4-Hydroxy-2(*E*)-nonenal enantiomers: (*S*)-selective inactivation of glyceraldehyde-3-phosphate dehydrogenase and detoxification by rat glutathione S-transferase A4-4

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The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase was irreversibly and (*S*)-selectively inactivated by the enantiomers of racemic 4-hydroxy-2(*E*)-nonenal (HNE), a reactive product released from biomembranes by lipid peroxidation in cells. Rates of the enzyme inactivations were 1.7, 3.0, and 6.0 M⁻¹·s⁻¹ for (*R*)-, racemic and (*S*)-HNEs respectively. In rat liver cytosol the HNE was detoxified 2.5-fold more (*S*)-selectively by GSH conjugation and 2.4-fold more (*R*)-selectively by NADH-dependent reduction mediated by alcohol dehydrogenase (ADH) than the opposite enantiomers. However, in the cytosol the GSH conjugation of (*R*)-HNE proceeded at a much higher

rate than did its ADH-mediated reduction. The minor glutathione S-transferase (GST) isoform, A4-4, in the rat (r) liver had a major role in the cytosolic (*S*)-selective GSH conjugation. The catalytic efficiency, k_{cat}/K_m , of purified rGSTA4-4 was 4-fold higher for (*S*)-HNE than for (*R*)-HNE; the K_m was 3-fold higher for (*R*)-HNE than for (*S*)-HNE. (*S*)-HNE was preferentially detoxified to (*R*)-HNE by rGSTA4-4 when racemic HNE was used as a substrate.

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

INTRODUCTION

4-Hydroxy-2(*E*)-nonenal (HNE), a major racemic product of lipid peroxidation, is known as the most cytotoxic and genotoxic product among a variety of aldehydic compounds that originate from the non-enzymic peroxidative breakdown of *n*–6 polyunsaturated fatty acid residues, such as linoleate and arachidonate [1,2]. The cytopathological effects of racemic HNE include the following: the inactivation of several enzymes such as, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [3], glucose-6-phosphate dehydrogenase [4], cytochrome *c* oxidase [5], adenylate cyclase [6] and glutathione reductase [7]; the lysis of erythrocytes [8]; the chemotactic activity of neutrophils [9]; and the inhibition of protein and DNA syntheses [2]. Racemic HNE also has genotoxic effects leading to sister chromatid exchange, micronuclei formation and DNA fragmentation [2]. These effects have been attributed to the ability of HNE to form Michael addition products through its olefinic C₃ atom as an electrophilic centre with the guanine residue of DNA and with the cysteinyl thiol, the lysyl primary amino and/or the histidyl imidazolyl groups of functional proteins to form stable adducts [1,2].

GAPDH, a classical glycolytic tetrameric protein composed of four identical 37 kDa monomers, has been most extensively studied with regard to its irreversible inhibition by racemic HNE. Uchida and Stadtman [3] demonstrated that rabbit muscle GAPDH was inactivated by racemic HNE in a concentration-dependent manner and that the loss of enzyme activity was associated with its reaction with the three nucleophilic amino acid residues, Cys, Lys and His, of the enzyme protein.

The metabolism of racemic HNE has been studied *in vitro* with

hepatocytes [2,10,11], hepatoma cells [2] and isolated heart [2,12], and *in vivo* in rats [2,13]. These studies indicate that the rat liver can rapidly convert racemic HNE into less toxic metabolites by means of conjugation with glutathione by cytosolic glutathione S-transferase (GST), oxidative metabolism by aldehyde dehydrogenase (ALDH), and reductive metabolism by alcohol dehydrogenase (ADH) or aldehyde reductase. Of these metabolic processes, GST-mediated GSH conjugation is recognized as the most effective for detoxifying racemic HNE [10,11]. HNE is neurotoxic in neuronal culture [14] and *in vivo* [15], and is elevated in Alzheimer's-disease brain [16] and cerebrospinal fluid [17]. Lovell et al. [18] demonstrated that significant decreases in 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole–GST activity and protein levels determined by anti-GSTM1 antibody were found in all brain areas and also in ventricular cerebrospinal fluid in patients with Alzheimer's disease.

