Involvement of Different Human Glutathione Transferase Isoforms in the Glutathione Conjugation of Reactive Metabolites of Troglitazone

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ABSTRACT:
Null mutation of glutathione transferase (GST) M1 and GSTT1 was reported to correlate statistically with an abnormal increase in the plasma levels of alanine aminotransferase or aspartate aminotransferase caused by troglitazone in diabetic patients (Clin Pharmacol Ther, 73:435–455, 2003). This clinical evidence leads to the hypothesis that GST conjugation catalyzed by GSTT1 and GSTM1 has a role in the elimination of reactive metabolites of troglitazone. However, the contribution of GST isoforms expressed in human liver to the detoxification of reactive metabolites of troglitazone has not yet been clarified. We investigated the involvement of human GST isoforms in the GSH conjugation of reactive metabolites of troglitazone using recombinant GST enzymes. Five reported GSH conjugates of reactive metabolites were produced from troglitazone after incubation with liver microsomes, NADPH, and GSH in a GSH concentration-dependent manner. Addition of human recombinant GSTA1, GSTA2, GSTM1, or GSTP1 protein to the incubation mixture further increased the GSH conjugates. However, the addition of GSTT1 did not show any catalytic effect. It is of interest that one of the reactive metabolites with a quinone structure was predominantly conjugated with GSH by GSTM1. Thus, we demonstrated that the GST isoforms contributed differently to the GSH conjugation of individual reactive metabolites of troglitazone, and GSTM1 is the most important GST isoform in the GSH conjugation of a specific reactive metabolite produced from the cytotoxic, quinone-form metabolite of troglitazone.

Introduction
Troglitazone is a first-generation thiazolidinedione insulin sensitizer for oral use and shows pharmacological effects through the activation of peroxisome proliferator-activated receptor gamma. Despite acceptable safety profiles in clinical trials, rare but lethal liver injury induced by troglitazone has been reported sporadically (Gitlin et al., 1998; Watkins and Whitcomb, 1998). Because of safety concerns over its use, troglitazone was withdrawn from the U.S. market in 2000. On the other hand, in preclinical species, it is very difficult to reproduce the troglitazone-induced liver injury, even when using a dose of troglitazone greater than the clinical dose (Watanabe et al., 2000; Fujimoto et al., 2009). This has hindered the clarification of the exact mechanisms of troglitazone-induced hepatotoxicity.

Idiosyncratic drug-induced liver injury (DILI) is one of the biggest clinical problems to be solved. Many drugs have been withdrawn from the market or clinical development of new drugs has suddenly been halted because of DILIs (Kola and Landis, 2004). In most cases, drugs are metabolized by cytochrome P450 (P450) for their detoxification, but metabolites sometimes show high chemical reactivity. In one of the hypothetical schemes, DILIs are triggered by the formation of reactive metabolites such as electrophilic intermediates (Walgren et al., 2005). As a mechanism to protect the liver against such reactive metabolites, they are efficiently conjugated with GSH, an abundant endogenous nucleophile in cytosol, by nonenzymatic and enzymatic reactions, and they lose their reactivity. The importance of detoxification by GSH conjugation for protection from DILIs has been previously proposed in (Shimizu et al., 2009), which became more sensitive to hepatic injury triggered by diclofenac, flutamide, and amodiaquine. Thus, the balance between the production of reactive metabolites catalyzed by P450 enzymes and the elimination of these metabolites by GSH.
SPECIFIC GSTs DETOXIFY REACTIVE METABOLITES OF TROGLITAZONE

conjugation is thought to determine the amount of reactive metabolites in the hepatocytes and the subsequent risk of DILIs.

