Impaired Sulfur-Amino Acid Metabolism and Oxidative Stress in Nonalcoholic Fatty Liver Are Alleviated by Betaine Supplementation in Rats

Do Y. Kwon, Young S. Jung, Sun J. Kim, Hee K. Park, Jae H. Park, and Young C. Kim

Nonalcoholic fatty liver disease (NAFLD) encompasses a broad spectrum of liver abnormalities ranging from simple accumulation of triglyceride in hepatocytes to nonalcoholic steatohepatitis (NASH) and in some patients, this is followed by the progression to fibrosis and cirrhosis (1). Because of its high prevalence in obesity, diabetes, and insulin resistance, NAFLD is increasingly appreciated as a hepatic manifestation of the metabolic syndrome (2,3). The mechanism involved in the development of NAFLD is poorly understood, although several hypotheses have been proposed. One generally accepted theory is the “two-hit” hypothesis, wherein the first hit involves simple accumulation of fat, rendering the liver more sensitive to a second, undefined insult that results in more severe liver damage (4).

The histology of NASH is identical to that of alcoholic hepatitis, with the primary damaging incident in the latter being lipid peroxidation and oxidative stress. The same mechanism has been proposed as the “second hit” in NASH, which causes the progression from simple steatosis to necroinflammation and subsequently leads to chronic liver injury (4,5). Oxidative stress in fatty livers is attributed to enhanced generation of reactive oxygen species via multiple intracellular pathways, such as cytochrome P450-mediated ω-oxidation of fatty acid, peroxisomal β-oxidation catalyzed by acyl-CoA oxidase, and impaired mitochondrial respiratory chain. Reactive oxygen species thus generated may induce liver damage via lipid peroxidation, cytokine induction, and Fas ligand expression. Recent studies...
have shown that hepatic and plasma oxidative stress-related variables are correlated with clinical and histological findings in NASH patients (6,7). Therefore, there is growing evidence that fat accumulation in liver plays a critical role not only in the initiation, but also in the progression, of NAFLD (8).

It has long been realized that chronic liver injury is associated with impairment of the metabolism of sulfur-amino acids in liver, which is attributed to abnormality in the activity of critical enzymes involved in the transsulfuration reactions and oxidative stress.

NAFLD. In addition, we examined the effect of betaine on the impairment of the metabolism of a high-fat diet. It was suspected that investigation of the early stages in ALD is the subject of many studies, its importance in

TABLE 1 Energy and betaine intakes and liver and body weights of rats fed NC, HF, or HFB for 3 wk

<table>
<thead>
<tr>
<th>Energy intake, kJ/3 wk</th>
<th>NC</th>
<th>HF</th>
<th>HFB</th>
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<tbody>
<tr>
<td>Total energy intake</td>
<td>6397 ± 138</td>
<td>6163 ± 314</td>
<td>6075 ± 159</td>
</tr>
<tr>
<td>Daily energy intake, kJ/d</td>
<td>306 ± 8</td>
<td>293 ± 17</td>
<td>287 ± 8</td>
</tr>
<tr>
<td>Betaine intake, g/(kg·d)</td>
<td>—</td>
<td>—</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Starting weight, g</td>
<td>189 ± 3</td>
<td>195 ± 4</td>
<td>197 ± 3</td>
</tr>
<tr>
<td>Final weight, g</td>
<td>346 ± 11</td>
<td>351 ± 5</td>
<td>353 ± 9</td>
</tr>
<tr>
<td>Weight gain, g/3 wk</td>
<td>157 ± 9</td>
<td>156 ± 7</td>
<td>157 ± 7</td>
</tr>
<tr>
<td>Terminal liver weight/body weight, %</td>
<td>4.09 ± 0.28</td>
<td>4.14 ± 0.20</td>
<td>4.02 ± 0.16</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n = 6.

TABLE 2 Serum ALT activity and TNF-α concentrations and hepatic triglyceride concentrations in rats fed NC, HF, or HFB for 3 wk

<table>
<thead>
<tr>
<th>ALT, U/L</th>
<th>NC</th>
<th>HF</th>
<th>HFB</th>
</tr>
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<tbody>
<tr>
<td>37.2 ± 3.3</td>
<td>39.8 ± 3.1</td>
<td>37.6 ± 1.8</td>
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<tr>
<td>Triglyceride, mmol/L</td>
<td>136.6 ± 1.1a</td>
<td>31.7 ± 3.0b</td>
<td>14.1 ± 1.4b</td>
</tr>
<tr>
<td>TNF-α, ng/g</td>
<td>36.0 ± 5.8b</td>
<td>1775 ± 10.4a</td>
<td>94.8 ± 10.4a</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n = 6. Means in a row with superscripts without a common letter differ, P < 0.05.

