Amelioration of D-Galactosamine-Induced Acute Liver Injury in Rats by Dietary Supplementation with Betaine Derived from Sugar Beet Molasses

Tomoko Okada,1 Sakura Kawakami,1 Yumi Nakamura,1 Kyu-Ho Han,1 Kiyoshi Ohba,2 Tsutomu Aritsuka,3 Hirokatsu Uchino,3 Ken-ichiro Shimada,1 Mitsuo Sekikawa,1 Hiroshi Ishii,1 and Michihiro Fukushima1,4

1Department of Food Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan
2Hokkaido Tokachi Area Regional Food Processing Technology Center, Obihiro, Hokkaido 080-2462, Japan
3Research Center, Nippon Beet Sugar MFG, Co., Ltd., Obihiro, Hokkaido 080-0831, Japan

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The effects of betaine supplementation on D-galactosamine-induced liver injury were examined in terms of hepatic and serum enzyme activities and of the levels of glutathione and betaine-derived intermediates. The rats induced with liver injury showed marked increases in serum enzyme activity, but those receiving dietary supplementation of 1% betaine showed enzyme activity levels similar to a control group without liver injury. Administration of betaine also increased both hepatic and serum glutathione levels, even following D-galactosamine injection. The activity of glutathione-related enzymes was markedly decreased following injection of D-galactosamine, but remained comparable to that of the control group in rats receiving 1% betaine. The concentrations of hepatic S-adenosyl methionine and cysteine showed similar trends to that observed for hepatic glutathione levels. These results indicate that 1% betaine has a hepatoprotective effect by increasing hepatic and serum glutathione levels along with glutathione-related enzyme activities in rats.

Key words: betaine; D-galactosamine; glutathione; S-adenosyl methionine

Betaine (glycine-betaine or trimethylglycine) is a non-toxic amino acid derivative from dietary sources or synthesized from choline. High levels of betaine can be found in animal products, especially shellfish, and some plants, members of the beet family (e.g., beetroot and spinach).1 It was first isolated from the sap of the sugar beet, Beta vulgaris,2 a betaine-accumulating dicotyledonous plant of the Chenopodiaceae family.3 It acts as an osmolyte in the inner medulla of the liver and kidney, preserving osmotic equilibrium while maintaining the tertiary structure of macromolecules. It was initially introduced to the feed industry as a replacement for methionine and choline in poultry and fish diets, where it is assumed to act both as a methyl donor and as an osmoprotectant.4

Previous study demonstrated that betaine is effective at reducing lipid accumulation in tissues in patients with fatty liver, coronary atherosclerosis, and hyperlipidemia, and it is therefore considered to be a lipotropic substance.5 Earlier studies also reported that patients with diabetes or renal failure have elevated betaine excretion as compared with healthy subjects.6,7 More recently, the role of betaine in preventing increases in plasma homocysteine concentrations has become a research focus.5–10 Betaines are also actively under investigation for their hepatoprotective activity,4,11 which can be especially beneficial for individuals with hepatitis.

The current study was undertaken to examine the hepatoprotective activity of betaine derived as a by-product from sugar beet molasses during sucrose production. Two experiments were carried out to verify the effects of betaine in animal models with and without acute liver injury. Liver injury was induced by D-galactosamine (D-GalN), a hepatotoxicant frequently used as a model agent because it causes diffuse hepatic necrosis in rodents, with effects that closely resemble those of human viral hepatitis.10 Several parameters were examined to explore the biological functions of betaine and determine their mechanisms, including serum enzyme activity; hepatic and serum glutathione (GSH) levels and GSH-related enzyme activities; and levels of sulfur-containing betaine-derived intermediates or products of trans-sulfuration reactions, including S-adenosyl methionine (SAM), S-adenosyl homocysteine (SAH), and free amino acids.

