(S)-Preferential detoxification of 4-hydroxy-2(*E*)-nonenal enantiomers by hepatic glutathione S-transferase isoforms in guinea-pigs and rats

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In guinea-pig liver cytosol, racemic 4-hydroxy-2(*E*)-nonenal (HNE), a reactive and highly toxic product released from biomembranes by lipid peroxidation, was detoxified (*S*)-preferentially by GSH conjugation mediated by glutathione S-transferases (GSTs) and (*R*)-preferentially by NAD⁺-dependent oxidation mediated by aldehyde dehydrogenase (ALDH). The GST-mediated detoxification of the HNE enantiomers proceeded at much higher rates than that mediated by ALDH in guinea-pig liver cytosol. All the major guinea-pig GSTs, A1-1, M1-1, M1-2 and M1-3*, isolated from guinea-pig liver cytosol also catalysed the (*S*)-preferential conjugation of the HNE enantiomers. The

liver and other major tissues of guinea-pigs had no immunologically detectable level of a putative GSTA4-4 orthologue, which exists as a minor GST protein in rat, mouse and human livers and exhibits extremely high catalytic activity towards HNE. All the hepatic rat GSTs, A1-1(2), A1-3, A4-4, M1-1, M1-2 and M2-2, also catalysed the (*S*)-preferential conjugation of HNE enantiomers.

Key words: alcohol dehydrogenase, aldehyde dehydrogenase, glutathione conjugation, lipid peroxidation, stereoselectivity.

INTRODUCTION

Peroxidation of membrane lipids in cells and tissues is known to produce various aldehydic compounds [1,2], which are strongly suggested to cause a wide range of chronic diseases, including atherosclerosis [3], Alzheimer's disease [4], cataractogenesis [5], Parkinson's disease [6] and cancer [2]. 4-Hydroxy-2(*E*)-nonenal (HNE), a racemic lipid peroxidation product, is recognized as being the most cytotoxic and genotoxic product among a variety of aldehydic compounds originating from the non-enzymic peroxidative breakdown of *n*-6 polyunsaturated fatty acid residues of membrane lipids, such as linoleate and arachidonate [1,2]. HNE is detoxified mainly by glutathione S-transferase (GST) [7–10] and at minor rates by alcohol dehydrogenase (ADH) [11,12] and aldehyde dehydrogenase (ALDH) [13,14] in liver cytosol of rodents.

In mammalian species, based on subcellular localization and primary structure, two distinct groups of GSTs have been defined. One is the cytosolic GSTs, and the other is the membrane-bound and designated microsomal GSTs [15–18]. The cytosolic GSTs occur as homo- and heterodimeric proteins with subunit masses of 23–28 kDa [15–17] and are classified into eight subfamilies, Alpha, Mu, Pi, Theta, Sigma, Kappa, Zeta and Omega classes, based on their primary structures [17,18]. Three distinct microsomal GSTs (MGSTs) have been identified in mammalian species and termed MGST-I, -II and -III [18,19]. The MGSTs grouped together with 5-lipoxygenase-activating protein and leukotriene C₄ synthase are collectively called MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) [19].

It has been shown that in rat (*r*) liver the minor Alpha-class GST (GSTA), rGSTA4-4, plays the most important role

among GSTs, by detoxifying racemic HNE [20]. GSTA4-4 orthologues, occurring as minor enzymes in liver cytosol of mice [9] and humans [10], also exhibit extremely high catalytic activity in the conjugation and detoxification of racemic HNE compared with other Alpha-class GSTs and GST isoforms of other classes [17].

It is of interest to investigate the enantioselective interactions of the potent endogenous toxicants, HNE enantiomers, with biomacromolecules. We recently demonstrated for the first time that the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase is irreversibly and (*S*)-preferentially inactivated by the HNE enantiomers [21]. We also showed that in rat liver cytosol, HNE enantiomers were detoxified (*S*)-preferentially by GSH conjugation and rather (*R*)-preferentially by NADH-dependent reduction mediated by ADH and that the minor rGSTA4-4 played a major role in the hepatic cytosolic (*S*)-preferential detoxification of HNE enantiomers [21]. However, nothing is known of which enantiomer of HNE occurs predominantly *in vivo* as a result of the metabolic inactivation of the racemate by these enzymes.

GSTA4-4 from the rat and mouse has a unique immunochemical property in that polyclonal antibodies to this isoform do not cross-react with any Alpha-class and other classes of GSTs, but only with corresponding antigens across and within species [9,22]. Therefore, it would be expected that the polyclonal antibody to rGSTA4-4 could be useful for detecting its orthologue specifically in tissues of other mammalian species including the guinea-pig.

It is well known that guinea-pigs have no detectable level of selenium-containing glutathione peroxidase (Se-GSH Px) in their tissues or organs but that their erythrocytes contain significant levels of this enzyme with activity comparable with that of

Abbreviations used: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione S-transferase; gpGST, guinea-pig GST; rGST, rat GST; GSTA, Alpha-class GST; GSTM, Mu-class GST; GSTP, Pi-class GST; HNE, 4-hydroxy-2(*E*)-nonenal; RP-HPLC, reversed-phase partition HPLC; GSH Px, glutathione peroxidase; Se-GSH Px, selenium-containing glutathione peroxidase; TBARS, thiobarbituric acid-reacting substances; PBE, polybuffer exchanger.