Mammalian cytosolic GSTs are homodimeric and heterodimeric proteins with molecular masses of 25–28 kDa [19,20] and are classified into seven subfamilies, Alpha (A), Mu (M), Pi (P), Theta, Sigma, Kappa and Zeta [19–23]. They are important in detoxifying a variety of electrophiles formed from exogenous and endogenous toxic or carcinogenic compounds in tissues [19,20]. Alpha-class GSTA4-4 orthologues occurring as minor enzymes in liver cytosol of the rat (r) [24,25], mouse [26] and human [27] have extremely high catalytic activity in the conjugation and detoxification of racemic HNE compared with other GST isoforms [20]. rGSTA4-4 is approx. 100-fold more active towards HNE than other rat liver GSTs, so it might have an essential role in protection of the animal tissue from the toxic aldehyde generated by oxidative stress *in vivo*. rGSTA5-5 has been reported by Hayes et al. [28] to have potent activity towards HNE

Abbreviations used: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione S-transferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPETE, hydroperoxy-5,8,11,13-eicosatetraenoate; HNE, 4-hydroxy-2(*E*)-nonenal; HPODE, hydroperoxy-9,11-octadecadienoate; r, rat.

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and aflatoxin B₁ 8,9-oxide. Its reported activity towards racemic HNE is about one-tenth that of rGSTA4-4. However, rGSTs bearing the subunit A5 occur only as a few minor heterodimers, one of which was demonstrated to have low activity towards the HNE [20]. The homodimer rGSTA5-5, expressed in bacteria, has not been found in the rat.

All previous studies on biological and toxicological effects of HNE have been performed with its racemate. Therefore nothing is known about which of the two enantiomers is the more toxic, the more reactive with biomacromolecules or the more readily detoxified by GST and ADH.

Here we provide the first evidence for the marked enantioselectivity by HNE enantiomers in the irreversible inactivation of the glycolytic enzyme rabbit muscle GAPDH, and in their detoxification by rat liver cytosolic rGSTA4-4 and ADH.

MATERIALS AND METHODS

Materials

1-Chloro-2,4-dinitrobenzene (CDNB), disulfiram, NAD⁺, NADP⁺ and NADPH were purchased from Wako Pure Chemicals Industries (Osaka, Japan); D,L-glyceraldehyde-3-phosphate, pyrazole, rabbit muscle GAPDH, and molecular-mass marker proteins were from Sigma; pKK223-3 DNA and λ gt11/*Eco*RI/CIP 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.). GSH, with purity higher than 99.8% and free from GSSG, was donated by Yamanouchi Pharmaceutical Co. (Tokyo, Japan). A rat liver λ gt11 cDNA library was prepared as described previously [29]. Other reagents used were of reagent grade.

(R)- and (S)-HNEs

(R)- and (S)-HNEs were prepared by the enzymic resolution of racemic HNE dimethylacetal [30] and purified by HPLC on a chiral column (Chiralcel OB; 10 μ m pore size, 4.6 mm $\times$ 250 mm; Daicel Chemical Industries, Tokyo, Japan) with a Jasco model 800 liquid chromatograph. (R)- and (S)-HNEs were eluted at retention times of 22.4 and 17.8 min respectively, with 2% (v/v) propan-2-ol/n-hexane at a flow rate of 0.8 ml/min. Chromatograms were monitored by measuring A_{223} . HPLC analysis with the chiral phase column indicated that the purified HNE enantiomers contained no detectable amount of the opposite enantiomers. Concentrations of racemic, (R)- and (S)-HNEs were determined by measuring A_{223} , assuming a molar absorption coefficient of 13 750 M⁻¹·cm⁻¹ for HNE (in water).

Preparation of cytosolic fraction

Male Sprague–Dawley rats weighing 200–230 g (7 weeks old) were anaesthetized with diethyl ether after overnight fasting, killed and used for preparation of the cytosolic fraction. The hepatic cytosolic fraction was prepared by centrifugation at 105 000 g for 60 min of a post-mitochondrial fraction obtained at 9000 g for 20 min from 3 vol. of a liver homogenate in 150 mM Tris/HCl buffer, pH 7.4, containing 5 mM EDTA, 193 mM KCl and 6.25 mM dithiothreitol, then dialysed against 50 vol. of 10 mM Tris/HCl buffer containing 1 mM EDTA and 2 mM 2-mercaptoethanol. The protein concentration of the cytosolic fraction was 15 mg/ml.

