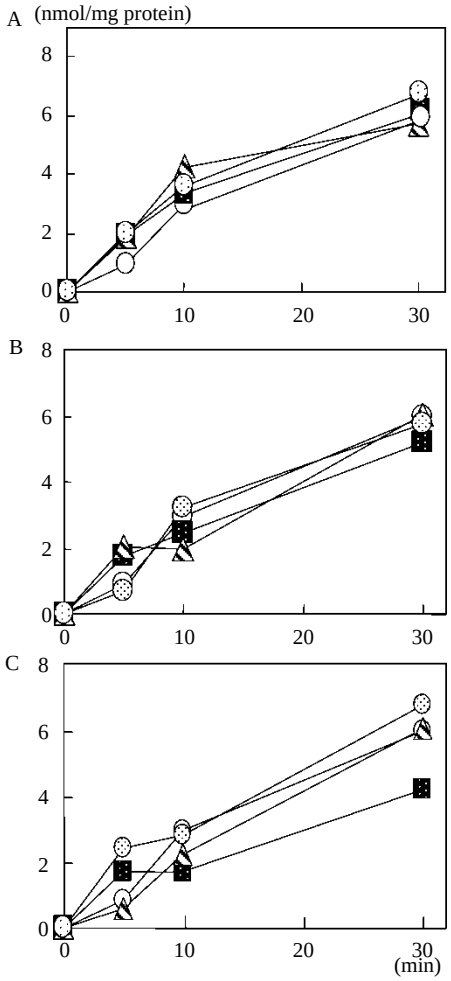

Figure 7. Time course of lipid peroxidation in microsomes pretreated with SAC (A), NAC (B) and Cys (C). The additions made were: None (open circle), 0.1 mM (square), 0.5 mM (triangle), and 1 mM (closed circle). All reactions were started with the addition of CCl_4.

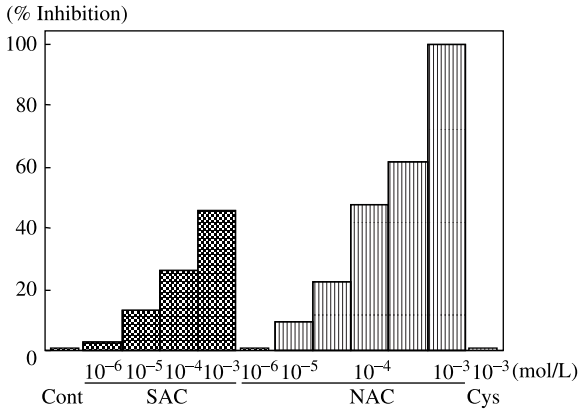

Figure 8. ROS generation by PMA-stumulated human polymorphonuclear cell (PMA). The generation of ROS in PMA was assayed to examine the ability to scavenge ROS. Cells were collected and incubated as described in Materials and Methods, then pre-incubated with NAC (10^{-6}–10^{-3} mol/l), SAC (10^{-6}–10^{-3} mol/l) or Cys (10^{-6}–10^{-3} mol/l) for 5 min at 37°C, before the reaction was started. Reaction was started by adding 5 nM PMA, and chemiluminescence intensity using an L-012 probe was recorded continuously using a Luminescence Reader. The chemiluminescence intensity was recorded continuously for 1–10 min using a Luminescence Reader (BLR-201; Aloka, Tokyo, Japan). The intensity was completely abolished following the addition of SOD (5 μl; 10,000 U/ml).

the hepatic and plasma GSH levels at 30 min after the treatment in the present study. Furthermore, although the thiol levels were decreased at 24 h after CCl_4 treatment in all groups, these levels did not differ significantly from those in the vehicle group. Meanwhile, a previous study [10] reported that SAC (0.1–10 mM) recovered oxidized LDL (0.1 g/l)-induced GSH depletion in bovine pulmonary arterial endothelial cells. In the present study, the concentration of SAC is unlikely to exceed 10 mM, even if all the administered SAC is present in the bloodstream. Furthermore, since SAC possesses part of the Cys structure but not the SH residue, it may be reasonable that the plasma and hepatic thiol levels did not increase. In other words, it is unlikely that SAC exerts its protective power by supplying its Cys component to the liver.