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The nucleotide data for guinea-pig GSTA1 reported in this paper have been deposited in the DDBJ, GenBank®, GSDB and EMBL Nucleotide Sequence Databases under the accession number AB016426.

erythrocyte Se-GSH Px in other mammals [23,24]. Se-GSH Px has potent specific activity towards hydroperoxides of arachidonic and linoleic acids [25], both of which are precursors of racemic HNE [2]. Therefore, in view of the defence against toxic racemic HNE formed in normal cells [1,2], it is of interest to know which enantiomer of the racemate is more readily detoxified by the cytosolic enzymes GST, ADH and ALDH in the guinea-pig. This paper deals with (i) the enantioselectivity in the GSH conjugation and oxidation of HNE enantiomers in guinea-pig liver cytosol, (ii) the existence of at least four GSTs catalysing the GSH conjugation of the toxic enantiomers, (iii) the absence of a putative GSTA4-4 orthologue in cytosol of the liver and other major tissues in the guinea-pig and (iv) the result of a comparative study on the GSH conjugation of HNE enantiomers by guinea-pig GSTs (gpGSTs) and rGSTs, both of which were purified from liver or kidney cytosol.

MATERIALS AND METHODS

Materials

1-Chloro-2,4-dinitrobenzene (CDNB), cumene hydroperoxide, disulphiram, 1,1,3,3-tetramethoxypropane, yeast GSH reductase, NAD^+ , NADP^+ , NADH and NADPH were purchased from Wako Pure Chemicals Industries (Osaka, Japan); iron-dextran, PMSF, pyrazole and molecular-mass marker proteins were from Sigma (St. Louis, MO, U.S.A.); epoxy-activated Sepharose 6B, polybuffer exchanger (PBE) 94, PBE 118, Pharmalyte 8-10.5 and Polybuffer 96 were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Goat anti-rabbit IgG was purchased from Organon Teknica Co. (West Chester, PA, U.S.A.) and peroxidase-conjugated rabbit anti-peroxidase IgG was from Seikagaku Kogyo Co. (Tokyo, Japan). Nitrocellulose membranes were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.), and Aquacide II was from Calbiochem-Novabiochem (La Jolla, CA, U.S.A.). GSH, with purity higher than 99.8% and free from GSSG, was donated from Yamanouchi Pharmaceutical Co. (Tokyo, Japan). Other reagents used were of reagent grade.

(R)- and (S)-HNEs with enantiopurity higher than 99% were prepared by the enzymic resolution of racemic HNE dimethyl acetal [26] and purified by HPLC on a chiral column (Chiralcel OB, 10 μm pore size, 4.6×250 mm; Daicel Chemical Industries, Tokyo, Japan) with a Jasco model 800 liquid chromatograph [21]. Concentrations of racemic, (R)- and (S)-HNEs were determined by measuring A_{223} , assuming a molar absorption coefficient of $13750 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for HNE (in water) [2].

Assay of thiobarbituric acid-reacting substances (TBARS)

Lipid peroxidation in liver homogenates of control and iron-dextran-treated animals was measured by TBARS assay according to a method described previously [27].

Animals and treatments

Male Sprague-Dawley rats, weighing 200–230 g (7 weeks old) and male Hartley guinea-pigs, weighing 300–350 g (5 weeks old), were each given a total of three intraperitoneal injections at 72 h intervals of an iron-dextran suspension (100 mg/kg of body weight) as reported previously [28]. A control group was injected with an equivalent volume of saline. The animals were anaesthetized with diethyl ether and killed 24 h after the third injection following an overnight fast, and their livers were excised, washed, perfused with cold isotonic KCl through the portal vein, and homogenized in 3 vol. of 10 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer,

pH 7.0, containing 1.4 mM 2-mercaptoethanol and centrifuged at 28000 g for 60 min.

Preparation of cytosol

Male Hartley guinea-pigs (5 weeks old) were anaesthetized with diethyl ether after an overnight fast, killed and their livers, kidneys, hearts and lungs were used for preparation of cytosolic fractions. Their isolated livers were perfused with cold isotonic KCl through the portal vein. The perfused livers and other tissues isolated from the animals were homogenized in 3 vol. of 150 mM Tris/HCl buffer, pH 7.4, containing 5 mM EDTA, 193 mM KCl, 0.25 mM PMSF and 6.25 mM dithiothreitol. Tissue cytosolic fractions were obtained by centrifugation of post-mitochondrial fractions of the homogenates at 105000 g for 60 min. The hepatic cytosolic fraction was dialysed twice for 6 h against 50 vol. of 10 mM Tris/HCl buffer, pH 7.8, containing 1 mM EDTA and 2 mM 2-mercaptoethanol.

Enzyme assay

GSH-conjugating activities of GSTs towards HNE [29] and CDNB [30], their glutathione peroxidase (GSH Px) activities towards cumene hydroperoxide [25], and also ALDH [31] and ADH [11] activities towards HNE, were determined as reported previously. For characterization of the cytosolic HNE-reducing and HNE-oxidizing enzymes, pyrazole (1 mM), an ADH inhibitor [32], or disulphiram (1 mM), an ALDH inhibitor [33], was added to the cytosolic fraction and allowed to incubate for 5 min before the addition of HNE (0.1 mM). Kinetic parameters, K_m and V_{max} , for the enzyme reactions of racemic, (R)- and (S)-HNEs were obtained from double-reciprocal plots at various substrate concentrations against apparent reaction rates obtained in the zero-order kinetics regions. Results are expressed as means of at least three experiments.

