

# Alterations in Sulfur Amino Acid Metabolism in Mice Treated with Silymarin: A Novel Mechanism of Its Action Involved in Enhancement of the Antioxidant Defense in Liver

## Authors

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## Key words

- *Silybum marianum*
- Asteraceae
- silymarin
- glutathione
- sulfur amino acid metabolism
- antioxidant

## Abstract

▼ It has been known that silymarin exhibits protective activity against oxidative liver injury induced by various hepatotoxicants, but the underlying mechanism of its beneficial action remains unclear. We determined the alterations in sulfur-containing amino acid metabolism induced by silymarin in association with its effects on the antioxidant capacity of liver. Male mice were treated with silymarin (100 or 200 mg/kg, p.o.) every 12 h for a total of 3 doses, and sacrificed 6 h after the final dosing. The hepatic methionine level was increased, but the activity and protein expression of methionine adenosyltransferase were decreased by silymarin in a dose-dependent manner. S-Adenosylmethionine or homocysteine concentration was not changed, whereas the sulfur-containing metabolites generated from homocysteine in the transsulfuration pathway including cystathionine, cysteine, and glutathione were increased significantly. Cystathionine  $\beta$ -synthase was induced, but cysteine dioxygenase was downregulated, both of which would contribute to the elevation of cysteine and its product, glutathione, in liver. Oxygen radical scavenging capacity of liver cytosol against peroxyl radical and peroxynitrite was increased, and also hepatic lipid peroxidation was diminished in the silymarin-

treated mice. Taken together, the results demonstrate that silymarin enhances hepatic glutathione generation by elevating cysteine availability via an increment in cysteine synthesis and an inhibition of its catabolism to taurine, which may subsequently contribute to the antioxidant defense of liver.

## Abbreviations

▼	BHMT:	betaine-homocysteine methyltransferase
	C $\beta$ S:	cystathionine $\beta$ -synthase
	CDC:	cysteine sulfinatase decarboxylase
	CDO:	cysteine dioxygenase
	CyL:	cystathionine $\gamma$ -lyase
	GCS:	$\gamma$ -glutamylcysteine synthetase
	GSH:	glutathione
	GSSG:	oxidized form of GSH
	KMBA:	$\alpha$ -keto- $\gamma$ -methylbutyric acid
	MAT:	methionine adenosyltransferase
	MS:	methionine synthase
	SAH:	S-adenosylhomocysteine
	SAM:	S-adenosylmethionine
	TBARS:	thiobarbituric acid reactive substance
	TOSC:	total oxyradical scavenging capacity

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## Bibliography

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## Introduction

▼ Silymarin, a mixture of four flavonolignan isomers (silybin, isosilybin, silydianin, silychristin), extracted from the seed of milk thistle (*Silybum marianum*; Asteraceae) has been used for the treatment of liver injury in traditional medicine. Numerous studies have shown that silymarin protects liver cells from various hepatotoxicants such as ethanol, diethylnitrosamine, and carbon tetrachloride [1–3]. Generation of oxidative stress

is accepted as the underlying mechanism of liver injury induced by these toxic substances, and, accordingly, the hepatoprotective effects of silymarin are frequently attributed to its antioxidant activity. In fact, silymarin was shown to inhibit lipid peroxide formation induced by various toxicants in hepatic mitochondria and microsomes [4]. Scavenging free radicals and chelating iron by the phenolic hydroxyl group of silymarin are suggested to be the key components of its antioxidant activity. It has been shown that silybin, a ma-

