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Biochemical Pharmacology 63 (2002) 2025–2036

Biochemical Pharmacology

Comparative expression of two alpha class glutathione *S*-transferases in human adult and prenatal liver tissues

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Received 12 June 2001; accepted 28 November 2001

Abstract

The ability of the fetus to detoxify transplacental drugs and chemicals can be a critical determinant of teratogenesis and developmental toxicity. Developmentally regulated expression of alpha class glutathione *S*-transferases (GSTs) is of particular interest, since these isozymes have high activity toward peroxidative byproducts of oxidative injury that are linked to teratogenesis. The present study was initiated to examine the expression and catalytic activities of alpha class GST isozymes in human prenatal liver. Northern analysis demonstrated the presence of hGSTA1 and/or A2 (hGSTA1/2) and hGSTA4 steady-state mRNAs in second trimester prenatal livers. Western blotting of prenatal liver proteins provided corroborating evidence via detection of an hGSTA1/2-reactive protein in both cytosol and mitochondria and of hGSTA4-4-reactive protein in mitochondria alone. Catalytic studies demonstrated that prenatal liver cytosolic GSTs were active toward 1-chloro-2,4-dinitrobenzene (a general GST reference substrate), δ^5-androstene-3,17-dione (relatively specific for hGSTA1-1), and 4-hydroxynonenal, a highly mutagenic α,β-unsaturated aldehyde produced during oxidative damage and a substrate for hGSTA4-4. Total GSH-peroxidase and GST-dependent peroxidase activities were 9- and 18-fold higher, respectively, in adult liver than in prenatal liver. Multiple tissue array analyses demonstrated considerable tissue-specific and developmental variation in GST mRNA expression. In summary, our results demonstrate the presence of two important alpha class GSTs in second trimester human prenatal tissues, and indicate that mitochondrial targeting of GST may represent an important pathway for removal of cytotoxic products in prenatal liver. Furthermore, the relatively inefficient prenatal reduction of hydroperoxides may underlie an increased susceptibility to maternally transferred pro-oxidant drugs and chemicals.

Keywords: Northern analysis; Prenatal liver; Mitochondria; Glutathione *S*-transferases; 4-Hydroxynonenal

1. Introduction

The GSTs (EC 2.5.1.18) constitute a major group of phase II detoxification proteins that protect against a variety of reactive chemicals and products of oxidative stress [1]. Currently, seven separate GST gene families (alpha, mu, pi, theta, sigma, kappa, and omega) have been identified that encode over twenty distinct soluble GST human proteins. The range of compounds detoxified by GSTs is remarkably diverse and includes a number of exogenous xenobiotic carcinogens, anticancer drugs, and environmental chemicals. Most of the enzymatic reactions catalyzed by GSTs involve conjugation of toxic electrophiles with the tripeptide, GSH. However, certain GST isozymes can both catalyze the reduction of cellular peroxides to their corresponding alcohols as well as conjugate endogenous genotoxic α,β-unsaturated aldehydes formed during the peroxidation of membrane lipids [2,3]. In this regard, the GST pathway mitigates the deleterious effects of reactive oxygen species and their toxic products and, thus, can be viewed as an integral component of the cellular antioxidant defense. Because a number of the compounds detoxified by GSTs are mutagenic, the level of GST isozyme expression can be an important determinant of sensitivity to carcinogenesis (reviewed by Hayes and Pulford in Ref. [1]).

Of the human GST isozymes, the alpha class GSTs are of particular interest due to their high specific catalytic

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Abbreviations: 4HNE, 4-hydroxynonenal; δ^5-ADI, δ^5-androstene-3,17-dione; CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GPX, glutathione peroxidase; GSH, glutathione; GST, glutathione *S*-transferase; HPRT, hypoxanthine guanine phosphoribosyl transferase; LDH, lactate dehydrogenase; Se-GPX, selenium-dependent glutathione peroxidases.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01017-1

activities toward lipid hydroperoxides and genotoxic α,β-unsaturated aldehydes [4,5]. The protein products of three alpha class GST genes (*hGSTA1*, *hGSTA2*, and *hGSTA4*) are present in human liver. The dimeric GST proteins hGSTA1-1, hGSTA1-2, and hGSTA2-2 have particularly high glutathione peroxidase (GPX) activity toward fatty acid hydroperoxides, phospholipid hydroperoxides, and CuOOH [5]. Because hGSTA1 and hGSTA2 subunits share extensive sequence homology, they are not easily resolved using northern or western blotting. Of the two subunits, hGSTA1 is the predominant form in human liver [6,7]. In contrast, the hGSTA4 subunit is considerably diverged from the other alpha GSTs [8] and displays a high specificity toward the 4-hydroxyalkenals, highly toxic products of peroxidative reactions [4]. In particular, the rate of hGSTA4-4-catalyzed conjugation of 4HNE is approximately 40-fold greater for hGSTA4-4 than for hGSTA1-1 or hGSTA2-2 [4]. The cellular removal of 4HNE by hGSTA4-4 is of particular significance due to the fact that 4HNE is highly genotoxic and relatively stable, and, therefore, can diffuse from its site of origin and attack distant intracellular or extracellular targets [9]. Because of their high activity toward 4HNE and related aldehydes, it has been suggested that hGSTA4-4 and its rodent orthologues (e.g. mGSTA4-4 and rGSTA4-4) have evolved to protect against oxidative injury *in vivo* [2,4,10].