SAC and SAC sulfoxide (SACS) may become *N*-acetylated to form *N*-acetyl-*S*-allyl-cysteine (NSAC) and *N*-acetyl-*S*-allyl-cysteine sulfoxide (NSACS), respectively, since NSACS has been detected in the urine of rats, mice and dogs [12,26] as well as in the urine of humans who consumed garlic [27]. In contrast, SAC is a much better substrate for flavin-containing monooxygenases than its corresponding mercapturic acid [28]. The metabolism of SAC has not yet been investigated in detail. However, a recent report pointed out that a number of garlic-derived Cys *S*-conjugates (including SAC) are beta-lyase substrates of liver gamma-cystathionase [29]. It is considered that the eliminated sulfur-containing fragment may interact with protein thiols, thereby altering the redox state of these proteins. Therefore, the metabolites of SAC may inhibit liver injury, and further investigations are required to elucidate the true target metabolites of SAC.

We evaluated the lipid peroxidation using the new biomarker tHODE [18]. The liver tHODE levels were dramatically decreased following administration of SAC and NAC compared to vehicle administration in CCl_4-induced liver injury. These changes in tHODE induced by SAC and NAC were well correlated with plasma ALT and LDH, and were better than the prevailing markers of lipid peroxidation, LPO and HNE. Therefore, tHODE may be a promising biomarker for assessing oxidative stress and evaluating antioxidant capacities *in vivo*.

In conclusion, the results of the present study have demonstrated that SAC can inhibit the experimental liver injury induced by CCl_4. Although the molecular masses and constitutional formulas of SAC and NAC are similar, they show different protective potencies against liver injury. Specifically, SAC (200 mg/kg) had the equivalent efficacy to NAC (600 mg/kg) for attenuating CCl_4-induced liver injury. SAC (200 mg/kg) also showed potent effects, whereas NAC did not at same dose. Furthermore, SAC may be a better therapeutic tool for long-term use to ameliorate chronic inflammatory diseases such as liver fibrosis, due to its 30-fold lower toxicity than other garlic components [2].

Acknowledgements

We gratefully thank Ms F. Kobayashi (Osaka City University Medical School) and Ms Y. Nishikawa (Okayama University) for their excellent technical assistance.

References

[1] Poli G, Parola M. Oxidative damage and fibrogenesis. Free Radic Biol Med 1997;22:287–305.
[2] Amagase H, Petesch BL, Matsuura H, Kasuga S, Itakura Y. Intake of garlic and its bioactive components. J Nutr 2001;131: 955S–962S.
[3] Ide N, Lau BH. Garlic compounds protect vascular endothelial cells from oxidized low density lipoprotein-induced injury. J Pharm Pharmacol 1997;49:908–911.
[4] Lawson LD. The composition and chemistry of garlic cloves and processed garlic. In: Koch HP, Lawson LD, editors. Garlic. The science and therapeutic application of *Allium sativum* L. and related species. Baltimore, MD: Williams & Wilkins; 1996. p 37–107.
[5] Kim KM, Chun SB, Koo MS, Choi WJ, Kim TW, Kwon YG, Chung HT, Billiar TR, Kim YM. Differential regulation of NO availability from macrophages and endothelial cells by the garlic component *S*-allyl cysteine. Free Radic Biol Med 2001; 30:747–756.
[6] Numagami Y, Ezura M, Takahashi A, Yoshimoto T. Antegrade recanalization of completely embolized internal carotid artery after treatment of a giant intracavernous aneurysm: A case report. Surg Neurol 1999;52:611–616.