Experimental

Animals and diets. Male Fischer 344 rats (7 weeks old) were purchased from Charles River Laboratories Japan (Yokohama, Japan). They were housed individually in plastic cages under a controlled 12-h light/dark cycle. Room temperature and relative humidity were kept at 23 ± 1 °C and 60 ± 5% There were no significant differences in body weight among the study groups at the beginning of the experiment. The rats were allowed free access to food and water throughout the experiments. Body weight and food consumption were recorded weekly and daily, respectively. The experimental design was approved by the Animal Experiment Committee of Obihiro University of
Agriculture and Veterinary Medicine, and adhered to the standard principles described in the “Guide for the Care and Use of Laboratory Animals.”

For experiment 1, the rats were randomly divided into three groups of five rats each and fed specific diets for a period of 4 weeks. The control group (CN-1) was fed a control diet based on the AIN-93G semi-purified rodent diet guidelines. The two experimental groups received the control diet supplemented with either 1 or 2% wt/wt sugar beet-derived betaine (groups B1 and B2, respectively). At the completion of the 4-week feeding period, the rats were anesthetized by intraperitoneal administration of sodium pentobarbital and blood samples were collected. The livers were surgically removed and washed with cold saline (9 g of NaCl/L deionized water), blotted dry on filter paper, and weighed before freezing at −80 °C for later analysis.

For experiment 2, the rats were randomly assigned to four groups of five rats each: a control group (CN-2), treated in the manner described above for CN-1, a control group with acute liver injury induced by d-GalN injection (group CG), and two groups with acute liver injury induced by d-GalN injection that were fed diets supplemented with 1 or 2% wt/wt sugar beet-derived betaine (groups B1G and B2G, respectively). The rats were fed the diets described above for 2 weeks. Acute liver injury was induced on the 14th day of the experiment by intraperitoneal injection of 400 mg/kg body wt d-GalN (Sigma, St. Louis, MO). For the CN-2 group, intraperitoneal injection was carried out with water instead of d-GalN. The rats were anesthetized by intraperitoneal administration for sodium pentobarbital, and blood samples were collected 22 h after liver injury. The livers were surgically removed, and were treated as described of experiment 1.

Serum collection. Blood samples (1 mL) were collected from the jugular veins of fasting rats anesthetized with intraperitoneal administration sodium pentobarbital. The samples were collected in tubes with no anticoagulant and were left at room temperature for 2 h, and then the serum was separated by centrifugation at 1,500 g for 15 min.

Serum enzyme activity measurement. Serum enzyme activity was measured for all the experimental groups. Measurement of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) was carried out with commercial assay kits for the TDX system, following the manufacturer’s instructions (Abbot Laboratory, Irving, TX).

Analysis of GSH concentration and related enzyme activities. Serum and hepatic GSH levels were determined by the method of Cohn and Lye using a fluorescence spectrophotometer (excitation wavelength 342 nm, emission wavelength 428 nm). To measure GSH-related enzyme activity, the liver cytoplasms was extracted by adding 2.8 mL of 0.25 M sucrase to 0.2 g of each liver sample, followed by homogenization on ice. Homogenates were further centrifuged at 104,000 g for 60 min at 4 °C using an ultracentrifuge (Optima TLX, Beckman Coulter Japan, Tokyo). A 1.5 mL aliquot of the supernatant from each sample was stored at −80 °C for enzyme activity assay. Glutathione reductase (GR) activity was determined using the method of Worthington and Rosemeyer, using glutathione peroxidase (GPx) activity was measured as described by Lawrence and Burk, and glutathione S-transferase (GST) activity was measured with the method of Habig. The total protein concentration was determined by the Lowry method using a Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

Measurement of hepatic sulfur amino acid metabolites. SAM and SAH were quantified as described by She, with some modifications. Liver samples (100 mg) were homogenized in 500 μL phosphate buffer, and the homogenate was centrifuged at 1,000 g for 5 min. The resulting pellet was resuspended in 500 μL of 0.5 M perchloric acid and centrifuged at 1,000 g for 15 min. The aqueous layer was quantitatively removed and neutralized with 3 M potassium hydroxide. These extracts were then analyzed for SAM and SAH by HPLC with a UV detector and integrator using a Partisil SCX 10-μm column (25 × 0.44 cm ID; Whatman, Clifton, NJ) at a flow rate of 1 mL/min. The mobile phase consisted of 0.05 M NH₄H₂PO₄ with 2% acetonitrile vol/vol, and the pH was adjusted to 2.6 using 2 M H₃PO₄. For quantification of SAM and SAH, the absorbance was measured at 254 nm, and the results were compared with standards prepared at the same time as the experimental samples.

