INTRODUCTION

Acetaminophen (APAP) is an analgesic and antipyretic drug, which is widely used as an over the counter drug. However, overdosing of APAP causes hepatotoxicity and it is the most frequent cause of drug-induced liver failure in the United States (Lee, 2003). APAP-induced hepatotoxicity is caused by the reactive metabolite, N-acetyl-p-benzoquinoneimine (NAPQI), which is produced by bioactivation after overdosing of APAP and is mainly catalyzed by CYP2E1 (James et al., 2003). Subsequently, NAPQI is detoxified by conjugation with glutathione (GSH) and excreted in urine and bile (James et al., 2003). It has been reported that GSH conjugation of NAPQI proceeds via both non-enzymatic and enzymatic reactions as a result of in vitro study, and enzymatic reaction is catalyzed by glutathione S-transferases (GSTs, EC 2.5.1.18) (Coles et al., 1988). Since the enzymatic reaction is effective only at low concentrations of NAPQI and GSH, the non-enzymatic reaction has been considered to be predominant in GSH conjugation of NAPQI (Coles et al., 1988). In the enzymatic reaction, involvement of GST Alpha, Mu and Pi has been shown in rat and human cytosol, and GST Pi seems to be the most effective isoform to catalyze GSH conjugation of NAPQI, since its conjugation activity is higher than that of GST Alpha or GST Pi.
It is difficult to reveal the contribution of the GST isoform which is responsible for metabolism and toxicity, especially in vivo, when normal animal models are used. However, generation of Gst-null (knockout) mice has enabled us to examine the contribution of the GST isoform in vivo (Board, 2007; Henderson and Wolf, 2011). Gstp1/p2-null mice are the first Gst-null model, and show little GST activity toward ethacrynic acid, a specific substrate for GST Pi (Henderson et al., 1998). APAP administration to Gstp1/p2-null mice unexpectedly results in resistance to APAP-induced hepatotoxicity (Henderson et al., 2000). Covalent binding of NAPQI to liver proteins and APAP-GSH conjugate concentration in bile are similar in wild-type and Gstp1/p2-null mice (Henderson et al., 2000). These results suggest that GSTP1/P2 does not contribute to the GSH conjugation of APAP but plays a novel and important role in APAP-induced hepatotoxicity. Although the mechanism of the resistance in Gstp1/p2-null mice appears to be an increased constitutive JNK activity (Elsby et al., 2003), the detailed mechanism has not been fully elucidated so far.

It has been believed that formation of NAPQI is a crucial step to cause APAP-induced hepatocyte necrosis, since NAPQI is highly reactive and covalently binds to macromolecules (Streeter et al., 1984). However, the importance of a cellular signal, particularly c-jun N-terminal kinase (JNK), has been recently reported (Gunawan et al., 2000; Henderson et al., 2007; Latchoumycandane et al., 2006, 2007). Even when covalent binding by NAPQI occurs, inhibition of the JNK signal by chemical inhibitor (SP600125) protects hepatocytes from APAP-induced necrosis in mice (Gunawan et al., 2006). In addition, in vivo knockdown of JNK using an antisense oligonucleotide also protects against APAP-induced hepatotoxicity in mice (Gunawan et al., 2006). Other groups show similar protective effect of JNK inhibition in mice (Henderson et al., 2007; Latchoumycandane et al., 2007) and immortalized human hepatocytes (Latchoumycandane et al., 2006). Therefore, activation of JNK is considered to play a central role in APAP-induced hepatotoxicity. Regarding upstream events of JNK activation, apoptosis signal-regulating kinase 1 (ASK1) and mitogen-activated protein kinase kinase 4 (MKK4) are well known as upstream kinases, which activate JNK through the phosphorylation cascade (Takeda et al., 2008). Recently, involvement of ASK1 (Nakagawa et al., 2008) and glycogen synthase kinase-3β (GSK-3β) (Shinohara et al., 2010) in APAP-induced hepatotoxicity has been demonstrated, and they seem to be crucial upstream protein kinases to activate JNK.