Glutathione transferases (GSTs) comprise a supergene family and catalyze GSH conjugation of a wide variety of chemically reactive compounds and metabolites (Hayes et al., 2005). In humans, seven distinct classes of cytosolic GSTs have been identified so far (classes A, M, P, S, T, Z, and O). A report indicated several genetic polymorphisms of these GST isoforms, some of which were confirmed to affect their enzymatic activities (Hayes et al., 2005). Among the genetic polymorphisms of GSTs, there is a growing interest in genetic polymorphisms of GSTM1 and GSTD1, which are predominantly expressed in the liver, as risk factors for DILIs (Simon et al., 2000; Ueda et al., 2007; Lucena et al., 2008). In the null variant allele (%0) of GSTM1 or GSTD1, 16 or 52 kilobases of their genomic regions are deleted from chromosome 1 or chromosome 22 by a homologous recombination, respectively (Xu et al., 1998; Sprenger et al., 2000), which causes their lack of protein expression. The frequencies of null alleles of GSTM1 and GSTD1 are very high, and the percentage of subjects with double null alleles (%0/%0) of both GSTM1 and GSTD1 is 10 and 25% in whites and Asians, respectively (Bolt and Thier, 2006). It is interesting to note that a previous report indicated that the double null variants of these two GST isoforms were associated with a higher risk of DILI caused by troglitazone (Watanabe et al., 2003). Among patients treated with troglitazone, the prevalence of an abnormal increase in the levels of amino transferases was much higher in the subjects with double null alleles of GSTM1 and GSTD1 than in the subjects with wild-type alleles. In the proposed metabolic pathways of troglitazone, previous reports showed that reactive metabolites are produced by P450 enzymes such as CYP3A4 and were conjugated with GSH to form five kinds of GSH conjugates (Kassahun et al., 2001; Yan et al., 2005). These reports suggest that GSTM1 and GSTT1 are involved in the detoxification of reactive metabolites of troglitazone. However, nobody has directly demonstrated which isoforms of GST enzymes are responsible for the GSH conjugation of each reactive metabolite of troglitazone.

In the present study, we investigated the role of GST isoforms in the GSH conjugation of reactive metabolites of troglitazone to find the reason for the increased risk of troglitazone-induced hepatotoxicity in the patients with double null mutations of GSTM1 and GSTT1.

Materials and Methods

Materials. Pooled human liver microsomes were purchased from KAC Co., Ltd. (Kyoto, Japan) and Charles River Laboratories, Inc. (Wilmington, MA). Troglitazone was purchased from LKT Laboratories (St. Paul, MN). GSH and NADPH were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals used were commercially available and of analytical grade.

Construction of Expression Plasmids for Human GSTs. Construction of Escherichia coli expression plasmids for GSTA2 and GSTM1b was performed as described previously with slight modifications (Zhao et al., 1999). In brief, each coding region of GSTA2 and GSTM1b was amplified by polymerase chain reaction from human liver Marathon-Ready cDNAs (Clontech, Mountain View, CA) using gene-specific oligonucleotide primers, subcloned into the BamHI/Ndel sites (GSTA2) and BamHI/HindIII sites (GSTM1b) of the pET-20b(+) vector (Novagen, Madison, WI), and introduced into E. coli BL21(DE3)pLysS (Novagen). The subcloned cDNA sequences determined by using an ABI Prism 310 DNA sequencer (PerkinElmer Life and Analytical Sciences, Waltham, MA) were in agreement with each of the published GSTA2 (GenBank accession no. NM_000846) and GSTM1b (GenBank accession no. NM_000561) cDNAs. The expression plasmids for GSTA1, GSTP1, and GSTT1 were constructed as described previously (Baker et al., 1994; Sherratt et al., 1997; Zhao et al., 1999).