Materials and Methods

Animals and treatments. Male Sprague-Dawley rats were purchased from Dae-Han Experimental Animal. The use of the rats was in compliance with the guidelines established by the Animal Care Committee of the College of Pharmacy, Seoul National University. Animals 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. Eighteen rats weighing 180–205 g were randomly divided into 3 groups: NC, standard liquid diet; HF, high-fat liquid diet; HFB, high-fat liquid diet supplemented with betaine. The NC group was fed a standard liquid diet ad libitum for 3 wk and was then killed by exsanguinations under ether anesthesia.

Hepatic triglyceride concentrations in rats fed NC, HF, or HFB for 3 wk were determined using commercially available kits from Sigma Chemical (catalog no. TR0100) and BioSource International (catalog no. KRC3014), respectively. For histopathologic evaluation, sections of frozen liver were sliced at 10 μm, immersed in propylene glycol for 5 min, and stained with Oil red O for 7 min before microscopic examination.

Measurement of S-containing metabolites and enzyme activities. We homogenized livers in a 4-fold volume of cold 1 mol/L perchloric acid. Denatured protein was removed by centrifugation. Total GSH concentrations were determined using an enzymatic recycling method (17). Cysteine concentrations were estimated by the acid-ninhydrin method (18). We used the method of She et al. (19) to determine S-adenosylmethionine (SAM) and SAH concentrations. The supernatant was directly injected into HPLC equipped with a UV detector and TSK-GEL ODS-80TM column (4.6 × 250 mm) (Tosoh).

Free amino acids hypotaurine and taurine were derivatized with O-phthalaldehyde/2-mercaptoethanol prior to quantification using HPLC with a fluorescence detector and a 3.5-m Kromasil C18 column (4.6 × 100 mm) (Tosoh). Free amino acids were separated using the method of Rajendra (20). The method of Ile (21) was used to quantify hypotaurine and taurine.

Livers were homogenized in a 3-fold volume of ice-cold buffer containing 1 mmol/L EDTA in 0.154 mol/L KCl and 30 mmol/L Tris-HCl (pH 7.4). The 10,000-g supernatant was centrifuged at 104,000 g for 60 min. The 104,000-g supernatant fraction (cytosol) was used to determine the enzyme activities. MAT activity was estimated by quantifying SAM and SAH production (19). QS activity was determined by cystathionine formation (22). We estimated cystathionine γ-lyase (Cyl) activity was estimated by α-ketobutyrate production (23). γ-Glutamylcysteine ligase (GCL) and cysteine dioxygenase (CDO) activities were measured by generation of γ-glutamylcysteine (24) and cysteinesulfinate (25), respectively. Activity of GSH S-transferase (GST) was measured using 1-chloro-2,4-dinitrobenzene as a substrate (26).

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The blots were incubated overnight with antibodies diluted in 5% bovine serum albumin at 4°C followed by incubation with secondary antibodies conjugated to horseradish peroxidase. Polyclonal antibodies against rat GCL, GST, MAT, and CDO were used as probes. Antibodies against MAT and CDO were kind gifts from Dr. María A. Pajares (Instituto de Investigaciones Biomédicas “Alberto Sols,” Madrid, Spain) and Dr. Yu Hosokawa (Faculty of Human Sciences, Jissen Women’s University, Tokyo, Japan), respectively. Proteins were detected by enhanced chemiluminescence.

**Statistical analysis.** All results were expressed as the mean ± SEM. Equality of variance was determined by Bartlett’s test. When the variances were unequal, the data were logarithmically transformed prior to analysis. The values presented in the tables and figures are in the original scale. Means of the different diet groups were compared using 1-way ANOVA followed by Newman-Keuls multiple range test. The acceptable level of significance was established at P < 0.05. All statistical analyses were conducted using GraphPad Prism version 4.0 software.

