Hepatic glutathione contributes to attenuation of thioacetamide-induced hepatic necrosis due to suppression of oxidative stress in diet-induced obese mice

Makoto Shirai, Miho Matsuoka, Toshihiko Makino, Kiyonori Kai, Munehiro Teranishi and Wataru Takasaki

Medicinal Safety Research Laboratories, Daiichi Sankyo Co., Ltd., 1-16-13 Kitakasai, Edogawa-ku, Tokyo 134-0081, Japan

(Received March 14, 2015; Accepted May 7, 2015)

ABSTRACT — We previously reported that hepatic necrosis induced by thioacetamide (TA), a hepatotoxicant, was attenuated in mice fed a high-fat diet (HFD mice) in comparison with mice fed a normal rodent diet (ND mice). In this study, we focused on investigation of the mechanism of the attenuation. Hepatic content of thiobarbituric acid reactive substances (TBARS), an oxidative stress marker, significantly increased in ND mice at 24 and 48 hr after TA administration in comparison to that in vehicle-treated ND mice. At these time points, severe hepatic necrosis was observed in ND mice. Treatment with an established antioxidant, butylated hydroxyanisole, attenuated the TA-induced hepatic necrosis in ND mice. In contrast, in HFD mice, hepatic TBARS content did not increase, and hepatic necrosis was attenuated in comparison with ND mice at 24 and 48 hr after TA dosing. Metabolomics analysis regarding hepatic glutathione, a biological antioxidant, revealed decreased glutathione and changes in the amount of glutathione metabolism-related metabolites, such as increased ophthalmate and decreased cysteine, and this indicated activation of glutathione synthesis and usage in HFD mice. Finally, after treatment with L-buthionine-S,R-sulfoxinine, an inhibitor of glutathione synthesis, TA-induced hepatic necrosis was enhanced and hepatic TBARS contents increased after TA dosing in HFD mice. These results suggested that activated synthesis and usage of hepatic GSH, which suppresses hepatic oxidative stress, is one of the factors that attenuate TA-induced hepatic necrosis in HFD mice.

Key words: Mouse, Oxidative stress, High-fat diet, Thioacetamide, Glutathione, Liver

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a very common disease in developed countries (Cohen et al., 2011). The hepatocytes of NAFLD patients or NAFLD model animals show abnormalities in mitochondrial function (Alberici et al., 2011; Mantena et al., 2009) and drug-metabolizing enzyme expression (Merrell and Cherrington, 2011; Videla et al., 2004). Hence, it is strongly suggested that NAFLD patients would be susceptible to a certain type of drug-induced hepatotoxicity. Basic research using animal models of NAFLD, to test whether the animals are susceptible to hepatotoxicants, is considered to be important to evaluate the risk of drug-induced hepatotoxicity in NAFLD patients. However, such research is limited.

Thioacetamide (TA) is a well-known hepatotoxicant, and it induces acute centrilobular necrosis by its reactive metabolite, TA-S,S-dioxide (Chilakapati et al., 2005). We previously reported that TA-induced hepatic necrosis was attenuated in mice fed a high-fat diet (HFD mice) in comparison with mice fed a normal rodent diet (ND mice) (Shirai et al., 2013). We also demonstrated that hepatic total glutathione (the sum of glutathione (GSH) and oxidized form of glutathione, glutathione disulfide (GSSG)) in HFD mice was significantly lower than that in ND mice after TA dosing (Shirai et al., 2013). GSH is a major biological antioxidant (Lu, 1999; Kretzschmar, 1996; Yuan and Kaplowitz, 2009), and it was reported that TA treatment depleted GSH and increased oxida-
tive stress in hepatocytes (Staňková et al., 2010). These results and reports suggested that the amount of hepatic GSH and oxidative stress are likely factors for the mechanism of the attenuated TA-induced hepatic necrosis in HFD mice. However there has been no report that confirmed the importance of GSH to attenuate TA-induced hepatic necrosis in mice. Moreover, the importance of GSH in the attenuation of TA-induced hepatic necrosis in HFD mice remains unclear because the amount of GSH itself was not investigated in the previous study (Shirai et al., 2013).

GSH is synthesized from glutamate, cysteine and glycine, and is converted to GSSG in the process of scavenging. Several metabolites are known to be involved in GSH metabolism (Kim et al., 2003; Soga et al., 2006; Wu et al., 2004; Lu, 1999), and hence, the status of GSH metabolism can be estimated by investigation of each of the metabolites using metabolomics analysis (Agudo-Barriuso et al., 2013). On the other hand, gamma-glutamlycysteine synthetase is the rate-limiting enzyme of GSH synthesis (Lu, 2000). A specific inhibitor of this enzyme, L-buthionine-S,R-sulfoxinine (BSO), can decrease the GSH level, even in vivo (Watanabe et al., 2003; Shimizu et al., 2009). By investigating hepatic metabolites of GSH synthesis and examining the effect of BSO treatment on hepatic oxidative stress and hepatic necrosis after TA dosing, therefore, we can confirm whether GSH is important in attenuation of TA-induced hepatic necrosis in HFD mice.

The purpose of this study was to investigate whether hepatic GSH contributes to the attenuated TA-induced hepatic necrosis due to suppression of oxidative stress in HFD mice. In this study, the level of hepatic oxidative stress and the synthesis and metabolism of hepatic GSH after TA administration were compared between HFD and ND mice. In addition, hepatic oxidative stress and hepatic necrosis were also evaluated in HFD mice given TA with and without BSO.