Bacterial Expression and Purification of Recombinant Human GSTs. Bacterial expression and purification of recombinant human GSTs by column chromatography were performed as described previously (Sherratt et al., 1997) with some modifications. A bacterial lysate (25 mg protein/ml) was loaded onto a His-bind metal chelation resin column (1 x 5 cm; Novagen) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.4) containing 5 mM imidazole and 500 mM NaCl (buffer I). After washing the column with 20 ml of the same buffer, the recombinant protein was eluted in a stepwise manner with 20 ml each of 25, 50, 100, and 200 mM imidazole in buffer I. Fractions containing the homogeneous enzyme protein were pooled and concentrated using Amicon Ultra-15 centrifugal filter devices (Millipore Corporation, Billerica, MA). The purified enzyme solution was desalted on a PD-10 column (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) pre-equilibrated with sodium phosphate buffer (20 mM, pH 7.4). Purification of recombinant GSTA1, GSTP1, and GSTT1 was performed as described previously (Baker et al., 1994; Sherratt et al., 1997; Zhao et al., 1999). Protein concentrations of the purified GSTs were measured by the Lowry method using bovine serum albumin as a standard as described previously (Lowry et al., 1951). Activities of purified GST isoforms were determined as described previously (Habig et al., 1974). The activities of GSH conjugation of chlorodinitrobenzene were 123.4 (GSTA1), 23.5 (A2), 103.9 (GSTM1), and 98.3 mmol.min⁻¹.mg⁻¹ (GSTP1), respectively. The activity of GSH conjugation of cumene hydroperoxide was 1.25 mmol.min⁻¹.mg⁻¹ (GSTT1). The purified GSTs used in this study showed comparable specific activities to those reported previously (Ross and Board, 1993; Baker et al., 1994; Sherratt et al., 1997; Zhao et al., 1999).

In Vitro GSH Conjugation of Reactive Metabolites of Troglitazone. To check the nonenzymatic GSH conjugation of the reactive metabolites of troglitazone, troglitazone (50 μM) was incubated with human liver microsomes (2 mg/ml) in the presence of GSH (5 mM), MgCl₂ (1 mM), and NADPH (1.2 mM) in potassium phosphate buffer (100 mM, pH 7.4) for 60 min at 37°C. The total incubation volume was 200 μl. The reaction was initiated by the addition of an NADPH solution after a 5-min preincubation and stopped by the addition of 200 μl of ice-cold acetonitrile. After centrifugation by a cooled centrifuge (Tomy MX-305; Tomy Seiko, Tokyo, Japan) at 15,000 rpm for 10 min, the supernatant was evaporated to dryness by a concentrative centrifuge (Tomy CC-105; Tomy Seiko). The residue was reconstituted in 30% acetonitrile in water (80 μl). After centrifugation (15,000 rpm for 10 min), aliquots of the supernatant were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). To check the GSH-concentration dependence of the GSH conjugation, the reaction was performed with 0.1, 0.3, 0.6, 1, and 5 mM GSH.

To observe the enzymatic GSH conjugation of reactive metabolites of troglitazone by GST isoforms, recombinant GST protein (0.1 mg/ml) was added to the incubation mixture in the presence of 0.6 mM GSH. The production of GSH conjugates mediated by each GST isoform was then estimated by the relative peak area calculated according to the following equation: relative peak area (%) = (peak area in the presence of recombinant GST)/(peak area in the absence of recombinant GSTs) × 100.

Time- and Enzyme Concentration-Dependent Formation of Specific GST Conjugates by Recombinant GST Isoforms. GSTM1-mediated GSH conjugation was further investigated at different GST concentrations (0.05, 0.1, and 0.2 mg/ml) in the presence of GSH (0.6 mM). The reaction was stopped by the addition of 200 μl of ice-cold acetonitrile after incubation for 7.5, 15, and 30 min. In addition, to test the involvement of other GST isoforms in the formation of metabolite 4 (Met4) (see Fig. 6), the enzymatic reaction was also performed with 0.2 mg/ml of recombinant GST proteins (GSTA1, GSTA2, GSTM1, GSTT1, and GSTP1) for 60 min at 37°C. For GSTA1, GSTM1, and GSTP1, the isoforms that produced a significant amount of Met4 at a concentration of 0.2 mg/ml dependence of the rate of Met4 formation on the protein concentration of GST isoforms was also examined at the concentration range from 0.025 to 0.2 mg/ml. The relative formation rate of Met4 by each GST isoform was estimated by the initial linear slope of the plots of the relative peak area (%) against the GST concentration in the incubation medium. The relative formation rate of Met4 mediated by each GST isoform in the liver was then estimated by multiplying the relative formation rate of Met4 by the protein expression level of each GST isoform in the liver reported previously (van Ommen et al., 1990). Thus, the relative contribution ratio of the formation rate of Met4 mediated by a specific GST isoform to that by GSTM1 in the liver was defined in the following equation: relative contribu-
Results