Purification of gpGSTs A1-1, M1-1, M1-2 and M1-3*

Hepatic Mu-class gpGSTs (gpGSTMs) were purified from guinea-pig liver cytosol as reported previously [34]. gpGSTA1-1 was purified from a pooled fraction passing through an S-hexyl-GSH-Sepharose 6B affinity column [35] to which dialysed guinea-pig liver cytosol (759 mg of protein) was applied directly to remove gpGSTMs. This pooled fraction, containing the first 60 ml of the column-washing solution, was concentrated to 20 ml (615 mg of protein) against Aquacide II, dialysed twice for 6 h against 1000 ml of 20 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, pH 7.0, containing 5 mM 2-mercaptoethanol, and then applied to a GSH-Sepharose 6B affinity column (1.5×6 cm) [36] pre-equilibrated with the same buffer used for dialysis. The column was washed with 280 ml of the same buffer and then eluted with 120 ml of 50 mM Tris/HCl buffer, pH 9.6, containing 5 mM GSH and 200 mM NaCl. The column effluent was collected into 7 ml fractions. The eluate containing 11 mg of protein in 28 ml was concentrated to 4 ml against Aquacide II and twice dialysed for 6 h against 1000 ml of 25 mM triethylamine/HCl, pH 11.0. It was then applied to a chromatofocusing gel (PBE 118) column ($1 \text{ cm} \times 30 \text{ cm}$) pre-equilibrated with the same buffer used for dialysis. The column was eluted at a flow rate of 13 ml/h with 400 ml of 1–80-fold diluted Pharmalyte adjusted to pH 8.0 with HCl. The column effluent was collected into 4 ml fractions. Fractions containing gpGSTA1-1 and two other very minor Alpha-class gpGST isoforms were pooled separately and concentrated against Aquacide II. GST activity of the fractions was determined with racemic HNE and CDNB, and GSH Px activity with cumene hydroperoxide used as substrates.

Preparation of GSH-affinity-purified GST pools from guinea-pig tissue cytosols

Purification of total GSTs from cytosols of liver, kidney, lung and heart was carried out using a GSH–Sepharose 6B affinity column according to a method published previously [36].

Purification of rGSTs and immunoblotting

rGSTs A1-1(2), A1-3, A3-3, M1-1, M1-2, M2-2 [25] and A4-4 [8] were purified from rat liver cytosol and rGSTP1-1 (a Pi-class rGST) [25] from rat kidney cytosol as reported previously.

The preparation of polyclonal antibodies against rGSTs A1-2, A4-4, M1-1 and P1-1 and Western-blot analysis using the polyclonal antibodies were as described previously [37].

Reversed-phase partition HPLC (RP-HPLC) and CNBr fission

Separation and isolation of GST subunits and CNBr-cleaved peptides were carried out on a μ Bondasphere C₁₈-300 Å column (5 μ m particle size, 3.9 $\times$ 150 mm; Nihon Waters, Tokyo, Japan) with an Atto Model Constametric-II liquid chromatograph as reported previously [34].

gpGST subunit A1 isolated by RP-HPLC was reduced, alkylated, and treated with CNBr according to a previously published method [34], and peptides obtained were separated and isolated from the reaction mixture by RP-HPLC.

Automated amino acid sequence analysis

N-terminal amino acid sequence analysis of purified gpGST subunit A1 and CNBr fragments obtained thereof was performed with an Applied Biosystems model 470A gas-phase protein sequencer (Foster City, CA, U.S.A.) as described previously [34]. C-terminal amino acid sequence analysis of purified gpGST subunit A1 was performed with an Applied Biosystems Procise C494 C-terminal amino acid sequencer.

Electrophoresis

SDS/PAGE was performed using the method of Laemmli [38]. Proteins were stained with Coomassie Brilliant Blue R-250.

Data analysis

Statistical significance was evaluated using Student's *t* test or Welch's *t* test, with *P* < 0.05 being taken to be significant. Values are expressed as means $\pm$ S.D.

Protein determination

Protein concentration was determined by the method of Bradford [39] with BSA as a standard.

RESULTS

Enantioselectivity in GSH conjugation and oxidation of HNE enantiomers in guinea-pig liver cytosol

A kinetic study using dialysed cytosol from guinea-pig liver indicated that (*R*), (*S*) and racemic forms of HNE were all detoxified much faster by GSH conjugation than by NAD⁺-dependent oxidation in the cytosol (Table 1). $V_{\max}/K_m$ values were 5.8-, 2.4- and 10-fold higher due to conjugation than oxidation for racemic, (*R*)- and (*S*)-HNEs, respectively. The kinetic parameters indicated that (*S*)-HNE was detoxified 1.7-fold faster than (*R*)-HNE by the conjugation, whereas (*R*)-HNE was 2.4-fold faster than (*S*)-HNE by oxidation. However, the K_m values for the GSH conjugation of (*R*)- and (*S*)-HNEs were 5.2-

and 11-fold higher than those for the NAD⁺-dependent oxidation of these enantiomers, respectively (Table 1). No detectable NAD(P)H-dependent reducing activity towards the HNEs was observed in the cytosol (results not shown).