MATERIALS AND METHODS

Chemicals

TA and dimethyl sulfoxide were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and 5-bromo-2-deoxyuridine (BrdU), butylated hydroxyanisole (BHA) and BSO were purchased from Sigma-Aldrich Co. (Tokyo, Japan).

Experimental design

In this study, three experiments were performed as described below. The timing of the treatments and the necropsies are outlined in Fig. 1.

Experiment 1 (comparison of TA-induced hepatotoxicity in ND and HFD mice)

Animals and In-Life Experiments

Three-week-old male C57BL/6J mice were obtained from Charles River Japan, Inc. (Yokohama, Japan), and were fed either a normal rodent diet (CRF-1; Oriental Yeast Co. Ltd., Tokyo, Japan) or a high-fat diet containing 60 kcal% fat (high fat diet 32; Clea Japan, Inc., Tokyo, Japan) and were given tap water ad libitum as described previously (Shirai et al., 2013). After 8 weeks of feeding, the HFD mice and the ND mice were allocated into groups for the toxicity studies and a separate group for the toxicokinetics study. From a preliminary study, high mortality rate was expected in ND mice at 24 and 48 hr after TA dosing. Hence, in the toxicity group, the number of ND mice necropsied at 24 and 48 hr was approximately double that of HFD mice. All mice were given either TA (100 mg/kg body weight; dissolved with saline) or saline once intraperitoneally. The dose level of 100 mg/kg was chosen based on an in-house preliminary study that showed severe hepatic necrosis in all the ND mice at 24 hr after administration at this dose. After the TA dosing, the mice in the toxicity study group were necropsied at 3, 8, 24 and 48 hr. Vehicle control mice were injected with saline and necropsied at 8 and 24 hr after administration for metabolomics analysis, or at 24 hr after administration for the other investigations described below. In the toxicity study groups necropsied at 8, 24 and 48 hr, mice were given BrdU (100 mg/kg body weight, dissolved with 1% dimethyl sulfoxide/saline) intraperitoneally 2 hr before necropsy for additional evaluations (which will be reported separately). The number of surviving animals was recorded at each necropsy time point. The animals found dead were necropsied immediately after they were found. For the toxicokinetic analysis group, plasma samples were collected from the vena cava under anesthesia by isoflurane (Pfizer Inc., Tokyo, Japan) at 5, 15, 30, 60, 120, 180 and 480 min after TA dosing.

Collection of liver samples and blood samples

At necropsy, all animals in the toxicity group were euthanized after blood collection for blood chemistry under anesthesia. After euthanasia, the livers were collected. The left lateral lobe and right and left medial lobes were trimmed and fixed in 10% neutral buffered formalin and embedded in paraffin for histopathology. To confirm fatty change of hepatocytes, the remaining portions of the
left lateral lobes of vehicle-treated mice were trimmed and fixed with glutaraldehyde followed by osmium tetroxide. After fixation, the liver samples were embedded in Poly/Bed 812 (Polysciences, Warrington, PA, USA), and the histopathological specimens were stained with toluidine blue. The remaining portions of the livers were frozen in liquid nitrogen and stored at -80°C in a deep freezer for the analyses described below.

Histopathological examination
Histopathological specimens were prepared and stained with hematoxylin and eosin (HE). As reported previously (Shirai et al., 2013), TA-induced centrilobular necrosis of hepatocytes and centrilobular swelling of hepatocytes were graded as described below.

Centrilobular necrosis of hepatocytes
Grade 1: Centrilobular necrosis that reached zone 2 was observed in less than 5 lobules in the 3 lobes (the left lateral, right medial and left medial lobes).
Grade 2: Centrilobular necrosis that reached zone 2 was observed in 5 or more lobules, or centrilobular necrosis that reached zone 1 was observed in less than 5 lobules in the 3 lobes.
Grade 3: Centrilobular necrosis that reached zone 1 was observed in 5 or more lobules in the 3 lobes, and the necrotic area was less than 33% of 1 or more of the 3 lobes.
Grade 4: The necrotic area was 33% to 66% of 1 or more the 3 lobes.
Grade 5: The necrotic area was more than 66% of 1 or more of the 3 lobes.

Centrilobular swelling of hepatocytes
Grade 1: Centrilobular swelling was observed, but it was limited to the centrilobular area in the hepatic lobe (zone 3).
Grade 2: Centrilobular swelling that reached zone 2 was observed in less than 50% of lobules.
Grade 3: Centrilobular swelling that reached zone 2 was observed in 50% or more of lobules.

Fig. 1. Schematic diagram of experiments 1 to 3. Each diagram outlines the timing of thioacetamide (TA), butylated hydroxyanisole (BHA) and L-buthionine-S,R-sulfoximine (BSO) treatments and necropsies. hr: hours, ND mice: mice fed a normal rodent diet, HFD mice: mice fed a high fat diet.
Measurement of oxidative stress

As an indicator of oxidative stress, lipid peroxidation in the frozen liver sample was estimated by detecting thiobarbituric acid reactive substances (TBARS) using the TBARS assay kit (TBARS kit; Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer’s instructions.