Although the expression of human alpha class GSTs has been reported in adult liver, relatively little is known regarding this important subgroup of detoxification proteins in human prenatal tissues. Like many biotransformation enzymes, GST isozyme expression in mammalian systems has been shown to be subject to both ontogenic and sex-specific regulation. Because a number of drugs and environmental chemicals may cross the placenta and accumulate in the fetus, GST isoenzyme expression in human prenatal tissues may be an important determinant of susceptibility of the fetus to chemical toxicity *in utero* [11]. In this regard, the pi class GST isoform hGSTP1-1 is a major GST in human prenatal liver, although its hepatic expression decreases rapidly after birth [7]. Alpha class GST proteins have also been detected in the developing human prenatal liver [7,12], and there is some evidence to suggest that the amounts of alpha class GST may vary among prenatal liver donors [13]. In general, however, little is known regarding expression of these important GSTs in human prenatal tissues. In the present study, we have investigated the tissue-specific expression of hGSTA1/2 and hGSTA4 steady-state mRNA in human tissues and specifically determined if these mRNAs were individually expressed among a panel of seven human prenatal liver donors. The comparative detoxification of alpha class GST substrates, including hydroperoxides and 4HNE, was examined in prenatal and adult liver donors along with the subcellular localization of hepatic hGSTA1/2 and hGSTA4-4 proteins.

2. Materials and methods

2.1. Chemicals

δ^5-ADI, CDNB, CuOOH, 4HNE, secondary antibodies, and other reagents were purchased from the Sigma Chemical Co. Ultrapure electrophoresis grade agarose, guanidinium isothiocyanate, nucleic acid grade phenol, and other molecular biology grade reagents as well as molecular weight standards and tissue culture media were purchased from Gibco/BRL. T4 polynucleotide kinase was purchased from Stratagene, *Taq* polymerase was obtained from Boehringer Mannheim, and [α-^{32}P]dATP and [α-^{32}P]dCTP were purchased from DuPont NEN. Nytran membranes were purchased from Immobilon Inc. and x-ray film from Kodak.

2.2. Tissue samples and subcellular fractionation

All human tissue work was approved by the University of Florida Health Center Institutional Review Board. Second trimester prenatal liver samples (all from caucasian donors, aged 20–24 weeks) were obtained from the Anatomical Gift Foundation (AGF), an independent non-profit human tissue bank that provides human tissue for biomedical research. All prenatal livers were obtained through elective termination of pregnancy. Because the specimens obtained by the tissue banks would otherwise be discarded and because they are from anonymous donors, the studies are considered to be exempted by regulatory provisions of human subject research (45 CFR 46). Adult human liver samples (caucasian male donors) from non-smokers without a history of drug abuse were also obtained from the AGF. Strict confidentiality of donor information was maintained for all human samples. The liver tissues were washed with ice-cold PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and minced prior to homogenization in 4 vol. of buffer containing 250 mM sucrose, 80 mM Tris–HCl, 1 mM EDTA, 0.2 mM dithiothreitol, pH 7.4, with six passes of a teflon pestle in a motorized homogenizer. The resulting liver homogenates were centrifuged at 600 *g* for 10 min at 4°, and the resulting supernatants were centrifuged at 10,000 *g* at 4° for 15 min to yield mitochondrial pellets and supernatants. The mitochondrial pellets were washed twice by suspendion in the homogenation buffer and centrifuged at 10,000 *g* for 10 min at 4° to remove adsorbed, non-mitochondrial proteins [14]. The resulting mitochondrial pellets were suspended in a volume of homogenization buffer equivalent to the original liver weight. The cytosolic fractions (100,000 *g* supernatants) were prepared from the 10,000 *g* supernatants. The mitochondrial and cytosolic fractions were stored at −80° until assayed. Mitochondrial and cytosolic protein concentrations were determined using a Bradford-based protein assay kit (Bio-Rad Inc.) and bovine serum albumin as a

standard. Purified GST protein concentrations were determined using the bicinchoninic acid method [15].