Isolation and purification of gpGSTs active towards HNE from guinea-pig liver cytosol

A dialysed cytosolic fraction from guinea-pig liver exhibited GST activities of 6.7 and 0.15 μ mol/min per mg of protein towards CDNB and HNE, respectively. 7.3 % of the CDNB- and 21 % of the HNE-conjugating activities of the hepatic cytosol passed through an *S*-hexyl-GSH–Sepharose affinity column (peak I) to which the cytosolic fraction was directly applied, and the remainder was eluted from the affinity column (peak II) with *S*-hexyl-GSH (Figure 1A). The HNE-conjugating activity in the fraction passing through from the *S*-hexyl-GSH affinity column was quantitatively retained on the GSH affinity column and eluted with GSH (peak III; Figure 1B). This peak III was separated by chromatofocusing into one major (IV) and two very minor (V and VI) peaks (Figure 1C).

The major gpGST, isolated as peak IV with an observed pH value of 9.2 by chromatofocusing, had GST activity towards HNE (3.3 μ mol/min per mg of protein). It was eluted at an approx. mass of 50 kDa from a TSK gel G3000 SW gel-filtration column calibrated with molecular-mass marker proteins, appeared as a single band at 24 kDa on SDS/PAGE (Figure 2A), and was eluted as a single peak at 50.5 min from a μ Bondasphere C₁₈-300 Å column by RP-HPLC, indicating that the enzyme protein was a homodimer. Western-blot analysis indicated that the homodimeric gpGST was an Alpha-class enzyme, as it cross-reacted with anti-rGSTA1-1(2)-antibody (Figure 2B), but not with antibodies to rGSTM1-1 (Figure 2C) or P1-1. N-terminal amino acid sequence analysis indicated that the N-terminus of this enzyme was blocked and that the two CNBr-fission fragments obtained thereof had sequences of CQEDLDKLNKNDGLL and VEEFDASLLANFTLLQALK, both of which were completely identical to local sequences reported previously for gpGSTa by Kamei-Hayashi et al. [40], who also demonstrated its N-terminus to be *N*-acetyl-Ser. In this study, we re-designated the homodimeric gpGSTa as gpGSTA1-1 according to the most recent nomenclature system.

The nucleotide sequence of the cDNA for gpGSTA1 coded for a polypeptide of 222 amino acid residues with a molecular mass of 25620 Da. The deduced amino acid sequence indicated that there was a minor error in the C-terminal amino acid sequence of the subunit A1 determined chemically by Kamei-Hayashi et al. [40], a deletion of two basic amino acids and one neutral one, Arg, Phe and Lys (Figure 3). Actually, C-terminal amino acid sequence analysis demonstrated that gpGSTA1-1 isolated from liver cytosol in our study had these three amino acid residues, as underlined in the observed C-terminal sequence of the subunit (see Figure 3).

The two very minor gpGSTs isolated as peaks V and VI by chromatofocusing (Figure 1C) appeared as single bands at an approx. subunit mass of 24 kDa on SDS/PAGE. Western-blot analysis indicated that they cross-reacted with the anti-rGSTA1-1(2) antibody, but not with antibodies to rGSTM1-1 or P1-1 (results not shown).

Hepatic cytosolic GST activity towards HNE, which was retained on the *S*-hexyl-GSH affinity column as mentioned above, was quantitatively eluted from the column with *S*-hexyl-GSH and separated by chromatofocusing into three peaks, VII, VIII and IX, with activity for HNE in the ratio 9.4:2:1, respectively (Figure 1D). The GSTs of peaks VII, VIII and IX

Table 1 Kinetic parameters for GSH conjugation and NAD-dependent oxidation of racemic, (*R*)- and (*S*)-HNEs in guinea-pig liver cytosol

Kinetic constants were determined by incubating racemic, (*R*)- or (*S*)-HNE with appropriate amounts of guinea-pig liver dialysed cytosolic protein in the presence of GSH (0.5 mM) at pH 6.5 or NAD (0.3 mM) at pH 7.5. Substrate concentrations ranging from 4 to 50 μ M were used for GSH conjugation and NAD-dependent oxidation. Reactions were performed at 25 °C for 3 min as described in the Materials and methods section. Data were obtained from double-reciprocal plots of initial reaction rates against substrate concentration and are expressed as means $\pm$ S.D. ($n > 5$). *, Significantly greater ($P < 0.01$) than NAD-dependent reduction. †, Significantly greater ($P < 0.01$) than (*R*)-HNE. ‡, Significantly less ($P < 0.01$) than (*R*)-HNE.