To measure free amino acids, liver samples (100 mg) were homogenized in about 400 μL of cold methanol and centrifuged at 10,000 g for 10 min. Free amino acids were derivatized with phenylisothiocyanate before injection into an HPLC (LC-VP, Shimadzu, Kyoto) equipped with a UV-VIS detector (SPD-10AVP) and column (STR-ODS-II 150 mm × 4.6 mm). Amino acid concentrations were calculated by comparison of their retention times with those of reference standards (standard amino acid mixture Type H Grade, Wako, Tokyo).

Statistical analyses. Data are presented as mean and standard deviation for the various sample groups. In experiment 1, significant differences were determined by analysis of variance (ANOVA) with the Tukey-Kramer test (p < 0.05, SAS Institute, Cary, NC). In experiment 2, significant differences between experimental group, and the control group were tested by Student’s t-test (*p < 0.05, **p < 0.01). Significant differences among the d-GalN injected groups in experiment 2 were determined by ANOVA with the Tukey-Kramer test as in experiment 1.

Results

Experiment 1

Body weights and food consumption

There were no significant differences in body weight gain (CN-1: 55.2 ± 3.90 g/4 weeks, B1: 59.1 ± 7.90 g/4 weeks, B2: 56.6 ± 12.7 g/4 weeks) or total food intake (CN: 423 ± 24.4 g/4 weeks, B1: 448 ± 25.6 g/4 weeks, B2: 439 ± 43.9 g/4 weeks) among the experimental groups at the end of the 4-week feeding period.

Serum enzyme activity

Figure 1 depicts changes in the enzymatic activity of ALT, LDH, and AST in rat serum for all treatment groups in experiment 1. The groups receiving 1% and 2% betaine (B1 and B2, respectively) showed significantly (p < 0.05) lower activity of all three enzymes at week 4 as compared to the CN-1 group. For instance, ALT activity in groups B1 and B2 was reduced by 34% and 24% respectively as compared to the activity in the CN-1 group at week 4.

Changes in GSH concentration and related enzyme activities

Hepatic and serum GSH levels are shown in Fig. 2. Hepatic GSH concentration increased significantly in a dose-dependent manner (group B1 = 3.35 ± 0.44 μmol/g wet liver; group B2 = 4.37 ± 0.34 μmol/g wet liver), as compared to the CN-1 group (2.83 ± 0.40 μmol/g wet liver). Group B1 showed the highest serum GSH concentration (119 ± 3.78 nmol/mL), followed by groups CN-1 (112 ± 3.93 nmol/mL) and B2 (109 ± 3.73 nmol/mL), with significant differences among the three groups. In terms of GSH-related enzymatic activity measured in this study, only hepatic GR showed significant differences among the three groups (Fig. 2C). Group B2 exhibited significantly higher GR activity (38.5 ± 5.87 mUnit/mg protein) than group CN (27.0 ± 3.58 mUnit/mg protein) or group B1 (31.1 ± 4.08 mUnit/mg protein).

Experiment 2

Body weights and food consumption

There were no significant differences in body weight gain (CN-2: 51.4 ± 4.10 g/2 weeks, CG: 48.3 ± 5.20 g/2 weeks, B1G: 49.3 ± 5.10 g/2 weeks, B2G:...
46.3 ± 4.4 g/2 weeks) among the experimental groups over the two weeks. The groups injected with d-GalN that received 1% and 2% betaine supplementation showed a significantly reduced food intake (B1G: 226 ± 15.0 g/2 weeks, B2G: 227 ± 14.0 g/2 weeks) compared to the CN-2 group (268 ± 32.0 g/2 weeks), however, there were no significant differences in terms of feed efficiency (CN-2: 0.19 ± 0.02, CG: 0.19 ± 0.03, B1G: 0.22 ± 0.01, B2G: 0.20 ± 0.02).