	Control	Silymarin (100 mg/kg)	Silymarin (200 mg/kg)
Methionine (nmol/g liver)	38.2 ± 1.4 <sup>a</sup>	46.7 ± 2.5 <sup>a,b</sup>	51.1 ± 6.2 <sup>b</sup>
SAM (nmol/g liver)	99.3 ± 5.2	95.5 ± 4.4	108.8 ± 8.6
SAH (nmol/g liver)	46.0 ± 2.4	49.1 ± 4.4	53.1 ± 4.6
Homocysteine (nmol/g liver)	6.8 ± 0.5	6.8 ± 0.2	6.4 ± 0.3
Cystathionine (nmol/g liver)	9.9 ± 1.4 <sup>a</sup>	13.5 ± 1.6 <sup>a,b</sup>	16.7 ± 1.2 <sup>b</sup>
Cysteine (nmol/g liver)	89.3 ± 9.3 <sup>a</sup>	84.4 ± 3.2 <sup>a</sup>	149.1 ± 10.3 <sup>b</sup>
Hypotaurine (μmol/g liver)	0.13 ± 0.02	0.14 ± 0.03	0.16 ± 0.02
Taurine (μmol/g liver)	13.0 ± 0.7	13.4 ± 0.7	14.7 ± 0.4
GSH (μmol/g liver)	5.7 ± 0.3 <sup>a</sup>	5.9 ± 0.3 <sup>a</sup>	6.9 ± 0.2 <sup>b</sup>
GSSG (μmol/g liver)	0.22 ± 0.01	0.20 ± 0.01	0.22 ± 0.01
GSH/GSSG	25.4 ± 0.6 <sup>a</sup>	29.9 ± 0.9 <sup>b</sup>	31.7 ± 1.2 <sup>b</sup>

Each value is the mean ± SE for 5 mice. Values with different letters (a, b) are significantly different from one another (one-way ANOVA followed by Newman-Keuls multiple range test,  $p < 0.05$ )

	Control	Silymarin (100 mg/kg)	Silymarin (200 mg/kg)
MAT (pmol/min/mg protein)	41.3 ± 3.2 <sup>a</sup>	38.5 ± 1.4 <sup>a,b</sup>	32.5 ± 1.8 <sup>b</sup>
BHMT (nmol/min/mg protein)	1.55 ± 0.14 <sup>a</sup>	1.34 ± 0.10 <sup>a,b</sup>	1.14 ± 0.10 <sup>b</sup>
CβS (nmol/min/mg protein)	7.1 ± 0.6 <sup>a</sup>	8.7 ± 0.8 <sup>a,b</sup>	10.1 ± 0.8 <sup>b</sup>
CγL (nmol/min/mg protein)	10.1 ± 0.1	10.4 ± 0.4	10.7 ± 0.5
CDO (nmol/min/mg protein)	0.62 ± 0.07 <sup>a</sup>	0.40 ± 0.02 <sup>b</sup>	0.32 ± 0.02 <sup>b</sup>
CDC (nmol/min/mg protein)	16.3 ± 0.8	16.1 ± 1.3	17.3 ± 1.1
GCS (nmol/min/mg protein)	3.8 ± 0.4	3.3 ± 0.1	3.2 ± 0.5

Each value is the mean ± SE for 5 mice. Values with different letters (a, b) are significantly different from one another (one-way ANOVA followed by Newman-Keuls multiple range test,  $p < 0.05$ )

**Table 1** Effect of silymarin on hepatic levels of sulfur amino acids.

**Table 2** Effect of silymarin on the enzyme activities involved in sulfur amino acid metabolism.

major constituent of silymarin, can directly scavenge hydroxyl radicals (HO·) [5] and hypochlorous acids (HOCl) [6]. Also, silybin-phosphatidyl choline complex was demonstrated to scavenge hydroxyethyl radicals in hepatic microsomes and bile of ethanol-fed rats [7].

Previous studies have shown that in experimental animals challenged with a hepatotoxic substance, silymarin administration attenuates the toxicant-induced GSH depletion [1–3]. This is usually considered to be a secondary effect, resulting from the preservation of GSH in the antioxidant defense against hepatotoxins owing to the direct radical scavenging activity of silymarin. However, an increase in hepatic GSH content was noted in rodents treated with silymarin only in a few studies conducted by others [8,9], and also in our laboratory (unpublished observation), suggesting that this substance might be capable of directly influencing the GSH concentration in liver. Therefore, it was of interest to examine the effects of silymarin on hepatic metabolism of sulfur-containing amino acids in association with the antioxidant capacity of liver using an animal model.