Metabolomics analysis of glutathione metabolism in the liver

To investigate the glutathione metabolism in the liver, a metabolomics analysis was performed at Metabolon Inc. (Durham, NC, USA). From surviving mice, we selected liver samples whose average necrotic score was approximately equal to the average score of all of the mice at each time point (n = 5); five of grade 0 in the vehicle-treated ND and HFD mice at 8 and 24 hr, four of grade 0 and 1 of grade 1 in the TA-dosed ND mice at 8 hr, two of grade 0 and 3 of grade 1 in the TA-dosed HFD mice at 8 hr, five of grade 4 in the TA-dosed ND mice at 24 hr, and three of grade 2 and 3 of grade 3 in the TA-dose HFD mice at 24 hr. The detailed conditions of sample preparation and analysis platform have been described in a previous publication (Evans et al., 2009). In brief, the samples from the frozen liver were prepared using an automated MicroLab STAR® system (Hamilton Company, Salt Lake City, UT, USA). The samples were then separated into three equal aliquots for analysis in three independent platforms: ultra-high performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) optimized for basic species, UHPLC/MS/MS optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). Metabolites were identified by automated comparison of the ion features in the experimental samples with a reference library of purified standards; these features included retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as their associated MS/MS spectra. Among the identified metabolites, metabolites relating to glutathione metabolism were analyzed.

Toxicokinetic analysis for TA and thioacetamide-S-oxide

Twenty microliters of each collected plasma sample was mixed with 100 μL of methanol and centrifuged at 20,000 rpm for 5 min at 4°C. The supernatant of the sample was analyzed by high-performance liquid chromatography as described previously (Shirai et al., 2013), and the concentrations of TA and thioacetamide-S-oxide (TASO) were determined.

Experiment 2 (the effect of antioxidant treatment on TA-induced hepatotoxicity in ND mice)

In-Life experiments and histopathological examination

Nine-week-old male C57BL/6J mice were obtained from Charles River Japan, Inc., and were fed a normal rodent diet (powdered CRF-1; Oriental Yeast Co. Ltd.) with or without 0.75% BHA for 14 days, as described in a previous report (Banerjee et al., 1995). At the 13th day of the feeding period, all of the mice were given either TA (100 mg/kg) or saline once intraperitoneally as described in Experiment 1, and necropsied 24 hr later. The livers were fixed in formalin, and histopathological examination including grading of hepatic necrosis and swelling was performed as described in Experiment 1.

Experiment 3 (the effect of GSH depletion on TA-induced hepatotoxicity in HFD mice)

Animals and In-Life experiments

As in Experiment 1, 3-week-old male C57BL/6J mice were obtained from Charles River Japan, Inc., and fed a high-fat diet (high fat diet 32) ad libitum to produce the HFD mice. After 8 weeks of feeding, all of the mice were allocated into groups for the toxicity studies and a separate group for the toxicokinetics study. All of the mice were given either TA (100 mg/kg) or saline once intraperitoneally as described in Experiment 1 (except for mice necropsied at 0 hr), and were necropsied at 1 hr after the 1st BSO treatment (represented as 0 hr after TA treatment) or were necropsied at 3, 8, 24 and 48 hr after the TA or saline administration. To decrease hepatic glutathione, as described previously (Shimizu et al., 2009), all of the mice were given BSO (700 mg/kg body weight; dissolved with saline) or saline twice intraperitoneally, once at 1 hr before and again at 5 hr after TA or saline administration (except for mice necropsied at 0 and 3 hr). Hence, there were 4 groups, vehicle-only treated group (vehicle/vehicle group), BSO- and vehicle-treated group (BSO/vehicle group), vehicle- and TA-treated group (vehicle/TA group) and BSO- and TA-treated group (BSO/TA group). After necropsy, formalin-fixed samples and frozen samples were collected from the livers as described in Experiment 1. For the toxicokinetic analysis group, plasma samples were collected from the vena cava under anesthesia by isoflurane at 5, 15, 30, 60, 120 and 180 min after TA dosing.
Histopathological examination, measurement of oxidative stress and toxicokinetic analysis

Histopathological examination including grading of hepatic necrosis and swelling, TBARS assay in the liver, and toxicokinetic analysis for TA were performed as described in Experiment 1.

Measurement of hepatic glutathione and glutathione disulfide

According to the manufacturer’s instructions, total glutathione contents and GSSG contents of the frozen liver samples were directly measured using a Bioxytech GSH: GSSG-412 assay kit (OXIS International Inc., Beverly Hills, CA, USA), and the content of glutathione was calculated from the obtained results.

Ethics

All of the studies were approved by the Ethics Review Committee for Animal Experimentation of Daiichi Sankyo Co., Ltd., and were conducted in compliance with the “Law Concerning the Protection and Control of Animals” (Japanese Law 105, October 1, 1973; revised on June 22, 2005).

Statistical analysis

The results are expressed as the mean ± S.D. In lipid peroxidation analysis and glutathione analysis, the F-t test was performed. The homogeneity of variance was evaluated by the F test, and the Student’s t test (for homogeneous data) or the Aspin-Welch t test (for heterogeneous data) was performed using Microsoft Office Excel 2003 (Microsoft, Redmond, WA, USA). In lipid peroxidation analysis, the parametric Dunn’s test for comparison among 5 groups was also performed using SAS Drug Development 2.1 (SAS Institute Inc., Cary, NC, USA). In histopathological analysis, the Mann-Whitney U test was performed using the common gateway interface program (http://aoki2.si.gunma-u.ac.jp/exact/utest/getpar.html). In metabolomics analysis, a log transformation was applied to the observed relative concentrations for each metabolite because, in general, the variance increases as a function of the average response of the metabolite. After log transformation, a three-way ANOVA was performed using R (http://cran.r-project.org/), and multiple comparisons were performed with the false discovery rate (FDR) method. Each FDR was estimated using the q-values. We used the conservative criteria of p < 0.05 and q < 0.05 to judge statistical significance in all statistical analyses.