Serum enzyme activity
Changes in the enzymatic activity of ALT, LDH, AST, and ALP in the serum for all treatment groups for experiment 2 are depicted in Fig. 3. As shown, the rats on the control diet that received a d-GalN injection (group CG) exhibited significant (p < 0.05) increases in serum enzymatic activity as compared to the rats on the control diet that did not receive d-GalN injection [(group CN-2: 6.70 ± 1.56 μmol/g wet liver), (Fig. 4A)]. However, the d-GalN-injected group that received 1% betaine supplementation (B1G) showed a level of hepatic GSH (6.14 ± 1.03 μmol/g wet liver) statistically similar to the CN-2 group. Serum GSH levels were also significantly lower in the CG group (10.4 ± 7.52 nmol/L), but not in the B1G group (134 ± 124.2 nmol/L), as compared with control [(191 ± 33.0 nmol/L), (Fig. 4B)]. The d-GalN-injected rats that received 2% betaine supplementation (group B2G) had serum GSH levels statistically similar to the CG group.

Hepatic GSH-related enzyme activity showed similar tendencies for all three enzymes across the experimental groups, with a few exceptions (Fig. 4C, D, and E). For the GR and GPx enzymes, activity was statistically
similar, between the CN-2 (13.0 ± 0.77 and 80.8 ± 18.9 Unit/total liver respectively) and the B1G group (13.5 ± 2.09 and 74.4 ± 12.8 Unit/total liver respectively), with significantly lower activity for the CG group (9.11 ± 0.35 and 51.7 ± 8.71 Unit/total liver respectively). Enzymatic activity for group B2G was statistically similar to group CG for GR and significantly higher than group CG for GPx.

Concentrations of hepatic metabolites and free amino acids

The changes in the levels of the major hepatic metabolites and products expected as a result of betaine administration are shown in Table 1. The levels of SAM in the liver were significantly higher for group B1G (0.72 ± 0.24 μg/mg liver) than for groups CN-2 (0.33 ± 0.07 μg/mg liver), CG (0.27 ± 0.07 μg/mg liver) and B2G (0.51 ± 0.19 μg/mg liver), while hepatic SAH concentrations were not significantly affected by betaine supplementation or d-GalN injection (data not shown). The levels of the amino acids known to be involved in betaine metabolism (methionine and cysteine) are shown in Table 1. The hepatic cysteine
concentration showed a trend similar to that observed for SAM, with significantly higher concentrations for B1G (0.03 ± 0.00 ng/mg liver) than for CG (0.01 ± 0.00 ng/mg liver). The levels of methionine were significantly lower for the control group (0.08 ± 0.01 μg/mg liver) compared with 0.15 ± 0.03 to 0.19 ± 0.02 μg/mg liver for the other experimental groups.

**Discussion**

Our study was designed to assess the relationship between betaine supplementation and serum enzyme activity (a marker of hepatic toxicity), hepatic/serum GSH concentrations, GSH-related enzyme activity, and betaine-GSH intermediate metabolites in rats with and without acute hepatic injury. Previous studies have shown that betaine has a protective effect against oxidative stress-mediated hepatic injury induced by a hepatotoxins such as ethanol, chloroform, lipopolysaccharide, and alpha-naphthylisothiocyanate, but this effect has not been demonstrated in relation to d-GalN-induced liver injury.

In our study, dietary betaine administration resulted in significant changes in various biochemical parameters in rats with and without d-GalN-induced liver injury. In experiment 1, we examined the effects of betaine treatment on 7-week-old male Fischer 344 rats without acute liver injury. Measurement of serum enzyme activities showed that betaine supplementation, regardless of concentration, significantly lowered serum ALT, LDH, and AST activity, suggesting a promising hepatoprotective effect. Another important factor to consider when examining the potential hepatoprotective effects of betaine is the GSH concentration in both the liver and the serum. GSH (L-glutamyl-cysteinylglycine) is a major antioxidant that helps eliminate peroxides and other oxidants. It plays a prominent role in the detoxification and antioxidant of exogenous and endogenous compounds, as well as maintaining intracellular redox status. The importance of GSH in the detoxification of chemically reactive metabolites has been comprehensively documented, with numerous examples of drug-induced toxicity after GSH depletion.