Inactivation of GAPDH by racemic, (R)- and (S)-HNEs

The reaction mixture (1 ml) containing 100 μ g of GAPDH was preincubated with various concentrations of racemic, (R)- or (S)-HNEs for 120 min or with 40 μ M of each of these substrates for various durations at 37 °C in 50 mM sodium phosphate buffer, pH 7.2. Aliquots (30 μ l) of these reaction mixtures were withdrawn and immediately assayed for their respective residual enzyme activities. GAPDH activity was measured by a coupled assay procedure as reported previously [3]. Control experiments were performed in parallel with test experiments in which the HNE solution was replaced with distilled water.

Enzyme assay

GSH-conjugating activities of GST towards HNE [31] and CDNB [32] were determined as reported previously. ALDH activity was measured in the dialysed cytosolic fraction by monitoring the increase in A_{340} due to NADH production during the oxidation of HNE enantiomers (5–500 μ M) with NAD⁺, as reported previously [33]. ADH activity was measured in the dialysed cytosolic fraction by monitoring the decrease in A_{340} due to NADH oxidation during the reduction of HNE enantiomers (25–500 μ M) [34]. The assay mixture (1 ml) for the measurement of these activities contained 0.3 mM NADH (for ADH) or 1 mM NAD⁺ (for ALDH) in 0.2 M potassium phosphate buffer, pH 7.0. For characterization of the cytosolic HNE-reducing and HNE-oxidizing enzymes, pyrazole (1 mM), an ADH inhibitor [35], or disulfiram (1 mM), an ALDH inhibitor [36], 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 for at least three experiments.

The assay of HNE enantiomers in the racemate remaining unreacted with GSH in the presence of rGSTA4-4 was performed as follows: a mixture consisting of GSH (0.5 mM), rGSTA4-4 (2.35 μ g/ml protein) and 0.1 M potassium phosphate buffer, pH 6.5 (2 ml), was incubated at 25 °C. The reaction was started by the addition of a solution of racemic HNE (100 μ M) in water (100 μ l). After the appropriate incubation period, the mixture was immediately chilled on an ice bath and shaken rapidly with diethyl ether (2 ml) to recover the unreacted substrate. The organic layer was evaporated at room temperature under vacuum. The residue obtained was dissolved in 0.1 ml of n-hexane for the subsequent determination of the HNE enantiomers by HPLC. HPLC analysis of HNE enantiomers was performed under the same chiral phase conditions as described above.

Purification of hepatic rGSTA4-4

rGSTA4-4 was isolated from the liver cytosol of the male Sprague–Dawley rats by the method of Ålin et al. [25]. Homogeneity of the GST preparation was confirmed by SDS/PAGE, reverse-phase partition HPLC, Western blotting and N-terminal amino acid sequence analyses, as reported previously [37,38]. The activities towards HNE, CDNB and ethacrynic acid were determined by the previously reported method [39].

Recombinant rGSTA4-4 preparation

For the expression of rGSTA4-4, a full-length cDNA encoding rGSTA4-4 was isolated from a female-rat liver λ gt11 cDNA library by using an antibody raised against rGSTA4-4; the insert

cDNA species of positive clones were subcloned into pUC19 at the *Eco*RI site for DNA sequencing. Determination of nucleotide sequences of the cDNA species was performed by the dideoxy chain-termination method [40]. The amino acid sequence deduced from the open reading frame was identical with the reported sequence for rGSTA4 [38]. The rGSTA4 cDNA in pUC19 was amplified by PCR with two oligonucleotide primers (5'-CCA GAA TTC ATG GAA GTC AAA CCC AAG CTC-3' as the sense primer, 5'-AGC AAG CTT TCA CTA GAA CTT CAG GAC GGT CCT-3' as the anti-sense primer) that were designed to include *Eco*RI and *Hind*III sites. The amplified product was digested with *Eco*RI and *Hind*III and inserted into the *Eco*RI and *Hind*III sites in pKK223-3. The orientation and integrity of the rGSTA4 cDNA were confirmed by sequence analysis. *Escherichia coli* JM101 was transformed with the pKK223-3-rGSTA4 vector as described previously [29]. The expression of the rGSTA4-4 in pKK223-3 and the extraction of the enzyme from the bacterial cells were performed with the previously described method [38]. The extract was centrifuged; the recombinant rGSTA4-4 was purified from the supernatant on a GSH-Sepharose 6B column (1 cm × 10 cm) as described previously [37]. The affinity-purified enzyme was dialysed against 50 vol. of 10 mM Tris/HCl buffer, pH 7.8, containing 1 mM EDTA and 2 mM 2-mercaptoethanol.