RESULTS

The effects of high fat diet on liver morphology (Experiment 1)

Similar to the previous study (Shirai et al., 2013), fatty change of hepatocytes was noted in all of the HFD mice treated with vehicle after feeding for 8 weeks (Table 1). The livers of the HFD mice did not show inflammatory or necrotic changes. In addition, the HFD mice reproducibly showed 1.33-fold heavier body weight, significantly higher plasma concentrations of glucose and total cholesterol, and lower plasma concentration of triglycerides in comparison to those of the ND mice after feeding for 8 weeks (data not shown).

Histopathological examination (Experiment 1)

From 24 to 48 hr after TA dosing, thirteen out of 31 ND mice treated with TA were found dead (Table 1).

Table 1. Lethality and histopathological scores of hepatic lesions after thioacetamide administration.

<table>
<thead>
<tr>
<th>Hours after TA administration</th>
<th>Control</th>
<th>3 hr</th>
<th>8 hr</th>
<th>24 hr</th>
<th>48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice fed a normal rodent diet +</td>
<td>6 (0)</td>
<td>6 (0)</td>
<td>6 (0)</td>
<td>19 (9)</td>
<td>12 (4)</td>
</tr>
<tr>
<td>Mean score of necrosis</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.2 ± 0.41</td>
<td>4.3 ± 0.58</td>
<td>4.7 ± 0.65</td>
</tr>
<tr>
<td>Mean score of swelling</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>1.0 ± 0.63</td>
<td>0.7 ± 0.99</td>
<td>1.0 ± 1.04</td>
</tr>
<tr>
<td>Mice fed a high fat diet +</td>
<td>6 (0)</td>
<td>6 (0)</td>
<td>6 (0)</td>
<td>8 (0)</td>
<td>6 (0)</td>
</tr>
<tr>
<td>Mean score of necrosis</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.5 ± 0.55</td>
<td>2.5 ± 0.76 **</td>
<td>3.2 ± 0.75 **</td>
</tr>
<tr>
<td>Mean score of swelling</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>3.0 ± 0.00 **</td>
<td>3.0 ± 0.00 **</td>
<td>3.0 ± 0.00 **</td>
</tr>
</tbody>
</table>

Control: The mice necropsied at 24 hr after vehicle administration. 3 to 48 hr: Animals necropsied at 3 to 48 hr after thioacetamide (TA) administration, respectively. Mean score is the mean of grades of hepatic necrosis and swelling (0 to 5 for hepatocellular necrosis and 0 to 3 for hepatocellular swelling) for individual mice ± S.D. + The number represents the total number of mice necropsied at each time point, and the values in parentheses show the number of dead mice. **: p < 0.01 for comparison versus the mice fed a normal rodent diet (Mann-Whitney U test).
All of the dead ND mice showed severe hepatocellular necrosis (grade 4 or 5). In the surviving ND mice, hepatic necrosis was slight at 8 hr, and was severe at 24 hr or more (Fig. 2-A, Table 1 and Supplementary Table 1). In contrast, none of the HFD mice was found dead after TA dosing, and the severity of hepatic necrosis was significantly less compared with that in the ND mice (Fig. 2-B, Table 1 and Supplementary Table 1). On the other hand, similar to the previous study (Shirai et al., 2013), severe swelling of hepatocytes was noted from zone 3 to zone 1 in the HFD mice at 8 to 48 hr, while slight swelling was noted in the ND mice (Fig. 2, Table 1 and Supplementary Table 1).

**Toxicokinetics of TA and TASO (Experiment 1)**

The TA and TASO concentrations in plasma reached peak at 5 and 60 min, respectively. At 480 min, neither TA nor TASO was detected in the plasma. The plasma concentration profiles of TA and TASO were not obviously different between HFD and ND mice (Fig. 3).

**Hepatic oxidative stress induced by TA administration (Experiment 1)**

There were no differences in the content of TBARS, a hepatic oxidative stress indicator, between the HFD mice and the ND mice treated with vehicle (Fig. 4). Compared with the vehicle-treated ND mice, hepatic TBARS content of the TA-treated ND mice significantly increased and approximately doubled at 24 and 48 hr, at which time TA-
induced hepatic necrosis was severe. On the other hand, hepatic TBARS content of the HFD mice did not increase after TA administration compared with the vehicle-treated HFD mice. At 48 hr after TA administration, hepatic TBARS content of the HFD mice was significantly decreased compared with the vehicle-treated HFD mice. However, the meaning of this decrease was unclear.

Metabolomics analysis of glutathione metabolism (Experiment 1)

Table 2 shows the results of the metabolomics analysis on hepatic GSH metabolism. Fig. 5 shows the metabolic pathway of GSH (Kim et al., 2003; Soga et al., 2006; Wu et al., 2004; Lu, 1999) and the changes in the amount of each metabolite at 8 and 24 hr after TA administration.