Detection of GSH Conjugates of Reactive Metabolites of Troglitazone Formed with Human Liver Microsomes and GSH. Full scan spectra were determined to detect five GSH conjugates of reactive metabolites of troglitazone as described previously (Kassahun et al., 2001). The extracted ion chromatograms by multiple reaction monitoring analysis were obtained from the incubation media with troglitazone, human liver microsomes, NADPH, and GSH (Fig. 1). We confirmed that these peaks were not detected when NADPH or GSH was removed from the incubation media (data not shown). In the full scan spectra of m/z 719, 779, 761, 735, and 745, a set of fragments were detected at designated retention times indicated by the arrows in Fig. 1 (Table 1). Considering the chemical structures of GSH conjugates of reactive metabolites of troglitazone presented in the previous report (Kassahun et al., 2001), these fragmentation patterns were identical to those of five metabolites (Supplemental Fig. 1), and we named these metabolites Met1, Met2, Met3, Met4, and Met5 as defined in Table 1.

GSH Concentration Dependence on the Formation of GSH Conjugates of Reactive Metabolites of Troglitazone. To confirm whether the peaks in Fig. 1 were derived from GSH conjugates of the reactive metabolites of troglitazone, the concentration dependence of GSH on the formation of five GSH conjugates by nonenzymatic reaction was observed. In Fig. 2, the peak area of each metabolite increased as the GSH concentration in the incubation media increased. Peak areas of all five metabolites linearly increased up to 0.6 mM GSH and further moderately increased at concentrations from 0.6 to 5 mM. On the other hand, the other peaks detected in Fig. 1 did not increase in a GSH concentration-dependent manner (data not shown).

Involvement of GST Isoforms in the Formation of Five GSH Conjugates of the Reactive Metabolites of Troglitazone. To investigate the involvement of GST isoforms in the formation of conjugated metabolites of troglitazone, troglitazone was incubated with human recombinant GSTs (0.1 mg/ml) purified from E. coli expression systems and human microsomes in the presence of NADPH and GSH. As shown in Fig. 3, A and C, at the same GSH concentration (0.6 mM), the amount of Met1 and Met3 increased largely by incubation with recombinant GSTA1, GSTA2, and GSTD1 proteins compared with incubation in the absence of any GST proteins. Met2 was increased largely by incubation with GSTA1, GSTM1, and GSTD1 (Fig. 3B). Met5 increased after incubation with GSTA1, GSTA2, GSTM1, and GSTD1 (Fig. 3E). Unlike other metabolites, the production of Met4 was specifically promoted 1.8-fold by the addition of GSTM1 under this experimental condition, whereas that production was 1.2-fold increased by GSTD1 (Fig. 3D). On the other hand, addition of GSTT1 did not enhance the production of these GSH conjugates of troglitazone compared with the control.