**Results**

**Body weight, liver weight, and liver injury.** The total energy intake, body weight change, and relative liver weight did not differ among the groups fed the 3 diets (Table 1). In the HF group, hepatic triglyceride and serum TNFα concentrations were higher than in the NC group, but serum ALT activity did not differ between the 2 groups (Table 2). Betaine supplementation blocked the elevation of hepatic triglyceride and serum TNFα concentrations in the HF group. Histopathological examination of liver showed similar results (Fig. 1). Fat accumulation in liver was greater in the HF group than in the NC group and this was prevented by betaine supplementation.

**Cytosolic TOSC.** The specific TOSC toward hydroxyl and peroxyl radicals was lower in the HF group than in the NC group, but peroxynitrite-scavenging capacity did not differ between the 2 groups (Fig. 2). Betaine supplementation prevented the HF-induced reduction of antioxidant capacities against hydroxyl and peroxyl radicals in liver cytosol completely.

**The concentrations of S-containing substances in liver.** Hepatic methionine concentrations were higher, but SAM and the SAM:SAH ratio were lower in the HF group than the NC group (Table 3). Hepatic GSH concentrations were lower, but hypotaurine and taurine were higher in the HF group. Hepatic methionine, SAM, and the SAM:SAH ratio were significantly greater in the HFB group than in the HF group. The reduction of hepatic GSH as well as the elevation of hypotaurine and taurine in the HF group was inhibited by betaine supplementation. Hepatic hypotaurine and taurine concentrations were lower in the HFB group than in the NC group.

**Enzymes involved in the transsulfuration pathway.** Hepatic MAT activity was significantly lower in the HF group than in the NC group (Table 4). The activity of CjS did not differ among the groups, but CyL activity was higher in the HF group than in the NC group. Intake of HF elevated the activities of CDO and GST. Betaine supplementation induced hepatic MAT activity to a level greater than that in the NC group. GST activity was normalized and that of CDO was depressed to a level less than that in the NC group by betaine intake. GCL activity did not differ between the NC and HF groups but was lower in the HFB group than in the HF group.

The level of MAT did not differ between the NC and HF groups (Fig. 3A), but concentrations of CDO (Fig. 3C) and GSTα (Fig. 3D) were greater in the HF group than in the NC group. Betaine supplementation of the HF diet induced MAT

**TABLE 3** Hepatic methionine, SAM, SAH, cysteine, GSH, hypotaurine, and taurine concentrations in rats fed NC, HF, or HFB for 3 wk.

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>HF</th>
<th>HFB</th>
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<tbody>
<tr>
<td>Methionine, nmol/g</td>
<td>39.4 ± 1.6a</td>
<td>49.2 ± 1.2b</td>
<td>102.4 ± 3.7f</td>
</tr>
<tr>
<td>SAM, nmol/g</td>
<td>83.0 ± 3.0b</td>
<td>61.4 ± 4.1f</td>
<td>239.5 ± 13.8c</td>
</tr>
<tr>
<td>SAH, nmol/g</td>
<td>21.2 ± 0.8a</td>
<td>20.1 ± 0.9f</td>
<td>34.3 ± 1.6b</td>
</tr>
<tr>
<td>SAM:SAH</td>
<td>4.0 ± 0.2b</td>
<td>3.0 ± 0.2e</td>
<td>7.1 ± 0.7c</td>
</tr>
<tr>
<td>Cysteine, nmol/g</td>
<td>90.8 ± 3.3b</td>
<td>84.5 ± 3.6e</td>
<td>99.4 ± 6.3c</td>
</tr>
<tr>
<td>GSH, µmol/g</td>
<td>6.8 ± 0.5b</td>
<td>3.8 ± 0.4f</td>
<td>5.9 ± 0.3b</td>
</tr>
<tr>
<td>Hypotaurine, µmol/g</td>
<td>0.23 ± 0.01b</td>
<td>0.33 ± 0.02e</td>
<td>0.14 ± 0.01a</td>
</tr>
<tr>
<td>Taurine, µmol/g</td>
<td>1.99 ± 0.23b</td>
<td>4.21 ± 0.41f</td>
<td>0.61 ± 0.09f</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 6. Means in a row with superscripts without a common letter differ, P < 0.05.
markedly. The increases in CDO and GSTα proteins in the HF group were prevented by betaine supplementation. The concentration of GCL did not differ between the NC and HF groups but was lower in the HFB group than in the HF group (Fig. 3B).