At 8 hr after TA dosing, cysteiny1-glycine was decreased both in the HFD and the ND mice. GSH, cysteine and taurine were decreased only in the HFD mice. Ophthalme, 5-oxoproline and glutamate were increased only in the HFD mice. The amount of gamma-glutamylaminoacids was changed only in the HFD mice.

At 24 hr after TA administration, GSH, glycine and gamma-glutamylaminoacids were decreased both in the HFD and the ND mice. An increase of GSSG and decreases of ophthalme, 5-oxoproline, cysteine, cysteiny1-glycine, and cystathionine were noted only in the ND mice. Increases of 2-aminobutyrate, ophthalme, 5-oxoproline and glutamate, and decreases of taurine and hypotaurine were observed only in the HFD mice.

The effect of treatment with antioxidant BHA on TA-induced hepatic necrosis in ND mice (Experiment 2)

To investigate whether oxidative stress causes TA-induced hepatic necrosis, an established antioxidant BHA was given to ND mice. With BHA treatment, none of the ND mice died, and hepatic necrosis was significantly attenuated (Table 3 and Supplementary Table 2).

TA-induced hepatic necrosis in HFD mice treated with GSH synthesis inhibitor BSO (Experiment 3)

To investigate whether GSH prevents TA-induced hepatic oxidative stress and hepatic necrosis in HFD mice, an established GSH synthesis inhibitor BSO was given to HFD mice by itself or together with TA. In the studies with BSO, 2 out of 25 HFD mice died from 24 to 48 hr after the dose of TA, while none of the HFD mice given TA without BSO died (Table 4 and Supplementary Table 3). In addition, BSO significantly increased the severity of TA-induced hepatic necrosis at 48 hr (Table 4 and Supplementary Table 3). BSO had no apparent effect on the severity of hepatocellular swelling in the HFD mice.

The effect of BSO on the amount of hepatic GSH in HFD mice (Experiment 3)

In comparison to the vehicle/vehicle group, hepatic GSH significantly decreased at 3 and 8 hr after vehicle treatment in the BSO/vehicle group (Fig. 6). At 8 and 24 hr after the dose of TA, hepatic GSH in the vehicle/TA group significantly decreased compared with the vehicle/vehicle group. At 3 to 48 hr after TA dosing, hepatic GSH in the BSO/TA group significantly decreased compared with that of the BSO/vehicle group. In comparison to the vehicle/TA group, hepatic GSH significantly decreased at 3, 8 and 48 hr in the BSO/TA group.

On the other hand, the toxicokinetics of TA in the BSO/TA group was almost the same as that in the vehicle/TA group (data not shown).

The change of TA-induced hepatic oxidative stress by BSO in HFD mice (Experiment 3)

At 24 and 48 hr after TA dosing in the HFD mice, no
statistically significant difference was observed in the hepatic TBARS content of the BSO/vehicle treated group or the vehicle/TA treated group compared with that of the vehicle/vehicle group (Fig. 7). In contrast, at 24 and 48 hr, the hepatic TBARS content in the BSO/TA group significantly increased in comparison to that in the BSO/vehicle group. Moreover, the hepatic TBARS content in the BSO/TA group significantly increased compared with that in the vehicle/TA group at 24 hr.

At 8 hr after dosing, hepatic TBARS content was significantly increased in the vehicle/TA group compared with the vehicle/vehicle group. However, this increase was judged to have little toxicological significance because TA-induced hepatic necrosis in the vehicle/TA group was slight, and hepatic TBARS content and hepatic necrosis were not significantly different between the vehicle/TA group and the BSO/TA group at this time point.

**DISCUSSION**

As shown in our previous report, TA-induced hepatic necrosis was attenuated in HFD mice compared with ND mice (Shirai et al., 2013). In addition, we found out that the amount of hepatic total glutathione, the sum of GSH and GSSG, was significantly lower in HFD mice than that in ND mice after TA administration. Therefore, we focused on investigation of whether hepatic GSH contributes to the attenuation of TA-induced hepatic necrosis in HFD mice.

In this study, the results of hepatic histopathology were confirmed to be similar to those in the previous study (Shirai et al., 2013). Compared with the ND mice, the HFD mice showed attenuated hepatic necrosis, and more severe swelling of hepatocytes after administration of 100 mg/kg TA. In addition, toxicokinetic analysis revealed that there was no apparent difference in the
toxicokinetics of TA and TASO in plasma between HFD and ND mice. Therefore, the attenuation of TA-induced hepatic necrosis in HFD mice could not be interpreted as due to decreased systemic exposure to TA and TASO.

As the first step to investigate the contribution of GSH to the hepatotoxicity, we investigated hepatic oxidative stress after TA administration, because GSH is a major biological antioxidant in the liver (Lu, 1999; Kretzschmar, 1996; Yuan and Kaplowitz, 2009). TBARS content is widely used as a marker of oxidative stress, and there was no significant difference in the hepatic TBARS content between vehicle-treated ND mice and vehicle-treated HFD mice. Therefore, it was indicated that basal oxidative stress level was unaltered by high fat diet feeding.

Table 2. Hepatic metabolites related to glutathione metabolism.