As experimental animal models, several lines of Gst-null (knockout) mice have been produced and utilized to examine the in vivo role of Gsts (Board, 2007; Henderson and Wolf, 2011). Gstm1- and Gstt1-null mice have been developed by our group (Fujimoto et al., 2006, 2007), since null genotypes of human GSTS have been reported to occur exclusively in GSTM1 and GSTT1 (Hayes et al., 2005). Gstm1- and Gsst1-null mice have the potential to be relevant models of humans with GSTM1- and GSTT1-null genotypes, since we showed the functional similarity between humans and mice for GSTM1 and GSTT1 toward some substrates (Arakawa et al., 2012). In addition, the resistance to APAP-induced hepatotoxicity in Gstp1/p2-null mice, which was described previously, prompted us to perform a study of APAP using Gst-null mice. Single administration of 1,2-dichloro-4-nitrobenzene (DCNB), a specific substrate toward Mu class GST, resulted in higher exposure to DCNB and more marked methemoglobinemia in Gstm1-null mice compared with wild-type mice (Arakawa et al., 2010). Furthermore, the null genotype of GSTM1 alone has been suggested to be a risk factor for drug-induced liver injury (DILI) induced by carbamazepine (Ueda et al., 2007) and antituberculosis drugs in humans (Roy et al., 2001). Based on these backgrounds, we investigated the in vivo role of GSTM1 in APAP-induced hepatotoxicity using Gstm1-null mice.

**MATERIALS AND METHODS**

**Generation and maintenance of Gstm1-null mice**

Gstm1-null mice were generated by homologous recombination in embryonic stem cells as described previously (Fujimoto et al., 2006). Wild-type and Gstm1-null mice were maintained in C57BL/6J and 129S1 mixed background.

**Animal care**

All mice described in these studies were kept in a controlled environment at a room temperature of 23 ± 2°C and humidity of 55 ± 10% with an illumination period of 12 hr (7:00 to 19:00) per day. Each mouse was housed individually in a cage and fed ad libitum with solid diet (Certified Diet CRF-1: Oriental Yeast Co., Ltd., Tokyo, Japan) sterilized by radiation (irradiated with a 60Co γ-ray of 3 kGy), and tap water was supplied by an automatic watering system. The studies were approved by the Ethics Review Committee for Animal Experimentation of Daiichi Sankyo Co., Ltd., and conducted in compliance with the “Law Concerning the Protection and Control of Animals”, Japanese Law No. 105, October 1, 1973, revised on June 22, 2005.
Resistance to APAP-induced hepatotoxicity in Gstm1-null mice

Study designs

Wild-type and Gstm1-null mice (males and females at 15-16 weeks of age) were orally administered with APAP (Sigma-Aldrich, St Louis, MO, USA), which was suspended in 0.5% methylcellulose solution. Since it has been reported that female mice are more resistant to APAP-induced hepatotoxicity than male mice (Dai et al., 2006; Masubuchi et al., 2011), we used both males and females in this study. For the study to investigate the role of GSTM1 in APAP-induced hepatotoxicity, the dose levels of APAP were 0, 125, 250 and 500 mg/kg in males and 0, 250, 500 and 1000 mg/kg in females. The number of animals per group used in this study was 5 animals in 0 mg/kg (control) groups and 10 animals in APAP-dosed groups. Autopsies were performed 24 hr after the dosing. Test items conducted in this study were plasma ALT and liver histopathology. To investigate the mechanism of resistance to APAP-induced hepatotoxicity in Gstm1-null mice, the dose levels of APAP were 0 and 500 mg/kg in both sexes. Four animals were used in each group. Autopsies were performed 2 and 6 hr after the dosing in the APAP-treated groups and 2 hr after dosing in the 0 mg/kg (control) groups. Test items were plasma ALT, liver histopathology, Western blot analysis and liver total GSH.

