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

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 β-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
- CBS: cystathionine β-synthase
- CDC: cysteine sulfinate decarboxylase
- CDO: cysteine dioxygenase
- CyL: cystathionine γ-lyase
- GCS: γ-glutamylcysteine synthetase
- GSH: glutathione
- GSSG: oxidized form of GSH
- KMBA: α-keto-γ-methylbutyric acid
- MS: methionine synthase
- MAT: methionine adenosyltransferase
- MS: methionine synthase
- SAH: S-adenosylhomocysteine
- SAM: S-adenosylmethionine
- TBARS: thiobarbituric acid reactive substance
- TOSC: total oxyradical scavenging capacity

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-
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.

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.

Table 1

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

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 2

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Silymarin (100 mg/kg)</th>
<th>Silymarin (200 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAT (pmol/min/mg protein)</td>
<td>41.3 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.5 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.5 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BHMT (nmol/min/mg protein)</td>
<td>1.55 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.34 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.14 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CPS (nmol/min/mg protein)</td>
<td>7.1 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.7 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.1 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CyL (nmol/min/mg protein)</td>
<td>10.1 ± 0.1</td>
<td>10.4 ± 0.4</td>
<td>10.7 ± 0.5</td>
</tr>
<tr>
<td>CDO (nmol/min/mg protein)</td>
<td>0.62 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.40 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CDC (nmol/min/mg protein)</td>
<td>16.3 ± 0.8</td>
<td>16.1 ± 1.3</td>
<td>17.3 ± 1.1</td>
</tr>
<tr>
<td>GCS (nmol/min/mg protein)</td>
<td>3.8 ± 0.4</td>
<td>3.3 ± 0.1</td>
<td>3.2 ± 0.5</td>
</tr>
</tbody>
</table>

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)
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 CBS enzyme and the cystathionine content were increased significantly. It is known that several factors are involved in the regulation of CBS. Exposure of H4IIE cells to triamcinolone, a synthetic corticosteroid, resulted in an increase in CBS 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 CBS 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 ascendency between the two enzymes determines the metabolic fate of cysteine in the transsulfuration pathway. CDO catalyzes the oxidation of cysteine to cysteinesulfenic acid, which is further metabolized to hypotaurine by the activity of CTC. 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 cysteinesulfenic 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 3 T3-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 silymarin 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 γ-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 (N. SNU-078531–1, 17/07/2007). The mice were acclimated to temperature (22 ± 2°C) and humidity (55 ± 5%) controlled rooms with a 12-h light/dark cycle for 1 wk before use. Mice were gavaged 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 HClO4 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°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-nitroprusside method [22]. An incubation mixture consisting of acid-nitroprusside solution, acetic acid, and liver sample was incubated at 100°C for 10 min. After
cooling, 95% ethanol (v/v) was added followed by the absorbance measurement at 560 nm. Methionine, cystathionine, threonine, homocysteine, and cysteinesulfonic acid were derivatized with O-phthalaldehydehydride-2-mercaptoethanol prior to quantification using the method of Rajendra [23]. An HPLC system installed with a 3.5-μm Kromasil C18 column (4.6 × 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 acetate, 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-μm Kromasil C18 column (4.6 × 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 HClO4 was added. After centrifugation, the supernatant was mixed with 0.167% homocysteine was incubated at 37°C for 60 min. The re-

### 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 MgCl2, 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 Eric- son 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. CPS activity was estimated 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 CuSO4, 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 nihydrin 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 α-keto- butyrate formation [28]. Cytosol was incubated in 0.1 M phospho-

### 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 centrifuga-

This document was downloaded for personal use only. Unauthorized distribution is strictly prohibited.
Data analysis
All results expressed as the mean ± 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.

Acknowledgements
This work was supported by National Research Foundation (NRF) grants (No. 2011–0016781 and No. 2009–0083533) funded by the Ministry of Education, Science and Technology (MEST), Korea.

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

References
2. Pradhan SC, Girish C. Dehmlow C, Murawski N, de Groot H. 5-Deoxy-5-(4-pyridyl)-1H-oxazol-2(3H)-one protects against acute ethanol-induced hepatotoxicity in mice. Evidence that silymarin acts both as an inhibitor of metabolic activation and as a chain-breaking antioxidant. Biochim Pharmacol 1990; 39: 2027–2034
14. Ueki I, Stipanuk MH. 3 T3-L1 adipocytes and rat adipose tissue have a high capacity for taurine synthesis by the cysteine dioxygenase/cysteine sulfinate decarboxylase and cysteamine dioxygenase pathways. J Nutr 2009; 139: 207–214