TABLE 1
GSH adducts of reactive metabolites of troglitazone identified by LC-MS/MS

<table>
<thead>
<tr>
<th>GSH Adduct</th>
<th>tR</th>
<th>Chemical Composition</th>
<th>MH</th>
<th>Fragment of GSH Adduct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met1</td>
<td>6.2</td>
<td>C33H44N4O10S2</td>
<td>719</td>
<td>446, 306, 272, 254</td>
</tr>
<tr>
<td>Met2</td>
<td>4.8</td>
<td>C34H42N4O11S2</td>
<td>779</td>
<td>650, 506, 474, 306, 272, 254</td>
</tr>
<tr>
<td>Met3</td>
<td>5.8</td>
<td>C34H42N4O11S2</td>
<td>761</td>
<td>488, 306, 272, 254</td>
</tr>
<tr>
<td>Met4</td>
<td>4.4</td>
<td>C33H44N4O11S2</td>
<td>735</td>
<td>606, 462, 430, 306, 272, 254</td>
</tr>
<tr>
<td>Met5</td>
<td>6.7</td>
<td>C34H44N4O11S2</td>
<td>745</td>
<td>616, 472, 306, 272, 254</td>
</tr>
</tbody>
</table>

tR, retention time; MH, m/z; of each metabolite in negative mode.
Time- and GST Concentration-Dependent Formation of Met4 Mediated by GSTM1. To further confirm whether the increase in the peak area of Met4 was caused by the enzymatic reaction mediated by GSTM1, the influence of incubation time (7.5, 15, and 30 min) and the amount of GSTM1 (0.05, 0.1, and 0.2 mg/ml) on the formation rate of Met4 were investigated (Fig. 4). At a GSTM1 concentration of 0.05 mg/ml, the peak area of Met4 increased in a time-dependent manner and was significantly higher compared with that in the absence of GSTM1. On the other hand, the increase in its peak area seems to reach a plateau at 15 min at a concentration of 0.1 or 0.2 mg/ml.

Furthermore, the formation of Met4 was accelerated as the GSTM1 concentration increased. After incubation for 15 min with 0.1 mg/ml GSTM1, the peak area of Met4 was significantly 1.3-fold higher compared with incubation with 0.05 mg/ml GSTM1 (Fig. 4).

Contribution of GSTM1 as the Major Isoform in the Formation of Met4 in Human Liver. To check whether GSTM1 mainly catalyzes the formation of Met4 in human liver, the concentration of each recombinant GST protein in the incubation media was increased from 0.1 to 0.2 mg/ml. As shown in Table 2, the addition of GSTA2 and GSTT1 never increased the formation of Met4. On the other hand, a significant increase in the formation of Met4 was observed by addition of 0.2 mg/ml of GSTA1, GSTM1, and GSTP1, and the amount of Met4 formed by GSTM1 for 60 min was 1.5- and 1.4-fold higher than that by GSTA1 and GSTP1, respectively.

Among the three GST isofoms catalyzing the formation of Met4, the conjugation activity was compared (Fig. 5). The formation of Met4 slightly and linearly increased in proportion to the concentration of GSTA1 and GSTP1. The formation of Met4 was also proportional to the GSTM1 concentration, but the linearity was no longer maintained over 0.05 mg/ml of GSTM1 in the incubation media. The initial slope of the GST concentration-dependent formation of Met4 shown in Fig. 5 was thought to correspond to the formation rate of Met4 per unit of GST protein. The initial slope for GSTA1 and GSTP1 was only 0.06- and 0.08-fold of that for GSTM1, respectively (Table 3). On the basis of the second equation, the relative contribution ratio of GSTA1 and GSTP1 was 22 and 1.9% of that of GSTM1, respectively (Table 3).

Discussion

Troglitazone was withdrawn from the market because of its severe hepatotoxicity. The clinical study indicated that double null alleles of GSTM1 and GSTT1 were associated with the onset of troglitazone-induced hepatotoxicity (Watanabe et al., 2003). However, nobody has clarified which GST isofoms are responsible for the conjugation of each reactive metabolite of troglitazone. In the present study, we sought to clarify the important GST isofoms involved in GSH conjugation of reactive metabolites of troglitazone produced in human liver using recombinant GST isofoms.