Measurement of plasma ALT activity

On the day of autopsy, the animals were anesthetized with isoflurane and blood samples were collected from the inferior vena cava. These samples were placed in blood sampling tubes (MICROTAINER: Nippon Becton Dickinson Company, Ltd., Tokyo, Japan) coated with heparin lithium. Plasma alanine aminotransferase (ALT) activity was measured using an autoanalyzer (TBA-200 FR, Toshiba Medical Systems Co., Ltd., Tochigi, Japan).

Liver histopathology

The liver was removed and then fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at a thickness of 2-3 μm and stained with hematoxylin and eosin. The presence and severity of hepatocellular necrosis was examined with a light microscope. The grading score of hepatocellular necrosis was determined based on the percentage of the hepatocytes with necrosis as follows. No change (Grade -) was defined as injury within normal limits. Minimal change (Grade ±) was defined as injury affecting < 10% of hepatocytes. Slight (Grade 1), moderate (Grade 2) and marked (Grade 3) changes were defined as injury affecting 10-25%, 26-50% and > 50% of hepatocytes, respectively.

Western blot analysis

For the Western blot analysis, liver cytosol was prepared using nuclear and cytoplasmic extract reagents (NE-PER: Thermo Fisher Scientific Inc., Rockford, IL, USA). The protein concentration of the cytosol was adjusted to 10 mg/ml with phosphate-buffered saline and subsequently diluted to 5 mg/ml with Tris-SDS beta-mercaptoethanol sample loading buffer (Cosmo Bio Co., Ltd., Tokyo, Japan). Then samples were heated at 95°C for 5 min, and 10 μl (50 μg) of each sample was loaded onto polyacrylamide gel (Funakoshi Corporation, Tokyo, Japan) and subjected to electrophoresis. Polyacrylamide concentrations in the gel were 7.5% for phosphorylated JNK (p-JNK) and total JNK (the sum of p-JNK and non-phosphorylated JNK) analysis, and 10% for phosphorylated GSK-3β (p-GSK-3β) analysis and 12.5% for phosphorylated MKK4 (p-MKK4) analysis. Electrophoresis was performed under constant current (26 mA) condition for 70 min (p-JNK, total JNK, p-GSK-3β, total GSK-3β and total MKK4) or 135 min (p-MKK4). The proteins were transferred from the gel to an Immobilon polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA, USA) using a blotting apparatus (Horizeblot: Atto Corporation, Tokyo, Japan). This membrane was blocked with enhanced chemiluminescence (ECL) blocking agent (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and treated successively with primary antibodies. Anti-human p-JNK (Tyr 183/Tyr 185), JNK, p-GSK-3β (Ser 9), GSK-3β, p-MKK4 (Ser 257), MKK4 antibodies (Cell Signaling Technology, Danvers, MA, USA) and p-GSK-3β (Tyr 216) (Abcam plc, Cambridge, UK) were used as primary antibodies. Then, the membrane was treated with biotin-labeled anti-rabbit IgG antibody (GE Healthcare) as the secondary antibody and finally treated with streptavidin-horseradish peroxidase conjugate (GE Healthcare). Protein-antibody complexes were detected using ECL Western blotting detection reagent (GE Healthcare) and the membrane was exposed to instant film (Fujifilm Corporation, Tokyo, Japan).

Measurement of total GSH

Total GSH in liver was measured using total glutathione quantification kit (Dojindo Laboratory, Kumamoto, Japan) in accordance with the product instructions.

Statistical analyses

The results are expressed as the mean ± standard deviation (S.D.). The values of the mean and S.D. in each group
were calculated with calculation software (Microsoft®
Office Excel 2003: Microsoft Corporation, Redmond,
WA, USA). A parametric Dunnett’s test was performed
to analyze the statistical significance of the differences in
the mean values compared to the control (0 mg/kg) group.
The mean values of two samples were excluded from the
statistical analysis. The statistical analysis was performed
with statistical software (SAS System version 6.1.2: SAS
Institute Inc., Cary, NC, USA). A 5% level of probability
was considered to be statistically significant.