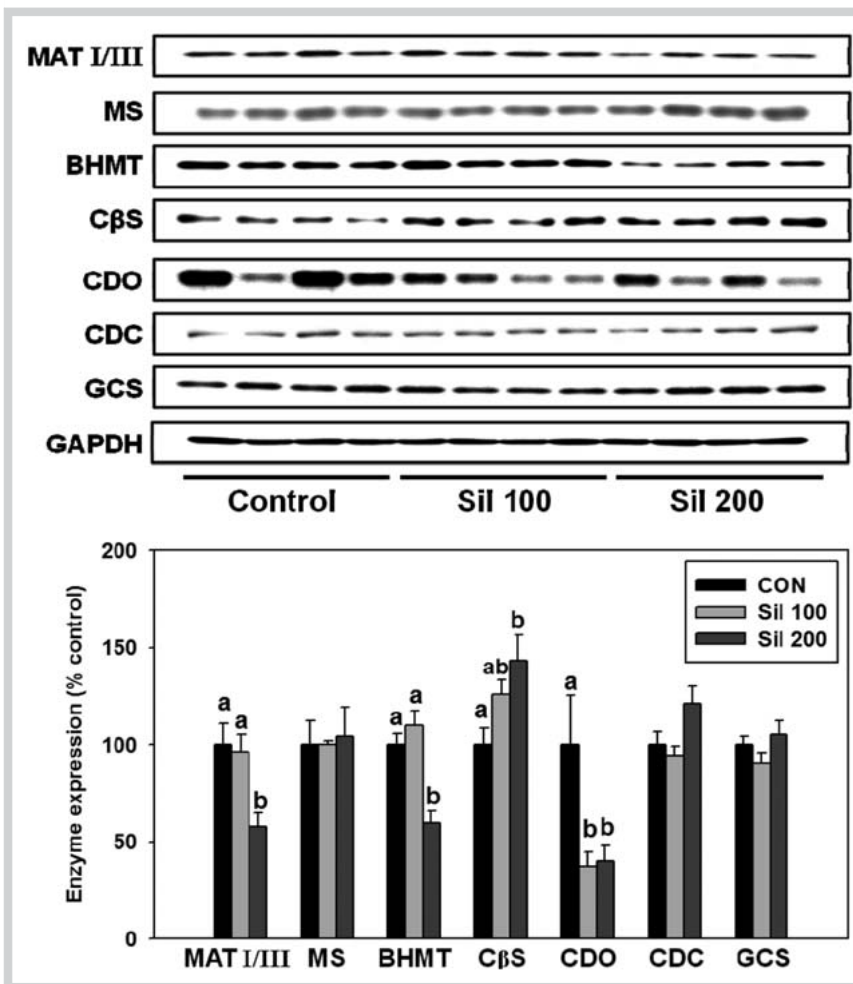
## Results

The hepatic methionine level was increased by silymarin treatment, but its metabolites in the methionine cycle, SAM, SAH, and homocysteine, were not influenced (Table 1). Silymarin treatment elevated the transsulfuration products from homocysteine, such as cystathionine, cysteine, and GSH, significantly. The GSH/GSSG ratio was also elevated, which might be due to the increase in *de novo* synthesis of GSH in liver. On the other hand, the other major metabolites of cysteine, hypotaurine and taurine, were not changed in the liver of the silymarin-treated mice.