GSTA1, GSTA2, GSTM1, GSTT1, and GSTP1 are mainly expressed in human liver cytosol (van Ommen et al., 1990). We focused on the role of these five GST isofoms in the formation of each GSH conjugate. It is interesting to note that our results indicated that specific GST isofoms were involved in GSH conjugation of each reactive metabolite of troglitazone to varying degrees (Fig. 3). In the case of Met4, especially, GSTM1 increased most strongly the formation of this metabolite, by 1.8-fold at the GST concentration of 0.1
mg/ml. We further confirmed the time- and GST concentration-dependent formation of Met4 mediated by GSTM1 (Fig. 4). The minor difference in the formation of Met4 for 30 min at 0.05 and 0.2 mg/ml GSTM1 would be due to the saturated production of or the loss of reactive metabolites of troglitazone in the liver microsomes. Regarding Met2 and Met3, Kassahun et al. (2001) have suggested that Met3 was directly formed from Met2. If so, GST isoforms involved in the formation of Met2 and Met3 are expected to be identical. However, our results indicated that the pattern of increase in the peak area of Met2 and Met3 by each GST isoform was different (Fig. 3). Therefore, it was possible that the precursor of Met3 was produced by other metabolic pathways that do not go through Met2 (Fig. 6), although we do not have any additional evidence.

To investigate which GST isoforms can catalyze the formation of Met4, we increased the GST concentration in the incubation media up to 0.2 mg/ml. As shown in Table 2, GSTA1, GSTM1, and GSTP1 catalyzed the formation of Met4 at a concentration of 0.2 mg/ml. However, GSTT1 did not show any conjugative reactions for all of the reactive metabolites of troglitazone, although a previous clinical study

**TABLE 2**

<table>
<thead>
<tr>
<th>GST Isoforms</th>
<th>Relative Peak Area</th>
<th>GST 0.1 mg/ml</th>
<th>GST 0.2 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>No GST</td>
<td>100 ± 3</td>
<td>100 ± 10</td>
<td></td>
</tr>
<tr>
<td>GSTA1</td>
<td>111 ± 6</td>
<td>129 ± 14**</td>
<td></td>
</tr>
<tr>
<td>GSTA2</td>
<td>114 ± 9</td>
<td>95.3 ± 12.4</td>
<td></td>
</tr>
<tr>
<td>GSTM1</td>
<td>182 ± 15**</td>
<td>209 ± 22**</td>
<td></td>
</tr>
<tr>
<td>GSTT1</td>
<td>89.4 ± 15.8</td>
<td>104 ± 15</td>
<td></td>
</tr>
<tr>
<td>GSTP1</td>
<td>123 ± 8**</td>
<td>141 ± 13**</td>
<td></td>
</tr>
</tbody>
</table>

**,** $p < 0.01$, significant difference (incubation sample in the absence of GSTs versus incubation sample in the presence of each GST isoform). Each value represents the mean ± S.E. (n = 6).
indicated that either GSTM1 null mutation or GSTT1 null mutation tended to increase the risk of troglitazone-induced hepatotoxicity (Watanabe et al., 2003). There is no doubt that the major function of GSTs is to conjugate GSH with electrophilic substrates; however, it was also reported that some GST isoforms possibly play a role in the control of the intracellular signaling pathway (Adler et al., 1999; Dorion et al., 2002). For example, cyclophosphamide-induced nephrotoxicity was potentiated in GST-null mice compared with wild-type mice (Conklin et al., 2009). A reactive metabolite of cyclophosphamide conjugated with mercapturic acid, which is derived from its GSH conjugates, was excreted in the urine to the same extent in both types of mice, whereas mitogen-activated protein kinase in bladder was more activated in GSTP-null mice than in wild-type mice. These results suggested that the GST function affected the onset of DILI by controlling the signaling pathway. From this evidence, although GSTT1 did not have any role in scavenging the reactive metabolites of troglitazone, it is possible that GSTT1 will modify the regulation of intracellular signaling and subsequent toxicological effects. Further studies to clarify the exact function of GSTT1 on troglitazone-induced hepatotoxicity will be needed.