RESULTS

Role of GSTM1 in APAP-induced hepatotoxicity

The role of GSTM1 in APAP-induced hepatotoxicity
was investigated in the single-dose study. Mortality 24 hr
after APAP administration is shown in Table 1. Mortali-
ty in wild-type males given 500 mg/kg was 4/10, but no
mortality was observed in \textit{Gstm1}-null males. Mortality
in wild-type females given 1,000 mg/kg was 8/10, while
2/10 in \textit{Gstm1}-null females. Mortality in \textit{Gstm1}-null
females given 500 mg/kg was 1/10, while no mortality
was observed in wild-type females. However, the mortal-
ity rate observed in \textit{Gstm1}-null females given 500 mg/kg
(1/10) was minimal, and an apparent difference com-
pared with wild-type females was not observed. In total,
the mortality rate in \textit{Gstm1}-null mice was lower than in
wild-type mice in both sexes. Plasma ALT activity in sur-
viving animals measured 24 hr after APAP administra-
tion is shown in Fig. 1. Although a statistically signifi-
cant increase was observed in both wild-type males and
\textit{Gstm1}-null males given 500 mg/kg, the magnitude of the
increase was attenuated in \textit{Gstm1}-null males. An increase
without statistical significance but judged as toxicologi-
cally significant was observed in wild-type males given
250 mg/kg, but not in \textit{Gstm1}-null males. Although sta-
tistical analysis was not performed due to limited sam-
ple numbers (n = 2), an increase in ALT was observed
in wild-type females given 1,000 mg/kg. An increase in
ALT without statistical significance but judged as toxico-

Table 1. Mortality 24 hr after administration of APAP

<table>
<thead>
<tr>
<th>APAP Dose</th>
<th>Strain</th>
<th>Wild-type</th>
<th>\textit{Gstm1}-null</th>
<th>Wild-type</th>
<th>\textit{Gstm1}-null</th>
</tr>
</thead>
<tbody>
<tr>
<td>125 mg/kg</td>
<td>0/10</td>
<td>0/10</td>
<td>Not done</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250 mg/kg</td>
<td>4/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>500 mg/kg</td>
<td>4/10</td>
<td>0/10</td>
<td>0/10</td>
<td>1/10</td>
<td></td>
</tr>
<tr>
<td>1000 mg/kg</td>
<td>Not done</td>
<td>8/10</td>
<td>2/10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Plasma ALT activity 24 hr after single administration of APAP. For males, white, light gray, gray and dark gray bars indicate
APAP dose levels of 0, 125, 250 and 500 mg/kg, respectively. For females, white, light gray, gray and dark gray bars indicate
APAP dose levels of 0, 250, 500 and 1,000 mg/kg, respectively. The values are depicted as the mean ± S.D. of surviving mice
per group. The value of wild-type females given 1,000 mg/kg is the mean of two animals and excluded from statistical analysis.
Significant differences from the control (0 mg/kg) group by Dunnett’s test are shown as * \( P < 0.05 \) and ** \( P < 0.01 \).
logically significant was observed in $Gstm1$-null females given 1,000 mg/kg, and the magnitude of the increase was slighter in $Gstm1$-null females than that in wild-type females. A statistically significant increase was observed in wild-type females given 500 mg/kg, but not in $Gstm1$-null females. Inter-individual variations in plasma ALT activity measured 2 and 6 hr after APAP administration were relatively large, and this may be due to the C57BL/6J and 129S1 mixed background of the animals. Liver histopathology, including dead animals, is shown in Table 2. In total, the grade of the hepatocyte necrosis was lower in $Gstm1$-null mice than that in wild-type mice in both sexes. Apparent sex difference in the role of GSTM1 in APAP-induced hepatotoxicity was not observed, and the resistance to APAP-induced hepatotoxicity in females compared with males was consistent with the previous reports (Dai et al., 2006; Masubuchi et al., 2011). In this study, $Gstm1$-null mice showed resistance to APAP-induced hepatotoxicity in both sexes.