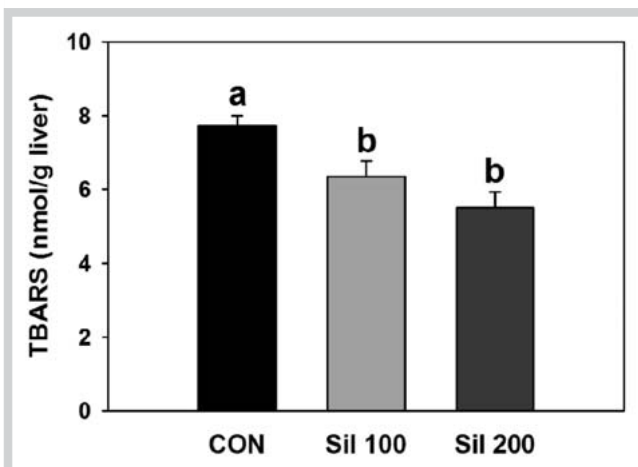
BHMT and MAT activities in liver were decreased by silymarin in a dose-dependent manner (Table 2). But CβS activity was increased significantly. The activity of CDO was inhibited markedly, while GCS was not altered by silymarin treatment. The alteration in the enzyme activities appeared to be directly related to the protein expressions because the tendency of the changes in activities was mostly equal to that of proteins (Fig. 1). BHMT and MAT I/III were decreased, whereas CβS was increased by silymarin treatment. CDO expression in the liver of the mice treated with silymarin was also lower than that found in the control animals. The hepatic level of TBARS was reduced by silymarin treatment significantly (Fig. 2). The specific TOSC of liver cytosol toward hydroxyl radicals and peroxynitrite was increased dose-dependently in the silymarin-treated mice (Fig. 3). Also, the cytosolic fraction of the liver from the silymarin-treated mice appeared to scavenge hydroxyl radicals more effectively than that of the control liver, although no statistical significance was noted. Therefore, the reduction of TBARS and the elevation of TOSC were nearly parallel to the increase in GSH and GSH/GSSG caused by silymarin treatment.

## Discussion

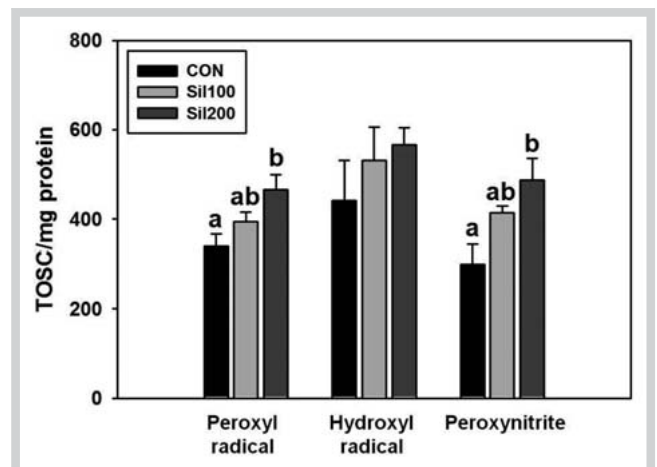
In this study, we examined the changes in hepatic sulfur-containing amino acid metabolism in the mice treated with silymarin acutely. The results show that silymarin alters the metabolism of sulfur amino acids extensively in normal mouse liver not exposed to extra oxidants. Particularly, silymarin increases the hepatic level of cysteine which is the essential substrate for GSH synthesis. It is thus suggested that the antioxidant activity of silymarin may be, at least in part, attributed to the increase in cysteine



**Fig. 1** Effect of silymarin on the protein expressions of enzymes involved in sulfur amino acid metabolism. Each value is the mean  $\pm$  SE for 4 mice. Values with different letters (**a**, **b**) are significantly different from one another (one-way ANOVA followed by Newman-Keuls multiple range test,  $p < 0.05$ ). Sil 100: silymarin 100 mg/kg; Sil 200: silymarin 200 mg/kg.



**Fig. 2** Effect of silymarin on hepatic lipid peroxidation. Each value is the mean  $\pm$  SE for 5 mice. Values with different letters (**a**, **b**) are significantly different from one another (one-way ANOVA followed by Newman-Keuls multiple range test,  $p < 0.05$ ).



**Fig. 3** TOSC of liver cytosol from the mice treated with silymarin. Each value is the mean  $\pm$  SE for 5 mice. Values with different letters (**a**, **b**) are significantly different from one another (one-way ANOVA followed by Newman-Keuls multiple range test,  $p < 0.05$ ).

availability, which results in an enhancement of GSH generation in liver.