Immunoblotting

Polyclonal antibodies against rGSTs A1-1(2), A4-4, M1-1, P1-1 and T2-2 were prepared as described previously [41]. Western blot analysis with these polyclonal antibodies was performed with a previously described method [41].

Protein determination

Protein concentration was determined by the method of Lowry et al. [42] with BSA as a standard.

Data analysis

Statistical significance was evaluated with Student's *t* test or Welch's *t* test, with the level of significance set at $P = 0.05$. Values are expressed as means $\pm$ S.D.

Electrophoresis

SDS/PAGE was performed by the method of Laemmli [43]. Proteins were stained with Coomassie Brilliant Blue R-250.

RESULTS AND DISCUSSION

Enantioselective inactivation of the glycolytic enzyme GAPDH by racemic, (*R*)- and (*S*)-HNEs

Rabbit muscle GAPDH was inactivated by various concentrations of (*S*)-, (*R*)- and racemic HNEs with a marked stereoselectivity when preincubated under physiological conditions. Of the three forms of HNE, (*S*)-HNE showed the highest inactivating activity towards GAPDH and (*R*)-HNE showed the lowest activity. Half of the activity was lost after the preincubations with 16, 32 and 57 μ M (*S*)-, racemic and (*R*)-HNEs for 120 min (Figure 1A). A time-course study indicated that the remaining activity of the GAPDH was only 29% for (*S*)-HNE, 65% for (*R*)-HNE and 48% for racemic HNE after 90 min of preincubation with the inhibitors at 40 μ M (Figure 1B). The rates of these second-order reactions were estimated from these data to

Figure 1 Dose dependence (A) and time courses (B) of inactivation of rabbit muscle GAPDH by racemic, (*R*)- and (*S*)-HNEs

Purified GAPDH at a concentration of 100 μ g/ml was preincubated with various concentrations of the HNEs for 120 min (A) and with the HNEs (40 μ M) for various durations (B) at 37 °C. Remaining activity is the percentage of the control value obtained in the absence of the HNEs. Each data point is the mean for three determinations. Symbols: ■, (*R*)-HNE; ●, racemic HNE; ▲, (*S*)-HNE.

be 1.7, 3.0 and 6.0 $\text{M}^{-1}\cdot\text{s}^{-1}$ for (*R*)-, racemic and (*S*)-HNEs respectively. The GAPDH inactivation by the HNE enantiomers was irreversible, as demonstrated previously with racemic HNE [3].

Crystal structures have been reported for a number of glycolytic homotetrameric GAPDHs isolated from eubacteria [44,45] and eukaryotes [46,47]. Two catalytic amino acids, Cys-149 and His-176, have been characterized; Cys-149 participates in the formation of a thioacyl intermediate, which in the catalytic mechanism finally leads to the formation and the release of 1,3-diphosphoglyceric acid [48]. In addition, it is known that His-176 and Lys-183 are located at the NAD^+ -binding site [49]. All α,β -unsaturated aldehydes, especially HNE, are strong electrophiles and therefore show high reactivity with nucleophiles such as the thiol and amino groups of nucleophilic amino acids. A previous study with racemic HNE (0.2–2.0 mM) showed that the loss of

rabbit muscle GAPDH activity is correlated with modification of about two Cys residues, three Lys residues and one His residue in the enzyme subunit [3] and that the enzyme inactivation was a result of selective modification of the nucleophilic amino acid residues of GAPDH by racemic HNE. However, it is still equivocal which nucleophilic amino acid residue is involved in the modification and inactivation of the GAPDH by racemic HNE. The present study strongly suggests that (*S*)-HNE interacts more readily than the (*R*)-enantiomer with the nucleophilic amino acid residue(s) located at the active site of the GAPDH. However, we have no data at present on which nucleophilic amino acid residue of the GAPDH would be specifically or selectively modified by (*S*)- and (*R*)-HNEs. Modification of the nucleophilic amino acid residues has also been reported for the irreversible inactivation of glucose-6-phosphate dehydrogenase [4], cytochrome *c* oxidase [5] and glutathione reductase [7] by racemic HNE. However, no information is available on the modified amino acid position(s) of these enzymes.