### Mechanism of resistance to APAP-induced hepatotoxicity in $Gstm1$-null mice

The mechanism of the resistance to APAP-induced hepatotoxicity in $Gstm1$-null mice was investigated in the single-dose study of APAP at dose levels of 0 and 500 mg/kg. Plasma ALT activity measured 2 and 6 hr after APAP administration is shown in Fig. 2. A statistically significant increase was observed in wild-type males 6 hr after administration. Although an increase without statistical significance but judged as toxicologically significant was observed in $Gstm1$-null males 6 hr after administration, the magnitude of the increase was slight compared with that in wild-type males. An increase without statistical significance but judged as toxicologically significant was observed in wild-type females 2 hr after administration, but not in $Gstm1$-null mice. An increase without statistical significance but judged as toxicologically significant was observed in wild-type females 2 and 6 hr after administration, but not in $Gstm1$-null mice at either observa-

#### Table 2. Histopathological grade of hepatocyte necrosis 24 hr after administration of APAP

<table>
<thead>
<tr>
<th>Sex and Dose</th>
<th>Grade</th>
<th>Number Examined</th>
<th>Dead Animals</th>
<th>Information of Dead Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male 125 mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>0</td>
<td>10</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>$Gstm1$-null</td>
<td>1</td>
<td>10</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>Male 250 mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>$Gstm1$-null</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Male 500 mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>$Gstm1$-null</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Female 250 mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>10</td>
<td>10</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>$Gstm1$-null</td>
<td>10</td>
<td>10</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>Female 500 mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>$Gstm1$-null</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Female 1000 mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>$Gstm1$-null</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

Grade 0: No change compared with the control (0 mg/kg), ±: Minimal change, Grade 1: Slight, Grade 2: Moderate, Grade 3: Marked
tion time. Inter-individual variations in plasma ALT after APAP administration were relatively large, and may be due to the C57BL/6J and 129S1 mixed background. Liver histopathology is shown in Table 3. The grade of the hepatocyte necrosis was lower in \textit{Gstm1}-null mice compared with that in wild-type mice in both sexes. Apparent sex difference in the role of GSTM1 in APAP-induced hepatotoxicity was not observed, and the resistance to APAP-induced hepatotoxicity in females compared with males was consistent with the previous reports (Dai \textit{et al.}, 2006; Masubuchi \textit{et al.}, 2011).

To gain insight on the molecular mechanism of resistance in \textit{Gstm1}-null mice, we examined phosphorylation of JNK by Western blot analysis 2 and 6 hr after administration of APAP. Results in males and females are shown Figs. 3 and 4, respectively. A marked increase in

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Sex} & \textbf{Dose, Sampling point} & \textbf{Strain} & \textbf{Grade} & \textbf{Number Examined} \\
\hline
\textbf{Male} & 500 mg/kg, 2 hr & & & \\
\textbf{Wild-type} & & & 4 & 4 \\
\textbf{Gstm1-null} & & & 4 & 4 \\
\textbf{Male} & 500 mg/kg, 6 hr & & & \\
\textbf{Wild-type} & & & 1 & 1 \text{±} 2 & 4 \\
\textbf{Gstm1-null} & & & 3 & 1 & 4 \\
\textbf{Female} & 500 mg/kg, 2 hr & & & \\
\textbf{Wild-type} & & & 3 & 1 & 4 \\
\textbf{Gstm1-null} & & & 4 & 4 \\
\textbf{Female} & 500 mg/kg, 6 hr & & & \\
\textbf{Wild-type} & & & 2 & 2 & 4 \\
\textbf{Gstm1-null} & & & 4 & 4 \\
\hline
\end{tabular}
\caption{Histopathological grade of hepatocyte necrosis 2 and 6 hr after administration of APAP}
\end{table}

Grade 0: No change compared with the control (0 mg/kg), ±: Minimal change, Grade 1: Slight, Grade 2: Moderate, Grade 3: Marked

