Glutaredoxin and Thioredoxin Can Be Involved in Producing the Pharmacologically Active Metabolite of a Thienopyridine Antiplatelet Agent, Prasugrel

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ABSTRACT:

A thienopyridine antiplatelet agent, prasugrel, is rapidly hydrolyzed to a thiolactone metabolite (R-95913, 2-[2-oxo-6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl]-1-cyclopropyl-2-(2-fluorophenyl)ethanone). R-95913 is oxidized by hepatic cytochromes P450 to the pharmacologically active metabolite R-138727 (2-[1–2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4-mercapto-3-piperidinylidene)acetic acid). One possible intermediate in the in vitro bioactivation pathway is a glutathione conjugate, R-133490, which could be reduced to generate R-138727 in the presence of a reducing agent such as glutathione. In this study, enzymes in human liver cytosols were found to accelerate reduction of R-133490 leading to the formation of R-138727. To explore the possible reductive enzymes, we separated the various proteins in human liver cytosol based on size using gel filtration chromatography. Two active peaks were detected and found to contain thioredoxin and glutaredoxin, respectively. In addition, recombinant human glutaredoxin and thioredoxin promoted the formation of R-138727 from R-133490 with much higher activity for glutaredoxin than for thioredoxin. This study is the first in vitro observation indicating that glutaredoxin and thioredoxin in human liver are active in reducing the mixed disulfide formed between xenobiotics and glutathione.

Introduction

Prasugrel (Effient in the United States and Efient in the European Union), clopidogrel (Plavix/Iscover), and ticlopidine (Ticlid) are thienopyridine antiplatelet agents. Results indicate that Prasugrel reduces the rate of thrombotic cardiovascular events and stent thrombosis in patients with acute coronary syndrome who are undergoing percutaneous coronary intervention (Wiviott et al., 2007) (Effient package insert). Likewise, clopidogrel is administered to patients after percutaneous coronary intervention and stent placement (Braunwald et al., 2000). The thienopyridines are prodrugs that are converted in vivo to their pharmacologically active metabolites via a corresponding thiolactone metabolite (Farid et al., 2010). Our previous in vitro studies suggested that one possible pathway for the hydrolysis of prasugrel to form the thiolactone, R-95913, during absorption (Williams et al., 2008), was via oxidation to a chemically unstable sulfenic acid or S-oxide intermediate. This intermediate could then be converted to a disulfide-type glutathione conjugate (R-133490) (Hagihara et al., 2009) and subsequently reduced to the pharmacologically active metabolite (R-138727) in the presence of another glutathione molecule (Fig. 1) (Hagihara et al., 2010). Addition of cytosolic fraction to liver microsomes has been shown to decrease the formation of R-133490 from R-95913 with an increase in the formation rate of R-138727. This result indicated that the glutathione conjugate generated could be immediately reduced to R-138727 in the presence of cystosol (Hagihara et al., 2010). In this study, we isolated and identified the enzymes in human liver cytosols catalyzing the in vitro glutathione-mediated reduction of R-133490 to R-138727 as glutaredoxin and thioredoxin.