It should be emphasized that the enantiomers of racemates, in general, interact separately with chiral biomacromolecules and are metabolized by enzymes in cells. HNE is formed non-enzymically as a racemate from the *n*–6 fatty acid residues of phospholipids in biomembranes throughout their oxidation followed by degradation [1,2]. (*S*)-HNE might be stereospecifically generated from arachidonate and linoleate via 15(*S*)-hydroperoxy-5,8,11,13-eicosatetraenoate [15(*S*)-HPETE] and 13(*S*)-hydroperoxy-9,10-octadecadienoate [13(*S*)-HPODE], formed from arachidonate and linoleate respectively, by 15-lipoxygenase in leucocytes and respiratory epithelial tissue [50], because racemic HNE is not only generated from 11-HPETE and 13-HPODE, autooxidation products of arachidonate and linoleate, but is also likely to be generated from 15-HPETE and 13-HPODE in accordance with the mechanism proposed by Pryor and Porter [51]. However, no information is available at present on the stereoselective formation of (*S*)-HNE in blood cells and respiratory tissue.

Our data strongly suggested that the (*S*)-enantiomer of the racemate released in the cytosol inactivates the glycolytic enzyme GAPDH more readily at a lower concentration than does the (*R*)-enantiomer. Therefore, in view of the defence against racemic HNE constantly formed in normal cells [1,2], it is of interest which enantiomer of the racemate is more readily detoxified by the cytosolic enzymes GST, ADH and ALDH. These enzymes have been demonstrated by Hartley et al. [10] and Siems et al. [11] to be important in the detoxification of racemic HNE in isolated hepatocytes.

Enantioselectivity in the GSH conjugation, reduction and oxidation of HNE enantiomers in rat liver cytosol

A kinetic study, performed with dialysed cytosol from rat liver cells, indicated that the GSH-conjugating activity by GST was much higher towards all of the three forms of HNEs than NADH-dependent reducing activity by ADH (Table 1). The marked difference in $V_{\max}/K_m$ between the enzyme reactions was attributable to the apparent K_m , i.e. the GSH conjugation of the three forms of HNEs proceeded with a much smaller K_m than did their NADH-dependent reduction. The racemate showed intermediate K_m and $V_{\max}/K_m$ values among the three forms of HNEs in reduction as well as in GSH conjugation. There existed a marked difference in enantioselectivity between the hepatic cytosolic GSH conjugation and NADH-dependent reduction of HNE enantiomers used as substrates (Table 1). The (*S*)-enantiomer of HNE was conjugated with GSH preferentially to the (*R*)-enantiomer at a ratio of 2.5:1. In contrast, the

(*R*)-enantiomer was reduced 2.4-fold more rapidly than the (*S*)-enantiomer. However, (*R*)-HNE was metabolized much more rapidly by GST than by ADH; the K_m was 7-fold larger for the latter than for the former, although $V_{\max}$ for the former was only double that for the latter.

As to the racemic substrate, cytosolic GSH conjugation proceeded at a 44-fold higher rate than the reduction, on the basis of their $V_{\max}/K_m$. The conjugation-to-reduction ratio was unexpectedly high compared with that previously demonstrated in isolated hepatocytes from the rat, which was reported by Siems et al. [11] to detoxify the racemate at a ratio of 4:1. The large discrepancy between the reaction ratios demonstrated by them and by us might be attributable to the rapid consumption of the cellular GSH level after exposure of the hepatocytes to racemic HNE as determined by them, so that the GST in the isolated cells could not exert maximal activity under the GSH-depleted condition as they suggested. However, they showed no data on the enzyme reactions in rat hepatic cytosol in the presence of a sufficient concentration of GSH.