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Plasma ALT activity 2 and 6 hr after single administration of APAP. Dose levels of APAP were 0 and 500 mg/kg in both sexes. White, light gray and gray bars indicate control, 2 and 6 hr after administration, respectively. The values are depicted as the mean ± S.D. of four mice per group. Significant differences from the control (0 mg/kg) group by Dunnett’s test are shown as ** \( P < 0.01 \).}
\end{figure}
p-JNK was observed in wild-type mice after doing with APAP, but it was markedly suppressed in Gstm1-null mice. Total JNK did not change after doing with APAP. Two bands of JNK isoforms, p46 and p54, were detected, consistent with previous reports (Hanawa et al., 2008; Nakagawa et al., 2008; Shinohara et al., 2010). The bands p46 and p54 were considered to represent JNK1 and JNK2, respectively. In accordance with p-JNK, marked increases in p-GSK-3β (Tyr 216) and p-GSK-3β (Ser 9) were observed in wild-type mice after doing with APAP, but they were suppressed in Gstm1-null mice. Total GSK-3β did not change after doing with APAP. An increase in p-MKK4 was observed after doing with APAP in wild-type mice, but not in Gstm1-null mice. Total MKK4 did not change after doing with APAP. Apparent sex difference in the role of GSTM1 in the phosphorylation of JNK, GSK-3β and MKK4 after dosing with APAP was not observed, since suppressed phosphorylation in Gstm1-null mice was observed in both sexes. In addition, sex difference between wild-type males and wild-type females in the phosphorylation of JNK after doing with APAP, which was evaluated in a same gel, was not observed (data not shown).

Total GSH in the liver measured 2 and 6 hr after APAP administration is shown in Fig. 5. A decrease in total GSH after administration of APAP has been considered to be an indicator of NAPQI generation (Boess et al., 1998; Latchoumycandane et al., 2007). In both sexes, a statistically significant decrease in total GSH was observed in both wild-type and Gstm1-null mice 2 and 6 hr after administration, with the exception of Gstm1-null females 6 hr after administration.

DISCUSSION

We investigated the role of GSTM1 in APAP-induced hepatotoxicity using Gstm1-null mice. It has been reported that NAPQI, the reactive metabolite of APAP, is detoxified by GSH conjugation (James et al., 2003), and enzymatic reactions catalyzed by GSTs are partially involved (Coles et al., 1988). In addition, the GSTM1-null genotype is a risk factor for DILI caused by carbamazepine (Ueda et al., 2007) and antituberculosis drugs (Roy et al., 2001). Therefore, we predicted that Gstm1-null mice...
may be sensitive to APAP-induced hepatotoxicity. However, Gstm1-null mice unexpectedly showed resistance to APAP-induced hepatotoxicity, since the increase in plasma ALT accompanied by the hepatocyte necrosis 24 hr after administration was attenuated in Gstm1-null mice compared with wild-type mice in both sexes (Fig. 1 and Table 2). In addition, mortality rate after a high dose with APAP was lower in Gstm1-null mice than in wild-type (Table 1). These results were unexpected, but similar to the report that showed resistance to APAP-induced hepatotoxicity in Gstp1/p2-null mice (Henderson et al., 2000).