Materials and Methods

Materials. The deacetylated (thiolactone) metabolite of prasugrel (R-95913), the active metabolite of prasugrel (R-138727), and its glutathione conjugate (R-133490) (shown in Fig. 1), and R-135766 (Hagihara et al., 2009) for the internal standard were synthesized by Ube Industries, Ltd. (Ube, Japan). Recombinant human glutaredoxin, human thioredoxin, goat anti-human glutaredoxin IgG, and goat anti-human thioredoxin IgG were purchased from American Diagnostica Inc. (Greenwich, CT). For goat anti-human glutaredoxin IgG, recombinant human glutaredoxin was used as the immunogen and IgG fraction of goat antiserum purified via human glutaredoxin-Sepharose immunoaffinity column chromatography. For goat anti-human thioredoxin IgG, recombinant human thioredoxin was used as the immunogen and IgG fraction of goat antiserum purified via human thioredoxin-Sepharose immunoaffinity column chromatography. Human liver microsomes and cytosols were obtained from XenoTech, LLC (Lenexa, KS) and Human and Animal Bridge Research Organization (Tokyo, Japan), respectively. Methoxyphenacetyl bromide was
Measurement of the activity to produce R-138727 with 0.1 mM R-133490 and glutathione conjugate (final concentration, 1 mg/ml) in 50 mM potassium phosphate buffer (pH 7.4) was preincubated for 5 min at 37°C. The reaction was initiated by addition of 10 µl of the glutathione conjugate of R-138727 solution (final concentration, 1 µM). After incubation at 37°C for 1, 2, 5, and 10 min (total volume, 1 ml), 100 µl of the incubation mixture was mixed with 200 µl of acetonitrile, which was treated with 1 µM methoxyphenylacridone to derivatize R-138727 for liquid chromatography equipped with tandem mass spectrometry (LC-MS/MS) assay. R-135766 was used as the internal standard. The assays of R-138727 and R-133490 were performed following methods reported previously (Hagihara et al., 2010). Separation of the analytes by high-performance liquid chromatography (HPLC) was conducted using an Alliance2690 Separations Module (Waters, Milford, MA). Mass spectra were determined with a Quattro LC-MS/MS system (Micromass Ltd., Milford, MA) in the positive ion detection mode using an ESI interface (Micromass Ltd.). A lower limit of quantification was set at 1.6 nM. Data acquisition and analyses were performed using MassLynx software (version 4.0; Micromass Ltd.).

Measurement of Enzyme Activity Producing R-138727 from Its Glutathione Conjugate. Twenty-five microliters of an aqueous glutathione solution (final concentration, 5 mM) was added to 200 µl of each fraction obtained after gel filtration of a cytosol mixture or 50 mM potassium phosphate buffer (pH 7.4). The mixture was preincubated for 5 min at 37°C. The reaction was initiated by addition of 25 µl of R-133490 solution in distilled water (final concentration, 0.1 mM). After incubation at 37°C for 0, 5, and 10 min (total volume, 250 µl), 30 µl of the incubation mixture was transferred to a centrifuge tube, and 90 µl of acetonitrile was added to the tube. The mixture was mixed well and centrifuged at 15,000g for 3 min at 4°C. In inhibition experiments with anti-human thioredoxin IgG, 70 µl of the gel-filtrated active fraction was mixed with 10 µl of anti-human glutaredoxin IgG (final concentrations, 33–300 µg/ml) and preincubated for 2 h at 37°C, followed by measurement of the activity to produce R-138727 with 0.1 mM R-133490 and 5 mM glutathione as final concentrations. In inhibition experiments with anti-human thioredoxin IgG, 30 µl of the gel-filtrated active fraction was mixed with 60 µl of anti-human thioredoxin IgG (final concentrations, 67–600 µg/ml) and preincubated for 1 h at 37°C, followed by measurement of the activity to produce R-138727 with 0.05 mM R-133490 and 5 mM glutathione as final concentrations. Mouse IgG was used as a control. Twenty-five microliters of supernatant was injected into an HPLC system for measurement of R-138727. The HPLC system (Shimadzu, Kyoto, Japan) consisted of a system controller (LC-10AD), auto injector (SIL-10AXL), UV-VIS detector (SRL-10A), column oven (CTO-10AL), and degasser (DGU-12A). A YMC-ODS A-302 column (4.6 × 150 mm; YMC Co., Ltd., Kyoto, Japan) was used as an analytical column, and a mixture of acetonitrile, isopropyl alcohol, distilled water, and trifluoroacetic acid (5:12:83:0.01, v/v/v/v) was used as mobile phase at a flow rate of 0.5 ml/min. Absorption at 220 nm was monitored to detect R-138727. The lower limit of quantification was 1 µM, and the intra-assay reproducibility was confirmed in the range of 1 to 100 µM. The stability of R-138727 in acetonitrile for 2 weeks at 4°C was 93.8% at a concentration of 5.7 µM.

Separation of Proteins in a Cytosol by Gel Filtration Chromatography. Crude human hepatic cytosols were centrifuged at 15,000g for 3 min at 4°C. Supernatant was filtered using MILLLEXR-GV (0.45 µm; Millipore) and was injected to the fast protein liquid chromatography (FPLC) system and separated on a gel filtration column (HiLoad 26/60 Superdex 75 prep grade; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) at room temperature. Potassium phosphate buffer (50 mM) containing 0.15 M NaCl (pH 6.8) was used as the mobile phase at a flow rate of 4 ml/min. UV absorption was monitored at 280 nm for detection of proteins. Each 8-ml fraction was collected, and the activity to produce R-138727 from R-133490 in the presence of glutathione was measured as described above.