Substrate	GSH conjugation			NAD-dependent oxidation		
	K_m (μ M)	V_{max} (nmol/min per mg of protein)	V_{max}/K_m	K_m (μ M)	V_{max} (nmol/min per mg of protein)	V_{max}/K_m
Racemic HNE	20.1 $\pm$ 0.2	103 $\pm$ 1.5	5.1 $\pm$ 0.02*	3.2 $\pm$ 0.1	2.83 $\pm$ 0.02	0.88 $\pm$ 0.033
(<i>R</i>)-HNE	20.7 $\pm$ 0.3	86.2 $\pm$ 2.0	4.1 $\pm$ 0.03*	4.0 $\pm$ 0.3	6.78 $\pm$ 0.12	1.7 $\pm$ 0.093
(<i>S</i>)-HNE	22.3 $\pm$ 0.2	155 $\pm$ 1.9	7.0 $\pm$ 0.11*†	2.1 $\pm$ 0.1	1.52 $\pm$ 0.03	0.7 $\pm$ 0.032‡

Figure 1 Isolation and purification of gpGSTs active towards 4-HNE from guinea-pig liver cytosol by column chromatography

(A) A pooled guinea-pig liver 105000 *g* supernatant fraction (759 mg of protein) was applied to an *S*-hexyl-GSH affinity column pre-equilibrated with 10 mM Tris/HCl buffer, pH 7.8, containing 1 mM EDTA and 2 mM 2-mercaptoethanol. The column was eluted stepwise with the buffer containing 200 mM NaCl and 5 mM *S*-hexyl-GSH. Horizontal arrows represent fractions I and II pooled for subsequent GSH affinity chromatography and chromatofocusing. (B) Pooled fraction I (615 mg of protein) obtained by *S*-hexyl-GSH affinity column chromatography was dialysed, concentrated and applied to a GSH affinity column. The column was eluted stepwise with 50 mM Tris/HCl buffer, pH 9.6, containing 200 mM NaCl and 5 mM GSH. Horizontal arrows represent the fraction pooled for subsequent chromatofocusing. (C) The pooled GST fraction III (11 mg of protein) obtained by GSH affinity column chromatography was dialysed, concentrated and applied to a chromatofocusing gel (PBE 118) column pre-equilibrated with 25 mM triethylamine/HCl, pH 11.0. The column was eluted with Pharmalyte adjusted to pH 8.0 with HCl. Pooled fraction IV contained homogeneous gpGSTA1-1. (D) The pooled fraction II (40 mg of protein) obtained by *S*-hexyl-GSH affinity column chromatography was dialysed, concentrated and applied to a chromatofocusing gel (PBE 94) column pre-equilibrated with 25 mM ethanolamine/HCl, pH 9.4. The column was eluted with Polybuffer 96 adjusted to pH 7.0 with HCl. Pooled fractions VII, VIII and IX contained homogeneous gpGSTs M1-1, M1-2 and M1-3*, respectively. Protein concentrations (broken lines) were determined by absorbance at 280 nm. GST activities were measured with CDNB and HNE as substrates and GSH Px activity with cumene hydroperoxide as substrate throughout the enzyme purification, as described in the Materials and methods section.

were identified as gpGSTs M1-1, M1-2 and M1-3*, respectively, by SDS/PAGE and Western-blot analyses (Figure 2C) and by RP-HPLC on μ Bondasphere C_{18} -300 Å with the corresponding authentic specimens purified from guinea-pig liver cytosol in our

laboratory as reported previously [34]. RP-HPLC analysis also indicated that the four major gpGSTs existed at concentrations of 10.5, 36.9, 7.9 and 4.0 μ g/mg of cytosolic protein for A1-1, M1-1, M1-2 and M1-3* in the liver, respectively.

Figure 2 SDS/PAGE (A) and Western-blot (B and C) analyses of purified guinea-pig liver cytosolic GSTs active towards CDNB and HNE

SDS/PAGE was performed on a 15% (w/v) acrylamide gel plate. For Western-blot analysis, protein samples were stained by rabbit antisera raised against purified rGSTA1-1(2) and rGSTM1-1. Lanes 1, 2, 3 and 4 represent gpGSTs (0.5 μ g each) isolated as peaks IV, VII, VIII and IX by chromatofocusing, as shown in Figures 1(C) and 1(D). The result of Western-blot analysis using polyclonal antibody to rGSTP1-1 is not shown, because none of the gpGSTs isolated cross-reacted with the antibody.

Figure 3 Alignment of amino acid sequences of gpGSTA1 determined by molecular cloning (upper line) and chemically (lower line)

Dashes in the chemically determined sequence represent amino acid residues identical to those of the molecularly cloned sequence. The underlined sequence represents the result of C-terminal amino acid sequence analysis of hepatic gpGSTA1-1. Asterisks represent the amino acid residues absent in the sequence reported by Kamei-Hayashi et al. [40].

A kinetic study indicated that all four major gpGST isoforms isolated from liver cytosol also catalysed the (*S*)-preferential GSH conjugation of the HNE enantiomers as observed in their hepatic cytosolic conjugation (Table 2).