As for the mechanism of the resistance to APAP-induced hepatotoxicity in Gstp1/p2-null mice, involvement of the increased constitutive JNK activity in Gstp1/p2-null mice is suggested (Elsby et al., 2003), although the detailed mechanism is not fully elucidated. In this case of Gstp1/p2-null mice, the increased constitutive JNK activity is thought to have a protective role in APAP-induced hepatotoxicity (Elsby et al., 2003; Henderson et al., 2000). Subsequently, it has been reported that JNK plays a central role to mediate the signal causing APAP-induced hepatocyte necrosis (Gunawan et al., 2006; Henderson et al., 2007; Latchoumycandane et al., 2006, 2007). Taken together, it is suggested that rapid JNK activation after APAP administration, rather than increased constitutive JNK activation before administration, is important in APAP-induced hepatocyte necrosis. Therefore, we investigated JNK activation (phosphorylation) 2 and 6 hr after administration of APAP to gain insight on the molecular mechanism of resistance in Gstm1-null mice. In this mechanistic study, Gstm1-null mice showed resistance to APAP-induced hepatotoxicity, as evaluated by plasma ALT and liver histopathology (Fig. 2 and Table 3). In accordance with the resistance to APAP-induced hepatotoxicity in Gstm1-null mice, Western blot analysis showed rapid and marked phosphorylation of JNK in both sexes of wild-type mice, whereas its magnitude was markedly attenuated in both sexes of Gstm1-null mice (Figs. 3 and 4). Constitutive activation of JNK, which has been reported in Gstp1/p2-null mice (Elsby et al., 2003), was not observed in Gstm1-null mice. However, the absence of rapid JNK phosphorylation after APAP administration seems to be a mechanism common to both Gstp1/p2- and Gstm1-null mice for the resistance to APAP-induced hepatotoxicity. To examine other possible mechanisms of the resistance to APAP-induced hepatotoxicity in Gstm1-null mice, we performed two-dimensional difference gel electrophoresis/mass spectrometry analysis in the liver of wild-type and Gstm1-null mice. However, notable changes except for the absence of GSTM1 protein expression in Gstm1-null mice were not observed (data not shown). Therefore, Gstm1-null mice are considered to be resistant to APAP-induced hepatotoxicity.

Fig. 5. Liver total GSH 2 and 6 hr after single administration of APAP. Dose levels of APAP were 0 and 500 mg/kg in both sexes. White, light gray and gray bars indicate control, 2 and 6 hr after administration, respectively. The values are depicted as the mean ± S.D. of four mice per group. Significant differences from the control (0 mg/kg) group by Dunnett’s test are shown as ** P < 0.01 and *** P < 0.001.
toxicity perhaps by the suppression of JNK phosphorylation, although other possible mechanisms cannot be eliminated.

Regarding upstream protein kinases activating JNK, rapid phosphorylation of GSK-3β and MKK4 was observed in both sexes of wild-type mice, whereas its magnitude was markedly attenuated in both sexes of Gstm1-null mice (Figs. 3 and 4). It has been reported that GSK-3β and ASK1 are key upstream protein kinases needed to activate JNK, since studies silencing of GSK-3β by antisense oligonucleotide (Shinohara et al., 2010) and using Ask1-null mice (Nakagawa et al., 2008) show the protection from APAP-induced hepatotoxicity accompanied by suppression of JNK activation. It has been reported that GSK-3β is activated by phosphorylation at tyrosine 216 and inactivated by phosphorylation at serine 9 (Eldar-Finkelman, 2002). Although an increase in both activated and inactivated forms of GSK-3β after dosing with APAP has been reported (Shinohara et al., 2010), which is in agreement with this study, it remains unclear so far which phosphorylated form is more important in APAP-induced hepatotoxicity. In addition, whether GSK-3β directly phosphorylates JNK or acts through other kinases remains unclear as well (Shinohara et al., 2010). MKK4, which is a downstream kinase of ASK1, is considered to mediate the signal from ASK1 to JNK (Takeda et al., 2008). Since commercially available anti-phosphorylated ASK1 antibodies could not detect endogenous phosphorylated ASK1 in our experimental conditions, we regarded phosphorylation of MKK4 as an indicator of ASK1 activation. Taking these facts into consideration, GSTM1 may act as an upstream factor that finally induces JNK activation through GSK-3β and ASK1-MKK4 signal pathways. However, further studies would be necessary, since a direct target protein of GSTM1 has not been identified in this study.