<table>
<thead>
<tr>
<th>Biochemical Name</th>
<th>KEGG</th>
<th>HMDB</th>
<th>8 hr ND</th>
<th>24 hr ND</th>
<th>8 hr HFD</th>
<th>24 hr HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>reduced glutathione (GSH)</td>
<td>C00051</td>
<td>HMDB00125</td>
<td>1.2654</td>
<td>0.7948</td>
<td>0.9547</td>
<td>0.2158 **</td>
</tr>
<tr>
<td>oxidized glutathione (GSSG)</td>
<td>C00127</td>
<td>HMDB03337</td>
<td>1.2612</td>
<td>1.5505</td>
<td>1.0289</td>
<td>1.0188</td>
</tr>
<tr>
<td>2-aminobutyrate</td>
<td>C02261</td>
<td>HMDB00650</td>
<td>1.2228</td>
<td>1.3474</td>
<td>0.6671</td>
<td>0.8998</td>
</tr>
<tr>
<td>ophthalmate</td>
<td>HMDB05765</td>
<td>1.566</td>
<td>1.8858</td>
<td>0.563</td>
<td>1.1334 **</td>
<td></td>
</tr>
<tr>
<td>gamma-glutamylvaline</td>
<td>HMDB11172</td>
<td>1.7219</td>
<td>1.7969</td>
<td>0.8736</td>
<td>1.3433 **</td>
<td></td>
</tr>
<tr>
<td>gamma-glutamylleucine</td>
<td>HMDB11171</td>
<td>1.411</td>
<td>1.2567</td>
<td>1.1111</td>
<td>0.8369</td>
<td></td>
</tr>
<tr>
<td>gamma-glutamylglutamate</td>
<td>1.3583</td>
<td>1.5947</td>
<td>0.989</td>
<td>1.207</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gamma-glutamyltyrosine</td>
<td>1.3496</td>
<td>1.3205</td>
<td>0.9626</td>
<td>0.6544 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-oxoproline</td>
<td>C01879</td>
<td>HMDB00267</td>
<td>1.0666</td>
<td>1.173</td>
<td>0.8696</td>
<td>1.3403 **</td>
</tr>
<tr>
<td>glutamate</td>
<td>C00025</td>
<td>HMDB03339</td>
<td>0.8792</td>
<td>1.3695</td>
<td>0.6578</td>
<td>1.9243 **</td>
</tr>
<tr>
<td>cysteine</td>
<td>C0097</td>
<td>HMDB00574</td>
<td>1.1578</td>
<td>0.839</td>
<td>1.2728</td>
<td>0.5225 **</td>
</tr>
<tr>
<td>cysteinylglycine</td>
<td>C01419</td>
<td>HMDB00078</td>
<td>1.3519</td>
<td>0.7326 **</td>
<td>0.8872</td>
<td>0.5702 **</td>
</tr>
<tr>
<td>glycine</td>
<td>C0037</td>
<td>HMDB00123</td>
<td>1.4823</td>
<td>1.438</td>
<td>0.9784</td>
<td>0.751</td>
</tr>
<tr>
<td>cystathionine</td>
<td>C02291</td>
<td>HMDB00099</td>
<td>1.2005</td>
<td>1.0451</td>
<td>1.067</td>
<td>0.8442</td>
</tr>
<tr>
<td>hypotaurine</td>
<td>C00519</td>
<td>HMDB00965</td>
<td>2.2075</td>
<td>3.4176</td>
<td>0.8986</td>
<td>0.5556</td>
</tr>
<tr>
<td>taurine</td>
<td>C0245</td>
<td>HMDB00251</td>
<td>0.9993</td>
<td>1.0219</td>
<td>1.3407</td>
<td>0.6637 **</td>
</tr>
</tbody>
</table>

Table 3. Histopathological scores of the hepatic lesions after thioacetamide administration with or without butylated hydroxyanisole treatment.

<table>
<thead>
<tr>
<th>Hours after TA administration</th>
<th>Control</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice fed a normal rodent diet without BHA ab</td>
<td>6 (0)</td>
<td>19 (9)</td>
</tr>
<tr>
<td>Mean score of necrosis</td>
<td>0.0 ± 0.00</td>
<td>4.3 ± 0.58</td>
</tr>
<tr>
<td>Mean score of swelling</td>
<td>0.0 ± 0.00</td>
<td>0.7 ± 0.99</td>
</tr>
<tr>
<td>Mice fed a normal rodent diet with BHA a</td>
<td>3 (0)</td>
<td>4 (0)</td>
</tr>
<tr>
<td>Mean score of necrosis</td>
<td>0.0 ± 0.00</td>
<td>1.8 ± 0.50 **</td>
</tr>
<tr>
<td>Mean score of swelling</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
</tbody>
</table>

Control and 24 hr: Animals necropsied at 8 or 24 hr after vehicle or thioacetamide (TA) administration, respectively (n = 5). ND: mice fed a normal rodent diet, HFD: mice fed a high fat diet KEGG: Kyoto Encyclopedia of Genes and Genomes, HMDB: Human Metabolome Database Each value was re-scaled to have median equal to 1. The orange shading shows values that were significantly higher than those of time- and diet-matched, vehicle-treated groups. The blue shading shows values that were significantly lower than those of time- and diet-matched, vehicle-treated groups. *, **: p < 0.05 or p < 0.01 versus time-matched vehicle-treated group (three-way ANOVA).