Measurement of Protein Concentrations by Lowry Method. Five microliters of each sample, 25 µl of De Protein Assay Reagent A (Bio-Rad Laboratories, Inc., Hercules, CA), and 200 µl of De Protein Assay Reagent B (Bio-Rad Laboratories, Inc.) were mixed in a 96-well plate. The plate was gently shaken for 20 min at room temperature, and the absorbance was measured at 750 nm using a Spectra Thermo (Tecan Group Ltd., Männedorf, Switzerland). The calibration curve was generated using bovine serum albumin as a standard (0, 0.13, 0.25, 0.5, 1, and 2 mg/ml in 50 mM potassium phosphate buffer, pH 7.4).

Western Blot Analysis of Human Hepatic Cytosol Proteins with Anti-Human Glutaredoxin IgG. Electrophoresis of the samples fractionated from human hepatic cytosols was performed on 15% Ready Gel J (Bio-Rad Laboratories, Inc.) using biotinylated molecular weight standard for SDS-polyacrylamide gel electrophoresis (PAGE) (broad range; Bio-Rad Laboratories, Inc.) were mixed in a 96-well plate. The plate was gently shaken for 20 min at room temperature, and the absorbance was measured at 750 nm using a Spectra Thermo (Tecan Group Ltd., Männedorf, Switzerland). The calibration curve was generated using bovine serum albumin as a standard (0, 0.13, 0.25, 0.5, 1, and 2 mg/ml in 50 mM potassium phosphate buffer, pH 7.4). Western blot analysis of human hepatic cytosol proteins with anti-human glutaredoxin IgG was performed on 15% Ready Gel J (Bio-Rad Laboratories, Inc.) using biotinylated molecular weight standard for SDS-polyacrylamide gel electrophoresis (PAGE) (broad range; Bio-Rad Laboratories, Inc.) in Tris/Glycine/SDS Buffer (Bio-Rad Laboratories, Inc.) at 200 V for 40 min. Before electrophoresis, 10 µg of protein of each sample was mixed with an equivalent volume of Sample Buffer (Bio-Rad Laboratories, Inc.), including 2-mercaptoethanol (final concentration, 5%). After electrophoresis, the gel was gently shaken in transfer buffer [a mixture of 10× Tris/glycine buffer, methanol, and distilled water (1:2:7, v/v/v)] for 30 min at room temperature and then electroblotted at 100 V for 1 h onto pure nitrocellulose membrane (Bio-Rad Laboratories, Inc.). The membrane was blocked with 1% rabbit serum in 0.05% Tween with Tris-buffered saline (TBS) mixture of 20 mM Tris and 500 mM NaCl, pH 7.5 (0.05% TBS) for 30 min at room temperature and was reacted with goat anti-human glutaredoxin IgG (2 µg/ml) in 0.05%...
TBS for 30 min. After washing with Sample buffer, the membrane was incubated with biotinylated rabbit anti-goat IgG (contained in Vectastain Elite ABC IgG Kit; Vector Laboratories, Inc., Burlingame, CA) in 0.05% TBS for 30 min and washed. The membrane was shaken in Vectastain Elite ABC Reagent for 30 min and washed, and substrate of horseradish peroxidase (HRP) was added to detect the protein bands. Twenty-five milliliters of 1 X HRP color development buffer, 150 μl of HRP color reagent B, and 5 ml of HRP color reagent A (Bio-Rad Laboratories, Inc.) were mixed as the substrate buffer.