Discussion

The present results show that administration of HF for 3 wk resulted in significant fat accumulation and reduction of antioxidant defense in rat liver. The serum TNFα concentration was also elevated, suggesting that the progression of liver injury reached a level beyond simple accumulation of fat in liver. The metabolism of S-containing substances in liver was disturbed profoundly, which was characterized mainly by reduction of SAM and GSH concentrations. The decrease in SAM and GSH appears to be responsible for the diminution of antioxidant defense in liver of the rats fed HF. These results indicate that impairment of hepatic transsulfuration reactions, which is suggested to serve as a causal factor in ALD, plays a critical role in the development of NAFLD as well.

In mammals, the liver plays a central role in the metabolism of sulfur-amino acids (28). The first step in the transsulfuration reactions is the formation of SAM from methionine and ATP, which is catalyzed by MAT. MAT is suggested to be regulated by cellular redox state. Molecular interaction between cysteine-121 residue of MAT and hydroxyl radical or NO results in inactivation of this enzyme (29,30). In this study, the HF intake did not affect the concentration of MAT but diminished its activity, suggesting the posttranslational regulation of MAT by the redox state. Aggravation of oxidative stress in fatty livers may result in inactivation of the critical site on MAT, leading to a decrease in the enzyme activity and SAM synthesis in liver.

SAM is the principal biological methyl donor, the precursor of aminopropyl groups used in polyamine synthesis, and a provider of cysteine for synthesis of GSH. Experimental evidence also suggests that an additional role of SAM may be its action as a direct antioxidant (31). SAM was more effective than GSH in directly scavenging hydroxyl radical (32). We recently observed that, in mice deficient in GSH peroxidase and catalase, hepatic SAM was reduced significantly, whereas GSH was unchanged, suggesting a potential role of SAM as an antioxidant under persistent oxidative stress (our unpublished data). These results imply that the reduction of hepatic SAM in this study may be associated with enhanced consumption of this substance in antioxidant defense as well as its reduced generation via inhibition of MAT activity. The depletion of SAM and the inactivation of the critical site on MAT would trigger a vicious cycle between the two, further aggravating the cellular redox state.

**TABLE 4** Hepatic MAT, CβS, CyL, GCL, CDO, and GST activities in rats fed NC, HF, and HFB for 3 wk

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>HF</th>
<th>HFB</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAT, nmol/(min·mg protein)</td>
<td>0.35 ± 0.02a</td>
<td>0.08 ± 0.01a</td>
<td>0.88 ± 0.07c</td>
</tr>
<tr>
<td>CβS, nmol/(min·mg protein)</td>
<td>21.0 ± 1.6</td>
<td>18.1 ± 1.1</td>
<td>18.3 ± 0.5</td>
</tr>
<tr>
<td>CyL, nmol/(min·mg protein)</td>
<td>50.2 ± 2.5a</td>
<td>68.2 ± 3.2b</td>
<td>72.6 ± 5.0a</td>
</tr>
<tr>
<td>GCL, nmol/(min·mg protein)</td>
<td>5.70 ± 0.48b</td>
<td>6.70 ± 0.37b</td>
<td>5.60 ± 0.51a</td>
</tr>
<tr>
<td>CDO, nmol/(min·mg protein)</td>
<td>0.52 ± 0.01b</td>
<td>0.75 ± 0.04b</td>
<td>0.39 ± 0.03a</td>
</tr>
<tr>
<td>GST, μmol/(min·mg protein)</td>
<td>4.00 ± 0.10a</td>
<td>4.90 ± 0.10b</td>
<td>3.73 ± 0.07a</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 6. Means in a row with superscripts without a common letter differ, P < 0.05.

**FIGURE 3** Concentrations of MAT, GCL, CDO, and GSTα proteins in rats fed NC, HF, or HFB for 3 wk. Values are means ± SEM, n = 3. Labeled means without a common letter differ, P < 0.05.
The hepatic GSH concentration is regulated by a balance among its synthesis, utilization, and export to other tissues. Synthesis of GSH in liver is limited mostly by 2 factors: availability of cysteine and activity of GCL (33). In the rats fed HF, neither cysteine availability nor GCL activity decreased; however, hepatic GSH was reduced significantly. Increased consumption of GSH in antioxidant defense may be responsible for the reduction of GSH in liver as reflected by the increment in GST activity and its concentration in the HF group.