V ol. 40 No. 4
under this study protocol. Hepatic TBARS content in the ND mice was increased at 24 and 48 hr after TA dosing, and it was significantly higher than that of the HFD mice. At this time point, massive TA-induced hepatic necrosis was observed, and the necrosis in the ND mice was significantly more severe than that in the HFD mice. In addition, we clarified that TA-induced hepatic necrosis was significantly ameliorated when BHA, a well-known antioxidant (Nair et al., 2006; Yuan et al., 2006), was given. These results suggested that increased hepatic oxidative stress enhances TA-induced hepatic necrosis in ND mice, and that suppression of TA-induced hepatic oxidative stress is responsible, at least in part, for the attenuation of necrosis in HFD mice.

As mentioned above, GSH is an important hepatic antioxidant, and oxidative stress is one of the causes of TA-induced hepatic necrosis. GSH is always intracellularly synthesized and metabolized in the livers of mice (the half-life is 145 min) (Sekura and Meister, 1974). Therefore if TA affects the metabolism of GSH and changes the level of GSH, then this could influence the severity of TA-induced hepatic necrosis. Hence, we investigated GSH metabolism at 8 and 24 hr after TA dosing to investigate its correlation with histopathological severity of the TA-induced hepatic necrosis, which was slight at 8 hr or severe at 24 hr. At 8 hr after TA administration, many of the hepatic metabolites related to GSH metabolism were changed in the HFD mice. Among these metabolites, ophthalmate increased only in the HFD mice. Ophthalmate is an indicator of the activity of glutathione synthesis because this is produced by the enzymes of glutathione synthesis, gamma glutamylcysteine synthetase and glutathione synthetase (Soga et al., 2006). Cysteine, which is a component of GSH (Kim et al., 2003; Wu et al., 2004) and a rate-limiting amino acid for GSH synthesis (Lu, 2000), was decreased only in the HFD mice. These facts indicated increased synthesis of GSH in the liver of the HFD mice treated with TA. Cysteine is unstable in extracellular spaces, so extracellular GSH serves as cysteine storage (Lu, 1999). Extracellular GSH is metabolized to cysteinyl-glycine by gamma-glutamyltranspeptidase (Lu, 1999). Cysteinyl-glycine also decreased in the HFD mice, and there is a possibility that this decrease was due to decreased metabolism of extracellular GSH. However, one of the hepatic gamma-glutamylaminoacids, which are made from extracellular GSH by glutamyltranspeptidase as with cysteine-glycine (Kim et al., 2003), was increased only in the HFD mice, although another of

Table 4. The effect of L-buthionine-S,R-sulfoxinine administration on thioacetamide-induced hepatic lesions in mice fed a high fat diet.

<table>
<thead>
<tr>
<th>Hours after TA administration</th>
<th>0 hr</th>
<th>3 hr</th>
<th>8 hr</th>
<th>24 hr</th>
<th>48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle/vehicle group</td>
<td>5 (0)</td>
<td>4 (0)</td>
<td>4 (0)</td>
<td>5 (0)</td>
<td>4 (0)</td>
</tr>
<tr>
<td>Mean score of necrosis</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>Mean score of swelling</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>BSO/vehicle group</td>
<td>5 (0)</td>
<td>5 (0)</td>
<td>5 (0)</td>
<td>5 (0)</td>
<td>5 (0)</td>
</tr>
<tr>
<td>Mean score of necrosis</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>Mean score of swelling</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>vehicle/TA group</td>
<td>5 (0)</td>
<td>5 (0)</td>
<td>8 (0)</td>
<td>7 (0)</td>
<td></td>
</tr>
<tr>
<td>Mean score of necrosis</td>
<td>0.0 ± 0.00</td>
<td>1.2 ± 0.45</td>
<td>2.4 ± 0.52</td>
<td>2.9 ± 0.69</td>
<td></td>
</tr>
<tr>
<td>Mean score of swelling</td>
<td>0.0 ± 0.00</td>
<td>3.0 ± 0.00</td>
<td>3.0 ± 0.00</td>
<td>2.4 ± 1.13</td>
<td></td>
</tr>
<tr>
<td>BSO/TA group</td>
<td>5 (0)</td>
<td>5 (0)</td>
<td>12 (0)</td>
<td>13 (2)</td>
<td></td>
</tr>
<tr>
<td>Mean score of necrosis</td>
<td>0.0 ± 0.00</td>
<td>0.8 ± 0.45</td>
<td>3.2 ± 1.03</td>
<td>4.2 ± 0.80**</td>
<td></td>
</tr>
<tr>
<td>Mean score of swelling</td>
<td>0.0 ± 0.00</td>
<td>3.0 ± 0.00</td>
<td>3.0 ± 0.00</td>
<td>2.9 ± 0.29</td>
<td>2.0 ± 1.41</td>
</tr>
</tbody>
</table>