**Protein Identification by Mass Spectrometry.** Five microliters of the gel-filtrated active fraction of the human hepatic cytosol (active peak 2 from gel-filtration separation) was mixed with 10 μl of Laemmli sample buffer (Bio-Rad Laboratories) containing 5% 2-mercaptoethanol. After heat-denaturing the enzymes for 2 min at 95°C, the mixture was loaded for SDS-PAGE (15% gel; Bio-Rad Laboratories). The gel was stained with a Zinc Stain Kit (Bio-Rad Laboratories). The protein in an excised gel piece was digested according to the procedure described by Wilm et al. (1996). Bands excised from the gel were destained with 2.5 mM Tris containing 19.2 mM glycine (pH 8.0) for 5 min each, and the collected extracts were eluted with FA/CH3CN buffer using the gradient program, and introduced into the ion trap mass spectrometer (LCQ; Thermo Fisher Scientific, Waltham, MA) with an electrospray ion source using an in-house-built spray mount. During a run, if an ion was present in the scan above a specified threshold, the mass spectrometer automatically alternated among full mass spectrometry, zoom scan (high resolution but narrow mass range), and MS/MS modes, so that the masses and the amino acid sequence information of the peptides could be obtained.

The resulting tandem mass spectra were searched against the Swiss-Prot database (http://www.expasy.ch/sprot/) using the Mascot program (MS/MS ion search mode; Matrix Science, London, UK). A Mascot score greater than 42 indicates that proteins analyzed by LC-MS/MS are the ones shown in Fig. 5b.

**Formation of R-138727 from R-133490 by Human Glutaredoxin and Thioredoxin.** Each incubation mixture (in triplicate) contained 1 μg/ml human glutaredoxin, or 2 μM human thioredoxin and 1 mM glutathione, or 1 mM dithiothreitol, respectively, and 1, 6, 3.1, 6.3, 13, 25, 50, and 100 μM glutathione conjugate of R-138727 in 50 mM potassium phosphate buffer (KB; pH 7.4). The mixture without glutaredoxin or thioredoxin was also prepared. A mixture without glutathione conjugate of R-138727 was preincubated at 37°C for 5 min, and the reaction was initiated by adding a solution of glutathione conjugate of R-138727 in KB. After incubation at 37°C for 5 min, the incubation mixture was collected and appropriately diluted with KBP. Two hundred microliters of methoxyphenacyl bromide (5 mM) acetonitrile solution was added to 100 μl of each sample to terminate the reaction and left at room temperature for 10 min to derivatize the thiol moiety of R-138727. Then, a solution of R-135766 (100 nM) was added as the internal standard (100 nM in acetonitrile). The concentrations of derivatized R-138727 were determined using LC-MS/MS as described above.

**Data Handling.** The formation of R-1338727 from glutathione conjugate of R-138727 by glutaredoxin and thioredoxin indicated a biphasic pattern in each Eadie-Hofstee plot (data not shown), suggesting the involvement of another factor other than glutaredoxin or thioredoxin, which was considered as non-enzymatic formation by glutathione or dithiothreitol, respectively. Therefore, the data were fitted to eq. 1 using WinNonlin Professional (version 4.0.1; Pharsight, Mountain View, CA).

\[
V = V_{\text{max}} \times \frac{S}{(K_m + S)} + k \times S
\]

where \(S\) is the substrate concentration; the Michaelis-Menten constant \(K_m\) and maximal reaction rate \(V_{\text{max}}\), are the kinetic parameters for the enzymatic
component (glutaredoxin or thioredoxin); and $k$ is the nonenzymatic component (glutathione or dithiothreitol, respectively). The $k$ values were determined in advance by fitting the data of nonenzymatic formation by glutathione or dithiothreitol alone to eq. 2, and the respective mean values were substituted into eq. 1.

$$V = k \times S$$

The intrinsic clearance ($\text{CL}_{\text{int}}$) was calculated as a ratio of $V_{\text{max}}$ to $K_m$. These parameters are expressed as mean ± S.D.

**Results**

**R-138727 Formation from Its Glutathione Conjugate in Human Liver Microsomes and Cytosols.** Addition of cytosols to potassium phosphate buffer or human liver microsomes increased the reduction rate of R-133490 to R-138727 in the presence of 5 mM glutathione (Fig. 2). In potassium phosphate buffer alone, the substrate R-133490 was converted to R-138727 and possibly other decomposition products because the loss of R-133490 was greater than the formation of R-138727. In the presence of human liver microsomes and/or cytosols, a higher percentage (at least 70%) of R-133490 was converted to R-138727 than that found with buffer alone, probably due to the increased formation of R-138727 in the presence of enzymes. Addition of heated human liver cytosols (for 1 h at 100°C) to potassium phosphate buffer did not increase the formation rate of R-138727 from R-133490 in the presence of 5 mM glutathione over that observed with buffer alone (data not shown), indicating that enzymes in human liver cytosols and microsomes likely mediated the reduction of R-133490 to form R-138727.