In our first experiment, hepatic and serum GSH levels were higher in the groups receiving 1% and 2% betaine than in the control group. It is known that moderate oxidation of GSH can cause the formation of glutathione disulfide (GSSG), and that GR catalyzes the reduction of GSSG back to GSH to maintain adequate levels of cellular GSH. In experiment 1, GR activity showed significant differences among the treatment groups. Supplementation with 2% betaine resulted in 43% higher hepatic GR activity compared with the control group. These results strongly suggest that betaine administration for four weeks promoted antioxidant activity in the liver by increasing hepatic and serum GSH levels. However, among the GSH-related enzyme activities that were measured in this experiment, only GR activity was found to increase significantly following betaine treatment. Therefore, a second experiment was conducted using a hepatitis animal model to further clarify the hepatoprotective effects of betaine administration.

In order to simulate hepatitis-like liver injury in experiment 2, we injected rats with d-GalN, an amino sugar that is selectively metabolized by hepatocytes and causes hepatotoxicity. Although d-GalN is known for inducing diffuse hepatic necrosis after a single intraperitoneal or parenteral administration, the biochemical mechanisms of this effect have not been fully elucidated. It has been suggested that d-GalN induces hepatotoxicity by decreasing the uridine 5′-triphosphate concentration in the hepatocytes, resulting in inhibition of mRNA and protein synthesis, ultimately leading to liver cell necrosis.

Previous studies have also found that endotoxins are involved in the induction of this hepatic injury and promote the secretion of proinflammatory cytokines. Another suggested mechanism of d-GalN-induced hepatic injury is through reactive oxygen species (ROS) produced by activated hepatic macrophages and it has been reported that d-GalN injection leads to liver damage by promoting reactions that generate ROS or oxidative stress. Although the detailed mechanisms following d-GalN injection are still under investigation, it is likely that liver injury occurs as a result of a combination of the events described above.

The results of experiment 2 showed detrimental effects of d-GalN on metabolic liver function, as indicated by increased serum activities of ALT, LDH, AST and ALP for the d-GalN-injected control group (CG). Increases in the activities of ALT and AST have previously been reported after d-GalN exposure. In contrast, these enzymatic activities were reduced by 76% (ALT), 81% (LDH), 77% (AST), and 28% (ALP) in the d-GalN-injected group that received 1% betaine supplementation (group B1G), which exhibited levels similar to the CN-2 group. These results indicate that betaine supplementation prevented the histopathological alterations and increases in enzymatic activity normally induced by d-GalN, demonstrating a significant hepatoprotective effect of betaine in this model of hepatic liver injury.

### Table 1. Changes in Hepatic SAM and Various Amino Acids Levels

<table>
<thead>
<tr>
<th></th>
<th>CN-2 (μg/mg, liver)</th>
<th>CG (μg/mg, liver)</th>
<th>B1G (μg/mg, liver)</th>
<th>G2G (μg/mg, liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM</td>
<td>0.33 ± 0.07</td>
<td>0.27 ± 0.07</td>
<td>0.72 ± 0.24</td>
<td>0.51 ± 0.19</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.08 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>0.19 ± 0.02</td>
<td>0.15 ± 0.03</td>
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<tr>
<td>Cysteine</td>
<td>0.02 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.03 ± 0.00</td>
<td>0.01 ± 0.00</td>
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*Mean values within a row with different superscript letters are significantly different (p < 0.05) by the Tukey-Kramer test.

**p < 0.01, "p < 0.05 versus CN-2 group by Student’s t-test."
damage. However, it should be noted that elevated levels of betaine (2% dietary supplementation) lessened this effect, possibly due to over-dosing. These findings highlight the importance of future research to determine the treatment level required to obtain maximum benefits and to prevent over-dosing.