It has been suggested that intracellular cysteine acts as an initial signal for regulation of CDO and GCL in liver (34). The upregulation of CDO, when cysteine concentration is high, allows more cysteine to be converted to cysteine sulfinate, a rate-limiting step in hypotaurine/taurine synthesis. In contrast, GCL is upregulated when cysteine availability is low, ensuring that more cysteine is conserved as GSH (35). In the rats fed HF, hepatic cysteine was unchanged, but both concentration and activity of CDO increased significantly, suggesting that a signal other than cysteine availability may also be involved in the regulation of CDO and GCL. Previously, we observed that cysteine catabolism to taurine was paradoxically increased in rats challenged with ethanol (36,37). The physiological importance of enhanced taurine synthesis under oxidative stress needs to be explored in future studies.

In this study, betaine supplementation completely blocked the fat accumulation and reduction of antioxidant capacity in liver of the rats fed HF. Our previous studies showed that betaine administration to mice and rats increased the MAT activity, hepatic methionine, and SAM and decreased homocysteine and cystathionine concentrations (38,39). However, cysteine and GSH concentrations were unaltered. Instead, cysteine catabolism to taurine was inhibited, suggesting that preserving the availability of cysteine from its use for taurine synthesis may be the mechanism for the maintenance of hepatic GSH. In the present study, betaine supplementation to the rats fed HF elevated methionine concentrations, which is apparently associated with enhanced supply of a substrate for methionine generation. But the other changes in the concentrations of S-containing substances in the HF group were prevented or reversed by betaine supplementation. Elevation of hepatic SAM is attributed to induction of methionine availability and MAT activity. Hepatic cysteine levels were not altered, but the depletion of GSH in the rats fed HF was prevented by betaine supplementation. Depression of cysteine catabolism to taurine appears to be responsible for the conservation of hepatic GSH. We previously showed that betaine administration was effective against oxidative stress-mediated hepatic injury induced by hepatotoxins such as alcohol, chloroform, lipopolysaccharide, and α-naphthylisothiocyanate (37,39–41). These results, in conjunction with the present data, suggest that the hepatoprotection provided by betaine is associated with the alleviation of oxidative stress via its effect on the metabolism of S-containing substances.

Although there are apparent etiological differences between ALD and NAFLD, both share many histological features and mechanistic factors associated with disturbance in hepatic metabolism. It has been suggested that depletion of hepatic SAM together with a concomitant elevation of homocysteine may be an etiologic contributor to ALD (12,13). Decreases in hepatic SAM and the SAM:SAH ratio result in serious functional consequences, including decreased essential biological methylations and reactions. Decreased activity of phosphorylalanine methyltransferase, isopenylcysteine carboxyl methyltransferase, and protein L-isoaspartate methyltransferase in alcoholic livers results in fat deposition, apoptosis, and accumulation of damaged proteins (42). Amelioration of ALD by betaine has been attributed to elevation of SAM and reduction of homocysteine (12,37,42,43). The hepatoprotective activity of betaine against the HF-induced fatty liver also appears to result from its ability to regulate the metabolism of S-containing substances in liver. The present results may explain the beneficial effects of betaine supplementation to NASH patients observed in several pilot studies (44,45).

In conclusion, the present results indicate that hepatic metabolism of sulfur-amino acids is significantly impaired in the early stage of NAFLD. This is the first report demonstrating that impairment of hepatic transsulfuration reactions is critically linked with the development of nonalcoholic fatty liver. Imbalance between prooxidants and antioxidant capacity in fatty livers appears to be responsible for the disturbance of hepatic transsulfuration reactions, which in turn further aggravates the oxidative stress associated with HF intake. Administration of betaine to the rats fed HF protects the liver from induction of oxidative stress and steatosis, most probably via its effects on the transsulfuration reactions. It is noteworthy that, in addition to elevating the MAT activity and SAM concentrations, betaine prevents the decrease in hepatic GSH by depressing cysteine catabolism to taurine. Further studies to determine the effects of betaine on the impaired sulfur-amino acid metabolism in chronic liver injury are being conducted in this laboratory.

**Literature Cited**