0 hr: Animals necropsied at 1 hr after single dosing of L-buthionine-S,R-sulfoxinine (BSO) without thioacetamide (TA) dosing. 3 to 48 hr: Animals necropsied at 3 to 48 hr after TA administration, respectively. vehicle/vehicle group: group treated only with vehicle, BSO/vehicle group: group treated with BSO and vehicle, vehicle/TA group: group treated with vehicle and TA, BSO/TA group: group treated with BSO and TA. Mean score is the mean of grades of hepatic necrosis and swelling (0 to 5 for hepatocellular necrosis and 0 to 3 for hepatocellular swelling) for individual mice ± S.D. #: The number represents the total number of mice necropsied at each time point, and the values in parentheses show the number of dead mice. #: Not applicable because the mice necropsied at 0 hr were not treated with TA. **: p < 0.01 versus the score of the time point matched group treated with vehicle and TA (Mann-Whitney U test).
the hepatic gamma-glutamylaminoacids decreased in the HFD mice. In addition, hepatic 5-oxoproline and glutamate, which are metabolites of gamma-glutamylaminoacids (Kim et al., 2003), were also increased only in the HFD mice. These data indicate that metabolism of extracellular GSH increased in HFD mice after TA dosing, and that the decrease in cysteinyl-glycine occurred due to increased usage of cysteine. Taken together, these data indicate that synthesis of intracellular GSH was up-regulated in HFD mice after TA dosing. At 24 hr after TA administration, the changes in the pattern of hepatic metabolites of GSH in HFD mice were mainly similar to those observed at 8 hr. In addition, hepatic hypotaurine and taurine, which are synthesized from cysteine as with GSH (Lu, 1999), decreased only in the HFD mice. It was reported that in rat hepatocytes, GSH synthesis was favored more than taurine synthesis when cysteine concentration was low (Stipanuk et al., 1992). Therefore, this decreased taurine metabolism indicates that hepatic cysteine in HFD mice was selectively used for GSH synthesis. In the ND mice,
on the other hand, ophtalmate, gamma-glutamylaminoacids, 5-oxoproline, glutamate, cysteine and taurine did not change at 8 hr. These data suggested that glutathione synthesis and extracellular GSH metabolism was not activated and cysteine usage for GSH synthesis was not prioritized in ND mice at 8 hr. At 24 hr, decreased ophtalmate showed that glutathione synthetase was inactivated. Moreover, 5-oxoproline decreased and cystathionine, a source of cysteine other than extracellular GSH, was also reduced as well as cysteine. This indicated that the supply of cysteine decreased in ND mice at 24 hr. Finally, hypotaurine and taurine did not change. Therefore, selective usage for GSH synthesis was still not observed in ND mice at 24 hr. Hence, it is suggested that glutathione synthesis, extracellular GSH metabolism and cysteine supply decreased while cysteine usage for GSH synthesis was not prioritized in ND mice at 24 hr. Overall, these results suggest that hepatic GSH synthesis and metabolism were more activated in HFD mice, compared with ND mice.

Although GSH metabolism and synthesis seemed to be activated, compared with the vehicle-treated HFD mice, the hepatic GSH content of the HFD mice significantly decreased at 8 and 24 hr after TA dosing. From these apparently paradoxical results, we hypothesized that actively synthesized GSH in the liver of HFD mice was used to reduce oxidative stress after TA dosing. To test our hypothesis, we investigated whether BSO, an established inhibitor of GSH synthesis (Shimizu et al., 2003), would increase oxidative stress and enhance hepatic necrosis after TA dosing. In this study, BSO treatment significantly decreased hepatic GSH of the HFD mice after treatment with vehicle (instead of TA). BSO itself did not induce an increase in hepatic TBARS content or any histopathological changes in the liver of the HFD mice. Therefore, we combined BSO treatment with TA dosing to confirm whether experimentally reduced GSH resulted in increased oxidative stress and enhanced hepatic necrosis in HFD mice after TA administration.

Compared with TA alone, hepatic oxidative stress after treatment with TA and BSO significantly increased at 24 and 48 hr after TA administration. In histopathology, TA-induced hepatic necrosis in the HFD mice was enhanced by BSO-treatment. As mentioned above, oxidative stress is thought to be a cause of TA-induced hepatic necrosis in mice. Moreover, BSO itself did not increase hepatic oxidative stress and did not result in hepatic necrosis, but it decreased the hepatic biological antioxidant, GSH. In addition, the plasma concentration of TA was almost the same whether TA was given with or without BSO in the HFD mice, and therefore, decreased GSH seemed not to influence TA absorption or metabolism. Taken together, our results strongly suggest that synthesis of GSH in the liver is activated in the HFD mice after TA dosing, which inhibits the increase in oxidative stress caused by TA, which in turn attenuates TA-induced hepatic necrosis.

In this study, hepatic GSH level and hepatic TBARS content of the HFD mice were almost the same as those of the ND mice at 24 hr after vehicle treatment. Hence, the balance among GSH synthesis, GSH usage, and oxidative stress level seemed not to be affected in the HFD mice. After TA dosing, however, it is suggested that the balance among these three factors changed, and the severity of hepatic necrosis was modified. Merrell and Cherrington (2011) reported that GSH content decreased in NAFLD patients whereas rodent NAFLD models did not show GSH depletion. Our study indicates the possibility that hepatic toxicity in NAFLD patients would change due to an altered state of glutathione metabolism, but it is unclear whether there are species differences in hepatic basal GSH state and changes in GSH metabolism after hepatotoxicant exposure between NAFLD patients and NAFLD model animals. Further investigation is required to clarify this possibility.

In conclusion, it is suggested that activated synthesis of hepatic GSH, which suppresses hepatic oxidative stress, is one of the factors that attenuate TA-induced hepatic necrosis in HFD mice.

ACKNOWLEDGMENTS

The authors would like to thank Mr. Takayuki Sato for his assistance for care of the mice, and Ms. Kimiko Hara, Shinobu Hakamata and Yoko Suzuki for their assistance for histopathological specimen preparation.

Conflict of interest— -- The authors declare that there is no conflict of interest.
REFERENCE