**Separation of Proteins in a Cytosol by Gel Filtration Chromatography.** To explore the enzymes in human liver cytosols mediating R-138727 formation from R-133490, proteins in a cytosol were gel filtered by FPLC, and the R-138727-producing activities and protein concentrations in each gel-filtrated fraction were monitored (Fig. 3). Two active peaks (designated as active peak 1 and 2) were detected in the presence of 5 mM glutathione. R-138727-producing activities of these two peaks were much lower (approximately 2.5-fold at most, relative to glutathione alone) than the activity found with human liver cytosol (Fig. 2, a and b), possibly indicating the decrease of enzymatic activities during the purification process.

**Contribution of Glutaredoxin to R-138727-Producing Activities in Human Liver Cytosols.** Because glutaredoxin has been found to be highly selective for glutathione containing mixed disulfides (Srinivasan et al., 1997), we investigated the involvement of glutaredoxin in R-138727 formation from R-133490 in human liver cytosols. A Western blot analysis using anti-human glutaredoxin antibody, showed that active peak 2, but not active peak 1, contained glutaredoxin (Fig. 4). Glutaredoxin was also identified from peak 2 by mass spectrometry of the trypsin digests of the bands extracted from the SDS-PAGE gels (Fig. 5).

The activity to produce R-138727 in active peak 2, but not in active peak 1, was significantly inhibited by anti-human glutaredoxin antibody in the presence of 5 mM glutathione (Fig. 6a). R-138727 formation by active peak 1 in the presence of 300 µg/ml anti-human glutaredoxin antibody increased for reasons that are unknown. The activity in peak 2 was not entirely inhibited, probably due to other enzymes present or to nonenzymatic reduction by glutathione.

**Contribution of Thioredoxin to R-138727-Producing Activities in Liver Cytosols.** Thioredoxin is well known as an enzyme similar to glutaredoxin in its structure and enzymatic characteristics. Therefore, we thought that thioredoxin may be the enzyme responsible for the activity found in peak 1. The activity to produce R-138727 in active peak 1 was significantly inhibited by 600 µg/ml anti-human thioredoxin antibody in the presence of 10 mM glutathione (Fig. 6b). R-138727 formation by active peak 1 in the presence of 67 µg/ml anti-human thioredoxin antibody increased for unknown reasons. The activity in active peak 1 was not completely inhibited by the antibody, possibly because of reduction by glutathione and/or by other enzymes present. In addition, the antibody titer used in this experiment may have been insufficient to completely neutralize the enzyme activity.

**Calculation of Kinetic Parameters on the Formation of R-138727 from Its Glutathione Conjugate.** The enzyme kinetic parameters, $K_m$, $V_{\text{max}}$, and $\text{CL}_{\text{int}}$, calculated by eq. 1 were 12.1 ± 8.0 µM, 600 ± 187 pmol/(min · µg), and 0.882 ± 0.649 µl/(min · pmol), respectively, by glutaredoxin in the presence of glutathione, and 817 ± 316 µM, 553 ± 228 pmol/(min · µg), and 0.00804 ± 0.00035 µl/(min · pmol), respectively, by thioredoxin in the presence of dithiothreitol (Table 1). In the presence of 1 mM glutathione or 1 mM dithiothreitol alone, R-138727 was proportionally formed depending on the concentrations of glutathione conjugate of R-138727 and the $k$ values were 6.06 ± 0.53 or 0.38 ± 0.01 µl/(min · µg), respectively (Fig. 7).

**Fig. 3.** R-138727-producing activity and protein concentration in each cytosol fraction separated by gel filtration chromatography. For each fraction, R-138727-producing activities (○) in the presence of glutathione (final concentration: 5 mM) and protein concentrations (A) were monitored. Background level of R-138727 was monitored. Background level of R-138727 was expressed as the mean ± S.D. ($n = 3$).