Among the three GST isoforms that could catalyze the formation of Met4, the formation rate of Met4 was compared (Fig. 5). Met4 was produced as the concentrations of GSTA1 or GSTP1 increased. On the other hand, the formation of Met4 reached a plateau at a GSTM1 concentration of not less than 0.05 mg/ml. Because the GSH concentration in the media increased (Kassahun et al., 2001; Yamamoto et al., 2002), the initial slope of GST concentration-dependent formation of Met4 was compared among GST isoforms. As a result, the relative peak area was calculated by the first equation as mentioned under Materials and Methods. The peak area for Met4 in the incubation mixture with GSTA1 ▲, GSTM1 ▼, or GSTP1 ▼ is shown. In the large graph, data are shown at enzyme concentrations of 0 to 0.05 mg/ml. The graph in the inset shows the data at the concentration of 0 to 0.20 mg/ml. Each point represents the mean ± S.E. (n = 6). Dotted lines represent the fitted lines to the data of which the GST concentration-dependent linear increase was maintained.

![Graph](https://example.com/graph.png)

**FIG. 5.** Concentration dependence of GSTA1, GSTM1, and GSTP1 on the formation of Met4. Relative peak area was calculated by the first equation as mentioned under Materials and Methods. The peak area for Met4 in the incubation mixture with GSTA1 ▲, GSTM1 ▼, or GSTP1 ▼ is shown. In the large graph, data are shown at enzyme concentrations of 0 to 0.05 mg/ml. The graph in the inset shows the data at the concentration of 0 to 0.20 mg/ml. Each point represents the mean ± S.E. (n = 6). Dotted lines represent the fitted lines to the data of which the GST concentration-dependent linear increase was maintained.

detect all kinds of a small amount of known metabolites even with the high-specification LC-MS/MS. The concentration of active metabolites in our incubation is considered to be much smaller than the $K_m$ values of typical substrates; thus, we think that the linearity of the GSH conjugation reaction was still maintained and our results can also be applied to the clinical situation.

In a previous report, several electrophilic intermediates of troglitazone were identified as GSH conjugates in the human liver microsomal extracts (Kassahun et al., 2001) (Fig. 6). Met4 was reported to be produced from the metabolite having a quinone structure. Previous in vitro experiments indicated that the quinone-form metabolite of troglitazone (precursor of Met4) enhanced cytotoxicity as their concentration in the media increased (Kassahun et al., 2001; Yamamoto et al., 2002). In the proposed metabolic pathway, Met4 was produced by conjugation at the thiazolidinedione ring with GSH. Furthermore, it was suggested previously that the thiazolidinedione moiety had an impact on the sensitivity of cytotoxicity (Saha et al., 2010), whereas Met4 still had a quinone moiety. These results implied that the thiazolidinedione ring showed a stronger chemical reactivity than the quinone structure. Regarding the substrate recognition of GSTM1, our results indicated that formation of Met4 was preferably catalyzed by GSTM1 compared with Met1, Met2, and Met3, which are also conjugated with GSH at the thiazolidinedione ring. This would indicate that the quinone moiety was important for substrate recognition by GSTM1 in the production of Met4. Our results suggested that GSTM1 had an impact on the detoxification of a specific reactive metabolite of troglitazone and, presumably, subsequent hepatotoxicity. Our in vitro results are in agreement with previous clinical observations, in which the deletion of the GSTM1 gene could be one of the risk factors for troglitazone-induced hepatotoxicity (Watanabe et al., 2003), although further studies will be needed to demonstrate the direct relationship between the formation of the precursor of Met4 and hepatotoxicity. However, the difference in the frequencies of null genotypes of GSTM1 between subjects suffering from troglitazone-induced hepatotoxicity and subjects with no symptoms was statistically not significant (Watanabe et al., 2003). Thus, it was thought that a decreased detoxification of reactive metabolites by GSH conjugation due to the null GSTM1 genotype was one of the risk factors, but a combination of this factor and other unknown factors should be critical for the onset of idiosyncratic toxicity.