In the first step of hepatic metabolism of sulfur amino acids, methionine sulfur is transferred to SAM by the activity of MAT. MAT

I and MAT III are liver-specific forms, and MAT II is an extrahepatic form. In this study, silymarin decreased MAT activity and MAT I/III protein expression, which may be responsible for the accumulation of methionine in liver. Regulation of MAT I/III pro-

tein expression is largely unknown, although our previous work shows that this enzyme is subject to post-translational regulation by oxidative stress [10]. SAM is demethylated to SAH, and SAH is further hydrolyzed to produce homocysteine, which is either remethylated to methionine or condensed with serine to yield cystathionine. The remethylation of homocysteine is catalyzed by BHMT and MS. In this study, the expression of MS was not changed, but BHMT was downregulated by silymarin (Fig. 1, Table 2), which could be due to the accumulation of methionine in liver. The reduction of BHMT activity would decrease the remethylation of homocysteine and instead enhance the condensation of this substance with serine into cystathionine. In fact, the *CβS* enzyme and the cystathionine content were increased significantly. It is known that several factors are involved in the regulation of *CβS*. Exposure of H4IIE cells to triamcinolone, a synthetic corticosteroid, resulted in an increase in *CβS* mRNA and protein expression [11]. It has been suggested that the structural similarity of silymarin to steroid hormones may account for the protein regulating action of these flavonoids [12]. Further studies need to be conducted to determine whether silymarin can affect the *CβS* expression through the steroid-like action.

CDO and GCS are the rate-limiting enzymes involved in the metabolic conversion of cysteine to taurine and GSH, respectively, and therefore the relative ascendancy between the two enzymes determines the metabolic fate of cysteine in the transsulfuration pathway. CDO catalyzes the oxidation of cysteine to cysteinesulfinic acid, which is further metabolized to hypotaurine by the activity of CDC. It has been demonstrated that cysteine itself is an important intracellular signal for upregulation of CDO [13]. A high level of cysteine allows more cysteine to be metabolically converted to cysteinesulfinic acid via induction of CDO. In contrast, GCS is upregulated when cysteine availability is low, ensuring that more cysteine is conserved as GSH [14]. In the present study, silymarin administration inhibited CDO activity and its protein expression despite the elevation of the cysteine level, which may in turn contribute to the accumulation of this sulfur amino acid in liver. This is in line with the results of a recent study demonstrating an increased intracellular cysteine level in predifferentiated 3T3-L1 cells, which have little CDO enzymes [15]. Therefore, not only the increased cysteine generation from homocysteine via cystathionine, but the reduced cysteine catabolism to taurine would lead to the increment in hepatic cysteine content. The inhibition of CDO did not result in a decrease in hypotaurine and taurine levels in this study. Considering the role of taurine as a major organic osmolyte, it is suggested that the necessity for maintenance of cellular homeostasis may inhibit a rapid change in the intracellular taurine concentration. In fact, a recent study revealed that hepatic CDO-knockout mice fed a taurine-free diet were able to maintain a normal taurine level in liver [16]. The mechanism involved in the inhibition of CDO by silymarin is not known. An earlier study showed that CDO activity was decreased by glucagon treatment via an induction of cAMP [17]. Meanwhile, the anticholestatic effect of silybin was suggested to be associated with an increase in cytosolic cAMP in isolated rat hepatocytes [18]. This raises the possibility that silymarin may affect CDO expression by regulating the cAMP content in liver. Additional studies are needed to clarify the underlying mechanism by which silymarin inhibits the activity and expression of CDO.