**Fig. 4.** Western blotting of each fraction separated by gel filtration chromatography of a cytosolic preparation. Electrophoresis was performed on 15% Ready Gel J in Tris/glycine/SDS buffer at 200 V for 40 min. After the gel was shaken in the transfer buffer for 30 min, it was electroblotted at 100 V for 1 h onto pure nitrocellulose membrane, which was blocked with 1% rabbit serum for 30 min. Immune complex was formed with goat anti-human glutaredoxin IgG (2 µg/ml) for 30 min. The membrane was reacted with biotinylated rabbit anti-goat IgG for 30 min.
In this study, we focused on the possible enzymes mediating R-138727 formation from R-133490. The primary purpose was to isolate and identify the reductive enzymes in human liver cytosol that could possibly mediate R-138727 formation from R-133490. Using gel filtration chromatography, two different peaks (active peak 1 and 2) that could catalyze the reaction were found in human liver cytosol. The R-138727-producing activities in active peak 1 and 2 from a cytosol were inhibited by anti-thioredoxin and anti-glutaredoxin antibodies, respectively, indicating thioredoxin and glutaredoxin as the main enzymes in active peak 1 and 2, respectively.

Contribution ratios of active peak 1 and active peak 2 (expected as thioredoxin and glutaredoxin, respectively) could be estimated based on the peak areas under the FPLC fraction number minus R-138727-producing activity curve (area under the curve) after subtracting the concentrations of R-138727 formed with 5 mM glutathione alone in potassium phosphate buffer (dotted line in Fig. 3). The R-138727-producing area under the curve of active peak 2 was approximately 3 times higher than that of active peak 1, indicating that glutaredoxin contributes to a greater extent to the formation of R-138727 in human liver than thioredoxin. Moreover, we compared intrinsic clearance values on the formation of R-138727 from R-133490 by human glutaredoxin and thioredoxin. The CLint values by human glutaredoxin and thioredoxin were 0.882 ± 0.0004 and 0.00035 ± 0.00003 l/(min·g), respectively, resulting in 110-fold higher clearance with glutaredoxin than thioredoxin. The results in the present study are consistent with the previous report showing 18 to 300-fold greater activity of glutaredoxin than thioredoxin in reducing glutathione-protein mixed disulfide (Jung et al., 1996). In spite of such efficient ability of recombinant glutaredoxin to reduce R-133490, active peak

**Table 1**

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isolated from human liver cytosol showed only 3-fold higher activity than active peak 1, possibly indicating that less glutaredoxin is present in human liver cytosols than thioredoxin. In this study, glutaredoxin showed higher substrate specificity than thioredoxin in reducing R-133490 ($K_m$ = 12.1 ± 8.0 and 817 ± 316 μM for glutaredoxin and thioredoxin, respectively). Previous reports have identified the structures of these enzymes in the presence of substrate: *Escherichia coli* glutaredoxin with glutathione bound to the active cysteine thiol (Bushweller et al., 1994) and human thioredoxin with a putative substrate peptide bound to its active cysteine thiol (Qin et al., 1995). Glutaredoxin has a small active site that uses Val59 to bind the cysteine moiety of glutathione and Tyr72 and Thr73 to bind the glutamate portion of glutathione by hydrogen bonds. The potential for interactions between the active site Tyr13 and the glutamate of glutathione also suggests that thioredoxin, which lacks Tyr13, would have less interaction with glutathione (Bushweller et al., 1994). Thioredoxin contains interactions of a large number of amino acids with a substrate peptide in a more open active site by hydrogen bonds, peptide bonds, and hydrophobic interactions (Qin et al., 1995).

Human cells contain three glutaredoxins: 1) the cytosolic Grx1 with the active site Cys-Pro-Tyr-Cys; 2) the primary mitochondrial Grx2 containing the active site Cys-Ser-Tyr-Cys; 3) and another mitochondrial Grx5 with only one Cys residue (Cys-Gly-Phe-Ser) in its active site (Berndt et al., 2007). For thioredoxin, human genome encodes one cytosolic Trx1 and one mitochondrial Trx2 (Meyer et al., 2009). Given this information, the thioredoxin and glutaredoxin in active peaks 1 and 2, respectively, are considered likely to be Trx1 and Grx1, respectively. Because reduction of R-133490 was also promoted by human liver microsomes (Fig. 2c), Grx2 or Grx5 and Trx2 may also be contributing to the activity.