Absence of a putative GSTA4-4 orthologue in guinea-pig liver cytosol

A comparative immunochemical study was performed using hepatic cytosols from guinea-pigs and rats to investigate whether

Figure 4 Western-blot analysis of hepatic cytosols from iron-dextran-treated guinea-pigs and rats

Lane 1, purified rGSTA4-4 (0.5 μ g) as a positive control. Lanes 2 and 3 represent untreated and iron-dextran-treated guinea-pig liver cytosols, respectively. Lanes 4 and 5 represent untreated and iron-dextran-treated rat liver cytosols, respectively. The same amount of cytosolic protein (100 μ g) was used for lanes 2–5.

guinea-pig liver has a putative GSTA4-4 orthologue with extremely high GST activity towards the toxic aldehyde, HNE, in rodents and in the human. Polyclonal antibody to rGSTA4-4 purified from rat liver cytosol could not detect any immunostainable band in guinea-pig liver cytosol by Western-blot analysis, whereas the very minor enzyme protein, rGSTA4-4, in rat liver cytosol was well stained under the same conditions (Figure 4). Western-blot analysis also indicated that intra-peritoneal treatment of guinea-pigs with iron-dextran, an inducer of hepatic lipid peroxidation and GSTA4-4 in rodents [28], could not induce expression of a GSTA4-4 orthologue in their liver cytosol, whereas a significant increase in the hepatic rGSTA4-4 level was observed in rats after the same treatment (Figure 4). There was no detectable amount of protein cross-reacting with anti-rGSTA4-4 antibody in cytosol from the kidney, lung and heart of the control and iron-dextran-treated guinea-pigs. Furthermore, the polyclonal antibody could not detect any immunostainable band in the GSH-affinity-purified GST pools obtained from the cytosols of liver, kidney, lung and heart of the control guinea-pigs.

No appreciable difference was observed in hepatic cytosolic GSH-conjugating activity for racemic HNE between the control (0.15 ± 0.01 μ mol/min per mg of protein) and iron-dextran-treated (0.14 ± 0.01 μ mol/min per mg of protein) guinea-pigs, whereas hepatic cytosol from rats treated with the lipid-peroxidation inducer showed 1.5-fold higher GST activity for racemic HNE than did the controls.

Table 2 Kinetic parameters for GSH conjugation of (*R*)- and (*S*)-HNEs by gpGSTs M1-1, M1-2, M1-3 and A1-1 purified from guinea-pig liver cytosol

To determine K_m and V_{max} values for GSH conjugation of (*R*)- and (*S*)-HNEs, substrate concentrations ranging from 4 to 25 μ M were used at pH 6.5 with purified gpGSTs M1-1 (1.8 μ g), M1-2 (2.1 μ g), M1-3 (1.3 μ g) or A1-1 (2.4 μ g) and a GSH concentration of 0.5 mM. Reactions were performed at 25 $^{\circ}$ C for 3 min, as described previously [29]. For calculation of k_{cat} values for gpGSTs, the molecular masses for gpGSTs M1-1, M1-2 and M1-3 were taken from Hiratsuka et al. [34] and for gpGSTA1-1 from this study. Values are means $\pm$ S.D. ($n > 5$).

GST isoform	(<i>R</i>)-HNE			(<i>S</i>)-HNE		
	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
A1-1	61 ± 2.2	3.7 ± 0.14	61 ± 0.5	20 ± 1.1	2.2 ± 0.11	110 ± 0.3
M1-1	12 ± 0.6	0.82 ± 0.01	68 ± 2.3	38 ± 1.3	3.7 ± 0.08	98 ± 0.7
M1-2	20 ± 0.9	0.78 ± 0.04	39 ± 2.6	12 ± 0.5	1.2 ± 0.05	100 ± 0.5
M1-3	66 ± 0.2	1.3 ± 0.05	19 ± 0.6	39 ± 1.1	2.7 ± 0.03	70 ± 1.1

Figure 5 TBARS levels in 28000 g supernatants from liver homogenates of untreated and iron-dextran-treated guinea-pigs and rats

Each bar represents means $\pm$ S.D. from three animals. TBARS were represented by malondialdehyde (MDA) values as described in the Materials and methods section.

Treatment of guinea-pigs and rats with iron-dextran resulted in hepatic TBARS levels that were 2.8- and 3.4-fold higher than those of the untreated animals, respectively (Figure 5).

GSH conjugation of HNE enantiomers by major GSTs isolated from rat liver and kidney cytosols

Our previous study using a rat liver cytosolic fraction and rGSTA4-4 purified thereof showed greater k_{cat}/K_m values with smaller K_m values towards (*S*)-HNE than towards (*R*)-HNE (Table 3) [21]. However, nothing has been published previously about the enantioselectivity in the GSH conjugation of the HNE enantiomers by other rGST isoforms. All the six major rGSTs, A1-1(2), A1-3, A3-3, M1-1, M1-2 and M2-2, isolated from the liver showed lower K_m values and greater k_{cat}/K_m values for (*S*)-HNE than for (*R*)-HNE, although the kinetic parameters for the GSH conjugation of the HNEs by these rGSTs were not significant compared with those by rGSTA4-4 (Table 3). rGSTA4-4 showed the lowest K_m and the highest k_{cat} values

towards (*S*)-HNE of all the rGST isoforms used. Another major rGSTP1-1 isolated from the kidney showed little difference in enantioselectivity.

The rGSTMs catalysed GSH conjugation of the HNE enantiomers more (*S*)-preferentially than did the major rGSTAs (Table 3). Of the three rGSTMs, M2-2 showed the highest (*S*)-selectivity.

DISCUSSION

The metabolism of racemic HNE has been studied *in vitro* using hepatocytes [2,41,42], hepatoma cells [2] and isolated heart [2,43,44], and *in vivo* in rats [2,45]. These studies indicate that rat liver can rapidly convert racemic HNE to less toxic metabolites by means of conjugation with GSH by GST, oxidation by ALDH and reduction by ADH or aldehyde reductase. Of these metabolic processes, GST-mediated GSH conjugation is recognized as being the most effective for detoxifying racemic HNE [41,42].