Further investigation was conducted to better understand the effects of betaine administration on GSH levels and related enzyme activities among the animal models in experiment 2 (Fig. 4). Both hepatic and serum GSH levels were significantly lower in the d-GalN treated group, but the group that was injected with d-GalN while receiving 1% betaine supplementation showed GSH levels similar to the CN-2 group. Hepatic GSH-related enzymatic activity was also examined, because these enzymes are essential to the overall regulation of GSH and the redox balance in the biological system. The enzyme GST is known to inactivate electrophiles produced by ROS and reactive nitrogen species by catalyzing the conjugation of electrophilic compounds with GSH, thus plays a vital role in drug conjugation and detoxification.30,31 Several experimental and clinical studies have shown that d-GalN ingestion alters the prooxidant-antioxidant balance in the organism,32 and as mentioned above, d-GalN has been suggested to increase the production of ROS.27 Therefore, the reduced levels of hepatic GST activity observed here following d-GalN injection are probably associated with d-GalN-induced liver injury and subsequent increases in ROS. However, our results suggest that supplementation with either 1% or 2% betaine can increase GST activity in rats with d-GalN-induced liver injury, with greater results observed for 1% betaine.

GPx is a potent antioxidant enzyme that reduces H₂O₂ and lipid peroxides and hydroperoxides through oxidation of GSH. Even with d-GalN treatment, the hepatic activity of GPx in both the 1% and 2% betaine-supplemented group was statistically similar to the control group, whereas the d-GalN-injected group without betaine supplementation showed significantly reduced levels of GPx activity. Similarly to the activities of GST, GPx may have a significant hepatoprotective role in terms of oxidative damage recovery by reducing the levels of ROS.

Finally, to further examine the mechanisms behind the positive effects of betaine on GSH levels and the activities of GSH-related enzymes, the levels of some sulfur-containing betaine intermediates and related free amino acids were determined for the animal models in experiment 2. Hepatic SAM levels were significantly elevated for the group receiving 1% betaine supplementation compared with all other groups (Table 1). While sulfhydryl-donating compounds, including SAM, have been found to protect against d-GalN induced liver injury, the underlying mechanisms have not been clarified. In general, the hepatoprotective effects of sulfhydryl-donating compounds are attributed to their capacity to stimulate the synthesis of GSH; however, d-GalN inhibits GSH re-synthesis in isolated hepatocytes.32,33 Besides being a sulfhydryl-donating compound, SAM is a universal biological methyl donor for a variety of acceptor substrates, including nucleic acids, proteins, phospholipids, and biologic amines.34 It is also the precursor of aminopropyl groups in polyamine biosynthesis and of GSH in the liver.3,11,35 Moreover, the hepatic cysteine concentration showed a trend similar to that found for the other parameters tested in this study, with significantly higher concentrations in the d-GalN-injected group receiving 1% betaine supplementation (0.03 ± 0.02 μg/mg liver) compared with the d-GalN-injected group without betaine supplementation (0.01 ± 0.00 μg/mg liver) and the B2G group (0.01 ± 0.00 μg/mg liver). It has been suggested that SAM can serve as a precursor to GSH through conversion to cysteine by the trans-sulfuration pathway.35

Together with previous studies, our findings indicate that betaine probably has protective effects on the liver by initiating a chain of events resulting in the promotion of enzymes and sulfur-containing metabolites that are important in reducing oxidative stress and ROS levels. Specifically, 1% betaine treatment was found to elevate the GSH concentration and related enzyme activities even following d-GalN injection. These results support the assertion that betaine is converted to SAM in the liver, then to SAH, and finally to GSH, a strong antioxidant that functions in concert with the enzymes GR, GPx, and GST to promote liver health.

In conclusion, this study indicates the potential of betaine to serve as a safe hepatoprotective agent and to help maintain the normal functions of the liver following injury. Although betaine shows promise for the amelioration of liver injury in a human health setting, additional analysis is necessary in order to determine the most suitable treatment levels.

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References