Cysteine availability is critical in GSH synthesis, because the hepatic cysteine level is much lower than that of glutamate or glycine. GCS catalyzes the ATP-dependent condensation of cysteine

and glutamate to form  $\gamma$ -glutamylcysteine. Therefore, cysteine availability and GCS activity are the two most important factors determining the generation of GSH. Silymarin treatment did not influence GCS expression or its activity, but elevated the cysteine level in liver significantly, which appeared to account for the increase in hepatic GSH generation. Several flavonoids were suggested to have positive effects on GSH synthesis. Quercetin was shown to induce both GCS activity and the GSH level in HepG2 cells [19]. Also, kaempferol and apigenin increased intracellular GSH by inducing GCS mRNA expression [20]. However, the present results indicate that silymarin may augment the generation of GSH in liver via an increase in cysteine availability without influencing GCS activity or its expression, which subsequently contributes to the enhancement of antioxidant capacity of liver tissues. This is consistent with the reduction of lipid peroxidation and the elevation of TOSC in the liver of the mice treated with silymarin.

In conclusion, the present results show that acute silymarin treatment increases the hepatic GSH level via an enhancement of cysteine availability in naïve mice. To our knowledge, this is the first study revealing that silymarin may induce intracellular GSH content via its direct effects on the metabolism of sulfur amino acids in liver. It is suggested that the elevation of hepatic GSH is associated with the increase in antioxidant capacity of liver in animals treated with this substance. The pharmacological significance of this finding is being evaluated in this laboratory.

## Materials and Methods



### Animals and treatments

Male C57BL/6 mice, weighing 25–30 g, were purchased from Orient-Bio. The use of these animals was in compliance with the guidelines established by the Animal Care Committee in College of Pharmacy, Seoul National University, and approved by the Ethical Animal Care and Use Committee of Seoul National University (No. SNU-070531-1, 17/07/2007). The mice were acclimated to temperature ( $22 \pm 2^\circ\text{C}$ ) and humidity ( $55 \pm 5\%$ ) controlled rooms with a 12-h light/dark cycle for 1 wk before use. Mice were gavage with silymarin (100 mg/kg or 200 mg/kg) every 12 h for a total of 3 doses. Silymarin was a kind gift from Bukwang Pharmaceuticals. This product contained silybin (40.9%) out of the total silymarin (81.6%) as determined by HPLC with 277 nm detection. Silymarin was dissolved in a solvent composed of 10% propylene glycol, 12% Tween-80, and 78% distilled water. Control mice received the vehicle only. At 6 h after the last dosing, the mice were sacrificed for collection of blood and liver samples.

### Determination of sulfur-containing amino acids

The liver was homogenized in 1 M  $\text{HClO}_4$  for detection of SAM, homocysteine, cysteine, and GSH, or in methanol for detection of methionine, cystathionine, hypotaurine, and taurine. GSH and GSSG were determined using the method of Griffith [21]. For measurement of total GSH, a mixture of NADPH, 5,5'-dithiobis(2-nitrobenzoic acid) and liver sample was preincubated at  $35^\circ\text{C}$  followed by the addition of GSH reductase. The change in absorbance at 412 nm was monitored using a UV/VIS spectrophotometer (V-530; Jasco Co.). GSSG was determined using the same procedure after masking GSH with 2-vinylpyridine. Cysteine was determined by the acid-ninhydrin method [22]. An incubation mixture consisting of acid-ninhydrin solution, acetic acid, and liver sample was incubated at  $100^\circ\text{C}$  for 10 min. After