Recently, we demonstrated that in rat liver cytosol HNE enantiomers were detoxified (*S*)-preferentially by GSH conjugation and (*R*)-preferentially by NADH-dependent reduction mediated by ADH, whereas there was little difference observed in the reaction rate of cytosolic oxidation mediated by ALDH between the enantiomers [21]. In guinea-pig liver cytosol, HNE enantiomers were also detoxified (*S*)-preferentially by GSH conjugation, and the oxidation of these enantiomers proceeded (*R*)-preferentially (Table 1). The cytosolic oxidation of the racemic and enantiomeric HNEs in guinea-pig liver cytosol was completely inhibited by 1 mM disulphiram (results not shown), an ALDH inhibitor, as demonstrated previously by us in the rat [21]. Although guinea-pig liver cytosol showed considerable ADH activity towards ethanol (1.5 nmol/min per mg of protein) and acetaldehyde (1.82 nmol/min per mg of protein) in the presence of NAD⁺ and NADH, respectively, compared with rat liver cytosol [11,46], HNE enantiomers were inactive substrates for guinea-pig liver ADH. Cytosolic ADHs have been purified from the livers of rats [47], mice [48] and humans [49], and their functions and primary structures have been reported. However, no information regarding ADH in the guinea-pig is available.

rGSTA4-4, a very minor GST component in rat liver cytosol, scavenges racemic HNE with the formation of a Michael-type of HNE-GSH conjugate at an extremely high rate compared with other rGSTs [17]. Recently, Hayes et al. [50] reported that rGSTA5-5 had potent activity towards racemic HNE, which is

Table 3 Kinetic parameters for GSH conjugation of (*R*)- and (*S*)-HNEs by rGST isoforms purified from rat liver and kidney cytosols

To determine K_m and V_{max} values for GSH conjugation of (*R*)- and (*S*)-HNEs, substrate concentrations ranging from 4 to 25 μM were used at pH 6.5 with appropriate amounts of the purified rGST isoforms and a GSH concentration of 0.5 mM. For calculation of k_{cat} values for rGSTs, the molecular mass of the enzyme was taken from Hayes and Pulford [17]. Reactions were performed at 25 °C for 3 min, as described previously [29]. Values are means $\pm$ S.D. ($n > 5$).

GST isoform	(<i>R</i>)-HNE			(<i>S</i>)-HNE		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \cdot \text{mM}^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \cdot \text{mM}^{-1}$)
A1-1(2)	165 $\pm$ 1.3	20.1 $\pm$ 0.1	122 $\pm$ 0.9	140 $\pm$ 1.9	422.7 $\pm$ 0.1	162 $\pm$ 1.1
A1-3	186 $\pm$ 0.5	14.3 $\pm$ 0.3	77 $\pm$ 1.2	93.9 $\pm$ 1.6	12.3 $\pm$ 0.1	131 $\pm$ 1.1
A3-3	317 $\pm$ 2.6	8.7 $\pm$ 0.4	27 $\pm$ 1.0	27.4 $\pm$ 1.1	1.9 $\pm$ 0.1	69 $\pm$ 2.5
A4-4*	20.4 $\pm$ 1.2	115 $\pm$ 2.00	5637 $\pm$ 336	6.7 $\pm$ 0.3	151 $\pm$ 4.0	22537 $\pm$ 736
M1-1	70.9 $\pm$ 0.8	2.36 $\pm$ 0.01	33 $\pm$ 0.6	9.7 $\pm$ 0.2	1.51 $\pm$ 0.02	156 $\pm$ 2.0
M1-2	34.6 $\pm$ 0.3	3.76 $\pm$ 0.05	109 $\pm$ 1.7	10.8 $\pm$ 0.3	5.58 $\pm$ 0.01	517 $\pm$ 12
M2-2	33.6 $\pm$ 0.3	3.83 $\pm$ 0.04	114 $\pm$ 1.8	10.6 $\pm$ 0.5	7.56 $\pm$ 0.01	713 $\pm$ 21
P1-1	34.7 $\pm$ 0.7	1.09 $\pm$ 0.08	31 $\pm$ 1.8	40.5 $\pm$ 1.8	1.36 $\pm$ 0.04	34 $\pm$ 0.5

* Values determined by Hiratsuka et al. [21].

about one-tenth that of rGSTA4-4, and towards aflatoxin B₁ 8,9-oxide. However, rGSTs bearing subunit A5 occur only as a few minor heterodimers, one of which was demonstrated to have low activity towards the HNE [50]. We demonstrated that rGSTA4-4 was most likely to be involved in hepatic cytosolic (*S*)-preferential GSH conjugation of HNE enantiomers [21]. As indicated in the present study, no GST protein with extremely high specific activity towards HNE was detected in guinea-pig liver cytosol throughout the chromatographic purification steps used for the gpGSTs (Figure 1). In addition, the guinea-pig had no putative GSTA4-4 orthologue stainable with anti-rGSTA4-4 antibody in the liver (Figure 2) and other major tissues, such as kidney, lung and heart, even when the animal suffered from high oxidative stress induced by iron-dextran. Furthermore, no immunostainable protein was detected in GSH-affinity-purified GST pools from these tissue cytosols by Western-blot analysis using the anti-rGSTA4-4 antibody. We also failed to isolate a cDNA coding for a gpGST subunit with identity higher than 60 % of that for rGSTA4 from the guinea-pig liver cDNA library (results not shown). The absence of a putative gpGSTA4-4 isoenzyme could be responsible for the observed low $V_{\max}/K_m$ value in the GSH conjugation of racemic HNE in guinea-pig liver cytosol (Table 1) compared with a high $V_{\max}/K_m$ value (28.8 ± 0.67) observed for that in rat liver cytosol [21].