[7] Numagami Y, Ohnishi ST. *S*-allylcysteine inhibits free radical production, lipid peroxidation and neuronal damage in rat brain ischemia. J Nutr 2001;131:1100S–1105S.
[8] Imada I, Sato EF, Miyamoto M, Ichimori Y, Minamiyama Y, Konaka R, Inoue M. Analysis of reactive oxygen species generated by neutrophils using a chemiluminescence probe L-012. Anal Biochem 1999;271:53–58.
[9] Sumioka I, Matsura T, Kasuga S, Itakura Y, Yamada K. Mechanisms of protection by *S*-allylmercaptocysteine against acetaminophen-induced liver injury in mice. Jpn J Pharmacol 1998;78:199–207.
[10] Ide N, Lau BH. Garlic compounds minimize intracellular oxidative stress and inhibit nuclear factor-kappa b activation. J Nutr 2001;131:1020S–1026S.
[11] Numagami Y, Sato S, Ohnishi ST. Attenuation of rat ischemic brain damage by aged garlic extracts: A possible protecting mechanism as antioxidants. Neurochem Int 1996;29: 135–143.
[12] Nagae S, Ushijima M, Hatono S, Imai J, Kasuga S, Matsuura H, Itakura Y, Higashi Y. Pharmacokinetics of the garlic compound *S*-allylcysteine. Planta Med 1994;60:214–217.
[13] Huang CN, Horng JS, Yin MC. Antioxidative and anti-glycative effects of six organosulfur compounds in low-density lipoprotein and plasma. J Agric Food Chem 2004;52: 3674–3678.
[14] Geng Z, Rong Y, Lau BH. *S*-allyl cysteine inhibits activation of nuclear factor kappa B in human T cells. Free Radic Biol Med 1997;23:345–350.
[15] Mostafa MG, Mima T, Ohnishi ST, Mori K. *S*-allylcysteine ameliorates doxorubicin toxicity in the heart and liver in mice. Planta Med 2000;66:148–151.
[16] Corcoran GB, Wong BK. Role of glutathione in prevention of acetaminophen-induced hepatotoxicity by *N*-acetyl-L-cysteine *in vivo*: Studies with *N*-acetyl-D-cysteine in mice. J Pharmacol Exp Ther 1986;238:54–61.
[17] Dobrzynska I, Skrzydlewska E, Kasacka I, Figaszewski Z. Protective effect of *N*-acetylcysteine on rat liver cell membrane during methanol intoxication. J Pharm Pharmacol 2000;52: 547–552.
[18] Yoshida Y, Niki E. Detection of lipid peroxidation *in vivo*: Total hydroxyoctadecadienoic acid and 7-hydroxycholesterol as oxidative stress marker. Free Radic Res 2004;38:787–794.
[19] Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70–77.
[20] Funae Y, Imaoka S. Simultaneous purification of multiple forms of rat liver microsomal cytochrome P-450 by high-performance liquid chromatography. Biochim Biophys Acta 1985;842:119–132.
[21] Mansuy D, Sassi A, Dansette PM, Plat M. A new potent inhibitor of lipid peroxidation *in vitro* and *in vivo*, the hepatoprotective drug anisyldithiolthione. Biochem Biophys Res Commun 1986;135:1015–1021.
[22] Recknagel RO, Ghoshal AK. Quantitative estimation of peroxidative degeneration of rat liver microsomal and mitochondrial lipids after carbon tetrachloride poisoning. Exp Mol Pathol 1966;5:413–426.
[23] Mitchell JR, Jollow DJ, Potter WZ, Gillette JR, Brodie BB. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. J Pharmacol Exp Ther 1973;187:211–217.
[24] Hazelton GA, Hjelle JJ, Klaassen CD. Effects of cysteine pro-drugs on acetaminophen-induced hepatotoxicity. J Pharmacol Exp Ther 1986;237:341–349.
[25] Miners JO, Drew R, Birkett DJ. Mechanism of action of paracetamol protective agents in mice *in vivo*. Biochem Pharmacol 1984;33:2995–3000.
[26] Kaye CM, Clapp JJ, Young L. The metabolic formation of mercapturic acids from allyl halides. Xenobiotica 1972;2: 129–139.
[27] de Rooij BM, Boogaard PJ, Rijksen DA, Commandeur JN, Vermeulen NP. Urinary excretion of *N*-acetyl-*S*-allyl-L-cysteine upon garlic consumption by human volunteers. Arch Toxicol 1996;70:635–639.
[28] Krause RJ, Glocke SC, Elfarra AA. Sulfoxides as urinary metabolites of *S*-allyl-L-cysteine in rats: Evidence for the involvement of flavin-containing monooxygenases. Drug Metab Dispos 2002;30:1137–1142.
[29] Cooper AJ, Pinto JT. Aminotransferase, L-amino acid oxidase and beta-lyase reactions involving L-cysteine *S*-conjugates found in allium extracts. Relevance to biological activity? Biochem Pharmacol 2005;69:209–220.