A decrease in liver total GSH after administration of APAP is considered to be an indicator of NAPQI generation (Boess et al., 1998; Latchoumycandane et al., 2007). Since a marked decrease in total GSH 2 hr after administration of APAP was observed in both sexes of wild-type and Gstm1-null mice (Fig. 5), exposure to NAPQI

![Fig. 6. Schematic diagram for the putative role of GSTM1 in APAP-induced hepatotoxicity. APAP is metabolized to NAPQI, which is the reactive metabolite of APAP, and causes covalent binding and a decrease in GSH. NAPQI is detoxified by non-enzymatic GSH conjugation, and GSTM1 is not involved in this metabolism. The occurrence of covalent binding and a decrease in GSH leads to generation of reactive oxygen species (ROS), and activate phosphorylation pathways to induce hepatocyte necrosis. Phosphorylation pathways via GSK-3β and ASK1-MKK4 are major pathways to activation of JNK, which plays a central role in APAP-induced hepatotoxicity. GSTM1 may act as an upstream factor that induces JNK activation through GSK-3β and ASK1-MKK4 signal pathways.](image-url)
recovery appeared to be due to the absence of hepatocyte necrosis in Gstm1-null females 2 and 6 hr after administration of APAP (Table 3). The animals with severe hepatotoxicity after administration of APAP showed a decrease in feeding behavior (data not shown), causing a decrease in intake of dietary cysteine, which is essential for the synthesis of GSH (Lu, 1999). Therefore, the occurrence of hepatocyte necrosis is considered to be associated with the delay of GSH recovery 6 hr after administration, which was observed in the groups except for Gstm1-null females. Taken together, it is suggested that GSTM1 is not involved in the enzymatic conjugation of NAPQI by GSH in vivo, consistent with the report of Gstpl/p2-null mice (Henderson et al., 2000). This observation indicates that the role of GSTM1 as a factor modulating cellular signaling is more important than its generally known role as a conjugation enzyme in APAP-induced hepatotoxicity.

The putative role of GSTM1 in APAP-induced hepatotoxicity is shown in Fig. 6. APAP is bioactivated to NAPQI, which causes hepatocyte necrosis. NAPQI is detoxified by non-enzymatic GSH conjugation, and GSTM1 seems not to be involved in this detoxification metabolism. Formation of NAPQI causes covalent binding to macromolecules and a decrease in GSH in liver. These steps lead to generation reactive oxygen species (ROS), which activate stress-induced phosphorylation pathways to cause hepatocyte necrosis. GSK-3β and ASK1-MKK4 signal pathways finally activate JNK, which mediates the central signal of APAP-induced hepatocyte necrosis. In these signal pathways, GSTM1 may act as an upstream factor that induces JNK activation through GSK-3β and ASK1-MKK4 signal pathways.

The GSTM1-null genotype, which lacks the whole gene due to homologous recombination, is frequently observed in humans (Moyer et al., 2007), and is considered a risk factor for DILI (Roy et al., 2001; Ueda et al., 2007). Therefore, the resistance to APAP-induced hepatotoxicity in Gstm1-null mice seems to be inconsistent with the report of the occurrence of DILI in humans with GSTM1-null genotype. However, not all patients with GSTM1-null genotype are predisposed to DILI (Roy et al., 2001; Ueda et al., 2007), suggesting the involvement of GSTM1 not only as a conjugation enzyme but also as a signal modulating factor. The novel function of GSTM1 as a factor mediating the cellular signal for hepatotoxicity is opposite to the function as a conjugation enzyme to detoxify a reactive metabolite, and might mask the occurrence of DILI in patients with GSTM1-null genotype. It would be necessary to investigate whether human GSTM1 also acts as a factor mediating the cellular signal for APAP-induced hepatotoxicity.

In conclusion, Gstm1-null mice are considered to be resistant to APAP-induced hepatotoxicity perhaps by the suppression of JNK phosphorylation, although other possibilities of the mechanism cannot be eliminated. GSTM1 may act as an upstream factor that induces JNK activation through phosphorylation of GSK-3β and MKK4. These observations indicate that GSTM1 has a novel function as a signal modulating factor in APAP-induced hepatotoxicity.

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**REFERENCES**


Resistance to APAP-induced hepatotoxicity in Gstm1-null mice

Dispos., 34, 1495-1501.
Masubuchi, Y., Nakayama, J. and Watanabe, Y. (2011): Sex difference in susceptibility to acetaminophen hepatotoxicity is reversed by buthionine sulfoximine. Toxicology, 287, 54-60.