It is well known that the guinea-pig has no detectable levels of Se-GSH Px in its major tissues, including the liver [23,24]. Oshino et al. [51] demonstrated that the homodimeric enzyme gpGSTa, a major hepatic GST re-designated as gpGSTA1-1 in the present study, had extremely high GSH Px activity towards cumene hydroperoxide and linoleic acid hydroperoxide. They isolated gpGSTa (A1-1) by a somewhat lengthy chromatographic method using anion exchange, gel filtration, cation exchange and GSH affinity columns in succession.

In this study, we established a simple chromatographic method for simultaneously and quantitatively isolating gpGSTA1-1 and other three major Mu-class gpGSTs in homogeneous form from guinea-pig liver cytosol (Figure 1). There was a distinct difference in affinity for the *S*-hexyl-GSH-Sepharose 6B column between the Alpha- and Mu-class gpGSTs under the conditions used in this study. The three Alpha-class gpGSTs could not be retained on the affinity column, whereas Mu-class enzymes were quantitatively retained on this column. However, the Alpha-class gpGSTs were quantitatively recovered on the GSH-Sepharose 6B column from the fraction passing through the *S*-hexyl-GSH affinity column. The gpGSTs retained on these columns were easily separated and purified to homogeneity by chromatofocusing after being eluted from the affinity columns with the corresponding ligands. The Alpha-class gpGST showed very high GSH Px activity towards cumene hydroperoxide and linoleic acid hydroperoxide, as reported previously by Oshino et al. [51] (results not shown).

In the present paper, we re-designated GSTa as gpGSTA1-1, a homodimeric GSTA with subunit 1. In addition, we corrected a minor error of the previously reported C-terminal amino acid sequence of GSTa [40]. Apart from the N-terminus and the deletion of three amino acids from the C-terminal sequence (Figure 3), the overall amino acid sequence reported for gpGSTa was identical to that of molecularly cloned gpGSTA1.

Our investigation, using CDNB, HNE and cumene hydroperoxide as substrates, showed that guinea-pigs had at least six GST isoforms in the liver (Figure 1). Based on the concentrations of the four gpGSTs, except for the two very minor GSTA isoforms in guinea-pig liver cytosol, and on their kinetic parameters for racemic HNE, the ratio of their contributions to the hepatic HNE-GSH conjugation was estimated to be

4.1:15.6:2.2:1 for gpGSTs A1-1, M1-1, M1-2 and M1-3*, respectively. This strongly suggests that gpGSTM1-1 plays a major part in the GSH-dependent detoxification of HNE in guinea-pig liver cytosol.

As estimated by the hepatic levels of TBARS, a mixture of aldehydic compounds, including HNE, from lipid peroxides, guinea-pig liver appeared to be oxidatively stressed to the same extent as rat liver (Figure 5). TBARS in the liver of both animals was also significantly induced to a similar extent by treatment with iron-dextran.

Kinetic studies using purified gpGSTs and rGSTs indicated that all the major hepatic gpGSTs and rGSTs catalysed the GSH conjugation of HNEs in the order (*S*) > (*R*), as observed in both hepatic cytosolic conjugations. The Alpha- and Mu-class rGSTs catalysed GSH conjugation of the HNE enantiomers more (*S*)-preferentially than did these classes of gpGSTs (Tables 2 and 3). Of the three Mu-class rGSTs, M2-2 showed the highest (*S*)-preferential GSH conjugation of HNE enantiomers (Table 3). It has also been shown that M2-2 plays the most important role among Mu- and other classes of rGSTs in the stereoselective GSH conjugation of epoxides such as *K*-region oxides of benzo[*a*]pyrene and benzo[*a*]anthracene [52], bay-region diol-oxides of benzo[*a*]pyrene [52], and 7,8-oxides of styrene [52,53] and 9,10-dihydrobenzo[*a*]pyrene [54].

It is recognized that the potent toxicity of racemic HNE is due to its strong electrophilic reactivity with the nucleophilic groups of protein and nucleic acids mainly by the mechanism of Michael-type addition of the alkenal C₃ to the biomacromolecules [2]. The enzymic GSH conjugation of HNE also occurs through the SH group on this carbon, which is adjacent to the asymmetric C₄ [2]. That may be the reason why HNE inactivated the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase enantioselectively and is enantiopreferentially metabolized by enzymes such as ADH, ALDH and GST. It is of interest that all isoforms of hepatic cytosolic GSTs in the guinea-pig and rat inactivated (*S*)-HNE preferentially to (*R*)-HNE and also that in contrast to the rat, mouse and human, the liver and other major tissues of the guinea-pig lack a putative GSTA4-4 orthologue with extremely high activity towards HNE. A study on the enantioselectivity in detoxification of HNE by GSTs in other species, including the human, is now in progress in our laboratory.

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