Glutaredoxin has been purified and identified from rat liver (Maellaro et al., 1994), human blood (Papov et al., 1994), human brain (Fernando et al., 1994), human placenta (Padilla et al., 1995), human

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### TABLE 1

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<tr>
<th>Incubation Time</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (μmol/min·μg)</th>
<th>$CL_{int}$ (l/min·μmol)</th>
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<td>Glutaredoxin</td>
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<td>12.1 ± 8.0</td>
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<td>817 ± 316</td>
<td>553 ± 228</td>
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<td>Thioredoxin</td>
<td>5</td>
<td>817 ± 316</td>
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Values are mean ± S.D. for $n = 3$. 

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Fig. 7. Kinetic analyses of the formation of R-138727 from its glutathione conjugate by human glutaredoxin and thioredoxin supplemented with glutathione and dithiothreitol, respectively. The formation rates of R-138727 from its glutathione conjugate in triplicate were determined in the presence of glutathione with (a) or without (b) recombinant human glutaredoxin or of dithiothreitol with (c) or without (d) recombinant human thioredoxin. In the presence of glutathione (b) or dithiothreitol (d) alone, the $k$ values were 6.06 ± 0.53 and 0.38 ± 0.01, respectively (mean ± S.D., $n = 3$).
lung (Peltoniemi et al., 2004), and human lens (Qiao et al., 2001). In addition, thioltransferase activities in human hepatic cell lines have been reported (Yuk-Young Lee, 2002). However, identification of glutaredoxin from human liver has not been reported. In addition, thioredoxin has been identified from human liver (EMBL/GenBank/ DDBJ databases, \url{http://www.ncbi.nlm.nih.gov/nuccore/af276919.1}), brain (EMBL/GenBank/DDBJ databases, \url{http://www.ncbi.nlm.nih.gov/nuccore/11345419}), and cervix (Strausberg et al., 2002), but not from liver. Therefore, the present study is the first report showing that glutaredoxin and thioredoxin are present in human liver cytosol. Moreover, human glutaredoxin and thioredoxin have not been reported to reduce the mixed disulfide of xenobiotics with glutathione even though these enzymes isolated from \textit{E. coli} are reported to be active on the reduction of S-S dimer xenobiotics (Verschraagen et al., 2004). Accordingly, this study is also the first report showing that glutaredoxin and thioredoxin in human liver functionally reduce the mixed disulfide of xenobiotics with glutathione.

In conclusion, glutaredoxin and thioredoxin in human liver cytosols could work as reductive enzymes in the formation of the pharmacologically active metabolite of prasugrel from its glutathione conjugate with much higher activity for glutaredoxin.

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Authorship Contributions

\textbf{Participated in research design:} Hagihara, Kazui, Kurihara, and Ikeda.

\textbf{Conducted experiments:} Hagihara, Kazui, and Kubota.

\textbf{Performed data analysis:} Hagihara, Kazui, and Kubota.

\textbf{Wrote or contributed to the writing of the manuscript:} Hagihara, Kazui, Kurihara, Kubota, and Ikeda.

References


Thioredoxin has been identified from human lens (EMBL/GenBank/DBJ databases, \url{http://www.ncbi.nlm.nih.gov/nuccore/af276919.1}), brain (EMBL/GenBank/DDBJ databases, \url{http://www.ncbi.nlm.nih.gov/nuccore/11345419}), and cervix (Strausberg et al., 2002), but not from liver. Therefore, the present study is the first report showing that glutaredoxin and thioredoxin are present in human liver cytosol. Moreover, human glutaredoxin and thioredoxin have not been reported to reduce the mixed disulfide of xenobiotics with glutathione even though these enzymes isolated from \textit{E. coli} are reported to be active on the reduction of S-S dimer xenobiotics (Verschraagen et al., 2004). Accordingly, this study is also the first report showing that glutaredoxin and thioredoxin in human liver functionally reduce the mixed disulfide of xenobiotics with glutathione.

In conclusion, glutaredoxin and thioredoxin in human liver cytosols could work as reductive enzymes in the formation of the pharmacologically active metabolite of prasugrel from its glutathione conjugate with much higher activity for glutaredoxin.

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