Siems et al. [11] also showed that in isolated hepatocytes, racemic HNE was oxidized to the corresponding carboxylic acid by ALDH in the presence of NAD⁺ at a 4-fold higher rate than it was reduced to the corresponding alcohol. However, our data on $V_{\max}/K_m$ indicated that, in hepatic cytosol, ALDH-mediated oxidation of the enantiomeric HNEs proceeded without enantioselectivity at a much lower rate (approx. 1/6) than did the ADH-mediated reduction of racemic HNE. Therefore little difference was observed in the reaction rate of cytosolic oxidation between the racemate and the enantiomers. There was little difference in K_m (21 μ M) and $V_{\max}$ (2 nmol/min per mg) of cytosolic protein for the cytosolic oxidation of the enantiomeric HNEs. As readily expected from the results of a previous study [36], the cytosolic oxidation of the racemic and enantiomeric HNEs was completely inhibited by disulfiram (1 mM), an ALDH inhibitor. It is unclear why the oxidation-to-reduction ratio of racemic HNE was much smaller in hepatic cytosol than in isolated hepatocytes.

Pyrazole (1 mM) completely inhibited the hepatic cytosolic reduction of all the three forms of HNE, strongly suggesting that ADH-3 was involved in the reduction. Of the known ADH isoforms 1, 2 and 3 in the rat, ADHs 1 and 2 have been demonstrated in purified preparations to be little affected by pyrazole concentrations lower than 20 mM [35]. However, ADH-3 purified from the rat liver and other tissues is completely inhibited by 1 mM pyrazole [35].

It is well known that rGSTA4-4, a minor enzyme in liver cytosol, has extremely high activity towards racemic HNE, and that the other rat hepatic GST isoforms have very weak activity towards the HNE [20,31]. We have found (A. Hiratsuka, K. Hirose, H. Saito and T. Watabe, unpublished work) that most of the total racemic HNE–GSH-conjugating activity in the rat liver was represented by cytosolic rGSTA4-4. Therefore the kinetic data shown in Table 1 suggest that rGSTA4-4 is most likely to be involved in the (*S*)-selective GSH conjugation of HNE enantiomers.

(*S*)-Selective GSH conjugation of HNE by rGSTA4-4

To avoid the use of many rats as subjects, recombinant rGSTA4-4 expressed in *E. coli* and purified therefrom was used instead of the very minor native enzyme from rat liver cytosol for the investigation of the enantioselective GSH conjugation of HNEs. The recombinant GST was identical with the hepatic enzyme in all respects, including chromatographic and electrophoretic mobilities, immunochemical properties and specific activities towards various substrates, as demonstrated in part by Stenberg

Table 1 Kinetic parameters for GSH conjugation and NADH-dependent reduction of racemic, (*R*)- and (*S*)- HNEs in rat liver cytosol

Kinetic constants were determined by incubating racemic, (*R*)- or (*S*)-HNE with 15 mg/ml of dialysed cytosolic protein from rat liver cells in the presence of GSH (0.5 mM) at pH 6.5, or NADH (0.3 mM) at pH 7.5. Substrate concentrations ranging from 5 to 100 μ M for GSH conjugation and from 25 to 500 μ M for NADH-dependent reduction were used. Reactions were performed at 25 °C for 3 min as described in the Materials and methods section. Results 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 NADH-dependent reduction. †Significantly smaller ($P < 0.01$) than (*R*)-HNE.

Substrate	GSH conjugation			NADH-dependent reduction		
	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	10.6 $\pm$ 0.2	305 $\pm$ 1.5	28.8 $\pm$ 0.67*	163.2 $\pm$ 0.1	105.6 $\pm$ 0.4	0.65 $\pm$ 0.003
(<i>R</i>)-HNE	18.0 $\pm$ 0.3	243 $\pm$ 2.0	13.5 $\pm$ 0.18*	134.8 $\pm$ 0.3	105.5 $\pm$ 0.4	0.78 $\pm$ 0.003
(<i>S</i>)-HNE	9.5 $\pm$ 0.4†	330 $\pm$ 1.8	34.8 $\pm$ 1.5*	340.7 $\pm$ 0.1	105.7 $\pm$ 0.5	0.31 $\pm$ 0.002

Table 2 Kinetic parameters for the GSH conjugation of racemic, (*R*)- and (*S*)-HNEs by rGSTA4-4

To determine K_m and V_{max} values for the GSH conjugation of racemic, (*R*)- and (*S*)-HNEs, substrate concentrations ranging from 2.5 to 40 μ M were used at pH 6.5 with rGSTA4-4 (2.35 μ g/ml) and a GSH concentration of 0.5 mM. For calculation of the k_{cat} for rGSTA4-4, the molecular mass of the enzyme was taken from Hayes and Pulford [20]. Reactions were performed at 25 °C for 3 min, as described previously [31]. Values are means $\pm$ S.D. ($n > 5$). *Significantly greater ($P < 0.01$) than (*R*)-HNE.