Until now, gene deletion of GSTM1 and GSTT1 has also been clinically associated with the susceptibility to DILIs caused by tacrine and carbamazepine (Simon et al., 2000; Ueda et al., 2007). Clinical studies have indicated that it was also associated with the susceptibility to troglitazone-induced hepatotoxicity and subjects with no symptoms was statistically not significant (Watanabe et al., 2003). Thus, it was thought that a decreased detoxification of reactive metabolites by GSH conjugation due to the null GSTM1 genotype was one of the risk factors, but a combination of this factor and other unknown factors should be critical for the onset of idiosyncratic toxicity.

**TABLE 3**

**Contribution of GSTA1, GSTM1, and GSTP1 to the formation of Met4 in the human liver**

The relative contribution ratio was calculated by eq. 2, as mentioned under Materials and Methods. Statistical analysis was performed using a one-way ANOVA followed by Dunnett’s test.

<table>
<thead>
<tr>
<th>Recombinant GST Isoforms</th>
<th>Slope</th>
<th>Expression (µg/mg protein)</th>
<th>Relative Contribution Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTA1</td>
<td>0.00</td>
<td>100 ± 19</td>
<td>22 ± 8</td>
</tr>
<tr>
<td>GSTM1</td>
<td>0.02</td>
<td>1530 ± 200</td>
<td>100 ± 56</td>
</tr>
<tr>
<td>GSTP1</td>
<td>0.04</td>
<td>127 ± 21</td>
<td>1.9 ± 1.5</td>
</tr>
</tbody>
</table>

***, p < 0.01, significant difference (relative contribution ratio of GSTM1 versus that of GSTA1 or GSTP1). Each value represents the mean ± S.E. (n = 6).**

**Protein expression levels of GST isoforms in human liver were previously reported by van Ommen et al. (1990) [n = 20 (GSTA1), n = 11 (GSTM1), and n = 18 (GSTP1)].**
the expression of DILIs and cancers are unknown, it is likely that GSTM1 and GSTT1 are generally involved as the major GST isoforms in detoxification of chemically reactive metabolites and promote their elimination from the body, and that either or both of two isoforms indirectly protect the cells by regulation of the intracellular signaling pathways.

In drug development, the total amount of reactive metabolites of drugs is considered to be one of the important indicators for the manifestation of drug-induced toxicity (Walgren et al., 2005). The positive relationship between the amount of reactive metabolites and frequency of DILIs is generally accepted. However, our present study would indicate a possibility that the amounts of particular reactive metabolites conjugated specifically by GSTM1 and/or GSTT1 is more important rather than the total amount of reactive metabolites as the indicator of the risk of DILIs. We further need to clarify the relationship between expression levels of GSTM1/T1 or genotypes of GSTM1/T1 and produced amount of GSH conjugates of active metabolites of troglitazone with human liver samples. Very recently, Dragovic et al. (2010) indicated that particular GST isoforms are involved in the GSH conjugation of specific reactive metabolites of clozapine. Further studies will be needed to reveal the importance of this concept.

In conclusion, we demonstrated that different GST isoforms are involved in GSH conjugation of specific reactive metabolites of troglitazone (Fig. 6), and that GSTM1 is responsible for the conjugation of the precursor of Met4.

Authorship Contributions

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Conducted experiments: Okada and Aoyama.

Contributed new reagents or analytic tools: Nishiyama, Aoyama, Tozuka, and Hiratsuka.

Performed data analysis: Okada, Maeda, and Sugiyama.

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References


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