cooling, 95% ethanol (v/v) was added followed by the absorbance measurement at 560 nm. Methionine, cystathionine, taurine, hypotaurine, and cysteinesulfinic acid were derivatized with *O*-phthalaldehyde/2-mercaptoethanol prior to quantification using the method of Rajendra [23]. An HPLC system installed with a 3.5- $\mu$ m Kromasil C18 column (4.6  $\times$  100 mm; Eka Chemicals), dual pumps (PU-980; Jasco Co.), and a fluorescence detector (FP-920; Jasco Co.) was used. Solvent A (0.1 M sodium acetic acid, pH 7.2) and solvent B [methanol:tetrahydrofuran = 97:3 (v/v)] were used as mobile phases. The flow rate was set at 1.0 mL/min and the gradient of solvent A was at 0 min 90%, 4–12 min 84%, 18–22 min 78%, 26–30 min 70%, 47–51 min 53%, 56 min 30%, 58–62 min 0%, and 63 min 90%. Peaks were detected at excitation and emission wavelengths of 338 nm and 425 nm, respectively. The method of She et al. [24] was employed to quantify SAM and SAH. A liver sample was injected into an HPLC system with a 3.5- $\mu$ m Kromasil C18 column (4.6  $\times$  250 mm; Eka Chemicals). The mobile phase used was 40 mM ammonium phosphate, 8 mM 1-heptane sulfonate, and 18% (v/v) methanol. The flow maintained at 1.0 mL/min was analyzed by a UV detector (UV-975; Jasco Co.). Homocysteine was determined using the method of Nolin et al. [25] with slight modifications. Oxidized and protein-binding forms of homocysteine in liver homogenate were reduced by 5 mM dithiothreitol and an equal volume of 1 M HClO<sub>4</sub> was added. After centrifugation, the supernatant was mixed with 1.55 M NaOH. The reaction mixture was incubated with 167 mg/L 7-fluorobenzofurazan-4-sulfonic acid at 60 °C for 1 h. An HPLC system with a 3.5- $\mu$ m Symmetry C18 column (4.6  $\times$  150 mm; Waters), dual pumps, and a fluorescence detector was used to analyze the effluent. Mobile phases were 0.1 M sodium acetic acid (solvent A; pH 4.0) and methanol (solvent B). With a flow rate of 0.8 mL/min, the gradient of solvent A was at 0–7 min 100%, 8–13 min 25%, and 14.5 min 100%. The peak of homocysteine was detected at excitation and emission wavelengths of 385 nm and 515 nm, respectively.

### Enzyme assays

The liver was homogenized in 0.154 M KCl/50 mM Tris-HCl and 1 mM EDTA (pH 7.4). The 104 000 g supernatant fraction (cytosol) was used to determine the enzyme activity and protein expression. MAT activity was estimated by quantifying the SAM production. Reaction mixtures consisted of 80 mM Tris-HCl/50 mM KCl (pH 7.4), 5 mM ATP, 40 mM MgCl<sub>2</sub>, 50 mM methionine, and 1.5 mg protein of cytosol in 1 mL. Incubation was carried at 37 °C for 30 min. BHMT activity was determined by the method of Ericson and Harper [26]. Cytosol containing 0.167% betaine and 0.167% homocysteine was incubated at 37 °C for 60 min. The reaction was terminated by cold methanol. After centrifugation, the supernatant was analyzed for methionine using the method described above. C $\beta$ S activity was quantified by the cystathionine production [27]. The reaction was carried in a mixture composed of 0.1 M Tris buffer (pH 8.3), 0.1 M serine, 0.12 mM pyridoxal phosphate, 0.5 mM CuSO<sub>4</sub>, 0.1 M homocysteine, and 2 mg protein of cytosol at 37 °C for 45 min. After centrifugation, the supernatant was incubated in 40 mM acid ninhydrin reagent at 100 °C for 5 min. The absorbance was detected at 455 nm using a UV/VIS spectrophotometer. CyL activity was estimated by the  $\alpha$ -keto-butyrate formation [28]. Cytosol was incubated in 0.1 M phosphate buffer (pH 7.5), 0.05 mM pyridoxal phosphate, 7.5 mM mercaptoethanol, 7 mM EDTA, and 32 mM homoserine for 30 min. After centrifugation, the supernatant was mixed with 0.1% 2,4-dinitrophenyl hydrazine followed by the addition of