Substrate	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
Racemic HNE	7.4 $\pm$ 0.2	144 $\pm$ 4	19459 $\pm$ 782
(<i>R</i>)-HNE	20.4 $\pm$ 1.2	115 $\pm$ 2	5637 $\pm$ 336
(<i>S</i>)-HNE	6.7 $\pm$ 0.3	151 $\pm$ 4	22537 $\pm$ 736*

et al. [38]. A kinetic study indicated that rGSTA4-4 catalysed the GSH conjugation of HNEs in the order (*S*) > racemic > (*R*), as observed in hepatic cytosolic conjugation (Table 2). The marked difference between k_{cat}/K_m values representing the catalytic efficiency of rGSTA4-4 for the enantiomers was attributable to the marked difference between K_m values for the enantiomers [(*S*) < (*R*)] as observed in hepatic cytosolic GSH conjugation; despite their small difference in k_{cat} , (*R*)-HNE showed 3-fold larger K_m for the GST than that of (*S*)-HNE.

In the GSH conjugation by rGSTA4-4, apparent K_m and k_{cat}/K_m values for racemic HNE were very close to those for (*S*)-HNE (Table 2). A time-course study indicated that the curve for the enzymic consumption of racemic HNE was almost superimposable on that for (*S*)-HNE at an initial stage of the reaction, but was quite different from that for (*R*)-HNE (Figure 2).

Direct evidence for the enantioselective consumption of the (*S*) component from racemic HNE was obtained by HPLC analysis of the substrate remaining unreacted on a chiral column from which (*S*)- and (*R*)-HNEs were eluted at 17.8 and 22.4 min respectively (Figure 3). The chromatogram indicated that, at an early stage of the enzyme reaction, (*S*)-HNE was consumed preferentially to (*R*)-HNE from the racemic substrate, and that at a stage of 50 % substrate consumption, the (*S*)-to-(*R*) ratio of the enantiomers remaining unreacted was 1:2.4. At a stage of 75 % substrate consumption, the (*S*)-to-(*R*) ratio was 1:4.4.

Thus the present study provides the first evidence for the enantioselective inactivation of an enzyme (GAPDH) by HNE and for the enantioselective detoxification of HNE by enzymes (rGSTA4-4 and ADH). We have found (A. Hiratsuka, K. Hirose, H. Saito and T. Watabe, unpublished work) that another rabbit muscle glycolytic enzyme, 3-phosphofructokinase, was also ir-

Figure 2 Time courses of enzymic glutathione conjugation of racemic, (*R*)- and (*S*)-HNEs by purified rGSTA4-4

Upper panel: HNEs (100 μ M each) were incubated with rGSTA4-4 (2.35 μ g/ml) in the presence of GSH (0.5 mM) at pH 6.5. The remaining substrate was determined directly by the decrease in A_{223} (the absorption maximum of the α,β -unsaturated aldehyde) of the reaction mixture. Lower panel: time-enlarged profiles of the early stage of the enzyme reactions. Symbols: ■, (*R*)-HNE; ●, racemic HNE; ▲, (*S*)-HNE.

reversibly inactivated stereoselectively by the three forms of HNE in the order of (*S*) > racemic > (*R*). A further study on the molecular mechanism of the enantioselective inactivation of GAPDH by HNE is now in progress in our laboratory.

Figure 3 HPLC separation of (*R*)- and (*S*)-HNEs in racemic HNE during incubation with rGSTA4-4

Incubation was performed under the same conditions as described in the legend to Figure 2. Chromatograms show the enantiomers remaining unreacted in the racemic HNE used as a substrate: from top to bottom, 0%, 25%, 50% and 75% of the substrate consumed. The two peaks eluted at 17.8 and 22.4 min represent (*S*)-HNE and (*R*)-HNE respectively. HPLC was performed on a chiral column (Chiralcel OB, 10 μ m pore size, 4.6 mm $\times$ 250 mm) in 2% (v/v) propan-2-ol/n-hexane (0.8 ml/min). The chromatograms were monitored by A_{223} . At pH 6.5, no appreciable conjugation of the HNEs with 0.5 mM GSH was observed in the absence of rGSTA4-4 during the incubation.

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