ethanol and 2.5 M NaOH. The absorbance was measured at 515 nm by a UV/VIS spectrophotometer. CDO activity was measured by quantification of cysteine sulfinic acid using the method described above. Cytosol was incubated in 0.5 mM Fe (NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>, 5 mM NH<sub>2</sub>OH-HCl, 2 mM NAD<sup>+</sup>, and 5 mM cysteine for 16 min at 37 °C. CDC activity was estimated by measuring the hypotaurine production as described above. Cytosol was incubated with 0.1 M phosphate buffer (pH 7.4) containing 0.1 mM pyridoxal 5-phosphate, 2.5 mM dithiothreitol, and 10 mM cysteinesulfinic acid at 37 °C for 30 min. GCS activity was estimated by the  $\gamma$ -glutamylcysteine formation. Reaction mixtures contained 0.1 M Tris buffer (pH 8.2), 10 mM L-glutamate, 10 mM ATP, 20 mM MgCl<sub>2</sub>, 2 mM EDTA, 5 mM cysteine, and cytosol. Incubation was continued for 15 min at 37 °C.  $\gamma$ -Glutamylcysteine was determined after *O*-phthalaldehyde derivatization using HPLC with a fluorescence detector and a 3.5- $\mu$ m Symmetry C18 column (4.6  $\times$  75 mm; Waters). The mobile phase consisted of 16% methanol and 0.21% acetic acid. The flow rate was set at 1.5 mL/min. Fluorescence was detected at excitation and emission wavelengths of 360 nm and 470 nm, respectively.

For Western blotting analysis, 20  $\mu$ g protein of each sample was loaded, separated by gel electrophoresis, and transferred to nitrocellulose membranes by electroblotting. The membranes were blocked in 5% nonfat dry milk in 0.1% Tween 20 in Tris-buffered saline. The blots were incubated overnight with primary antibodies in 5% bovine serum albumin followed by incubation with secondary antibodies. Polyclonal antibodies against rat MAT I/III, C $\beta$ S, glyceraldehydes-3-phosphate dehydrogenase (Santa Cruz Biotechnology), GCS (NeoMarkers), BHMT, MS (Everest Biotech), CDC (Abcam plc), and CDO were used as probes. The CDO antibody was a kind gift from Dr. Yu Hosokawa (Faculty of Human Sciences, Jissen Women's University, Tokyo, Japan). Proteins were detected by enhanced chemiluminescence.

### Measurement of lipid peroxidation and TOSC

Hepatic lipid peroxidation was quantified by measuring TBARS [29]. The liver was homogenized in 0.154 M KCl. After centrifugation, the supernatant was incubated in a mixture containing trichloroacetic acid (10%), 2-thiobarbituric acid (0.025%), and HCl (167 mM) at 90 °C for 20 min. After centrifugation at 10 000 g, the absorbance was measured at 535 nm using a UV/VIS spectrophotometer. The method of Regoli and Winston [30] was employed to determine the TOSC of liver cytosolic fraction. This assay is based on the ethylene-yielding reaction of KMBA with peroxyl, hydroxyl radicals, and peroxyxynitrite. For the TOSC against the peroxyl radical, cytosol was incubated in 1 mL of a mixture consisting of 0.2 mM KMBA and 20 mM 2,2'-azobisamidinopropane in 200 mM potassium phosphate buffer (pH 7.4) at 37 °C using a sealed 20-mL glass bottle. For estimation of TOSC against the hydroxyl radical or peroxyxynitrite, ABAP was substituted for 0.3 mM ascorbic acid, 6  $\mu$ M EDTA, and 3  $\mu$ M ferrous ammonium sulfate or 0.1 mM diethylene triamine pentaacetic acid and 70  $\mu$ M 3-morpholinopyridone N-ethylcarbamide, respectively. During incubation, an aliquot of upper air in the bottle was taken at intervals and injected into GC (Model 3300; Varian) equipped with a flame ionization detector and porapack N column (Sigma-Aldrich) for measurement of ethylene. TOSC values were quantified from the equation TOSC = 100 - (JSA/JCA  $\times$  100), where JSA and JCA were the integrated ethylene peak areas obtained from the sample and control reactions, respectively. The specific TOSC was obtained by dividing the experimental TOSC by the weight of the cytosolic protein used.

## Data analysis

All results expressed as the mean  $\pm$  SEM were analyzed by a one-way ANOVA followed by the Newman-Keuls multiple range test. The acceptable level of significance was established at  $p < 0.05$ .

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## Conflict of Interest

The authors declare that they have no financial or nonfinancial competing interests.

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