2.3. Affinity purification of prenatal liver cytosolic GST

Dithiothreitol (5 mM) and phenylmethylsulfonyl fluoride (1 mM) were added to 600 μL of liver cytosol prior to incubation with 60 μL GSH linked to Sepharose 4B matrix. The cytosol/GSH matrix was mixed at room temperature for 10 min in a spin column, and washed twice with 0.5 mL of Tris-buffered saline (TBS); the purified GSTs were obtained by eluting with 150 μL of elution buffer (50 mM Tris–HCl, 150 mM glutathione, 1.4 mM β-mercaptoethanol, pH 9.6). Sample pH was adjusted immediately to neutrality with the addition of 100 mM Tris–HCl, pH 7.2. Then samples were dialyzed for 40 hr at 4° against 500 vol. of TBS.

2.4. Northern analysis of GST mRNA expression

Total liver RNA was isolated from approximately 100 mg of snap-frozen liver tissue from seven prenatal liver samples using Trizol (Gibco/BRL). The RNA pellets were suspended in diethyl pyrocarbonate (DEPC)-treated water, and total RNA concentrations were determined by UV spectroscopy before being stored at −80°. The integrity of the RNA was evaluated by visual inspection of the 18S and 28S ribosomal RNA bands following ethidium bromide staining. Twenty micrograms of the total liver RNA was separated on a 1.0% agarose-formaldehyde gel and transferred to Nytran membranes. The immobilized RNA was stained with 0.03% methylene blue in 0.3 M sodium acetate and scanned densitometrically for inspection of 18S and 28S ribosomal RNA. The isolation and construction of hGSTA1-pGEM-T and hGSTA4-pGEM-T recombinant plasmids have been described previously [16]. The hGSTA1 and hGSTA4 cDNA inserts were excised from pGEM-T plasmid using *Sph*I and *Pst*I and labeled using [α-^{32}P]dCTP and random primers. Following labeling, the probes were purified, using NACS prepack columns (Gibco/BRL), prior to hybridization. The blots were hybridized with radiolabeled probes for hGSTA1/2 and hGSTA4 at 68° in Quik-Hyb (Stratagene) using a Robbins hybridization oven (Robbins Scientific Corp.). Following hybridization, the blots were washed once at room temperature and twice at 2° below the T_H for 15 min in 2× sodium citrate solution (SSC) containing 0.1% SDS. Autoradiography was performed at −70° for 24 hr (for hGSTA1/2 mRNA expression) or for 7 days (for hGSTA4 mRNA expression). The autoradiographs were scanned using Adobe Photoshop 5.0 software for the Macintosh.

2.5. Western analysis

Proteins were resolved by 14% SDS–PAGE and electroblotted onto polyvinylidene fluoride (PVDF) membranes. Non-specific binding was blocked by overnight incubation of the membranes in 5% non-fat dried milk in TBS containing 0.1% (v/v) Tween 20 (TBS-T). The membranes were incubated with either a 1:1000 dilution of rabbit anti-hGSTA1-1 (Oxford Biochemical Inc.), a 1:10,000 dilution of chicken anti-hGSTA4-4 [14], or a 1:2000 dilution of mouse anti-human LDH (Sigma Chemical Co.) in 5% non-fat dried milk/TBS-T for 1 hr. This was followed by incubation with horseradish peroxidase conjugated to goat anti-rabbit IgG (1:10,000), rabbit anti-chicken IgY (1:200,000), or goat anti-mouse IgG (1:10,000) as the secondary antibody. The purity of the mitochondrial fractions was determined by western analysis using a cytosolic LDH antibody. Antibody–antigen complexes were visualized using an enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech) followed by exposure to Kodak X-Omat film (Eastman Kodak Co.), except for analyses using anti-hGSTA4-4 in which ECL Plus™ was utilized.

2.6. Hepatic GST catalytic activity assays

Hepatic cytosolic GST activities toward CDNB (a general GST reference substrate [3]) and δ^5-ADI (a substrate largely conjugated by hGSTA1-1 [3]) were determined at 30° using the spectrophotometric assays of Habig and Jakoby [17] in a 96-well microplate format. Cytosolic GPX activity was measured in dual assays by the method of Esworthy *et al.* [18]. The reaction mixtures contained 50 mM $NaPO_4$ (pH 7.0), 1 mM GSH, 0.4 mM NADPH, 1 U/mL of glutathione reductase, 11.25 mM sodium azide, and 5 μL of a protein sample per well. The assays were conducted at 30°, reactions were initiated by the addition of either 0.25 mM H_2O_2 or 1.5 mM CuOOH, and the oxidation of NADPH was followed at 340 nm. Under the conditions of the assay, the substrate H_2O_2 is reduced exclusively by Se-GPX1, the major GPX enzyme in liver cytosol. In contrast, NADPH oxidation resulting from CuOOH reduction reflects the combined contributions to GPX activity from both Se-GPX1 and soluble GST, primarily hGSTA1-1 and hGSTA2-2 [3,5]. Because all substrates are saturating and H_2O_2 is not a substrate for GST, the calculated difference in activity toward H_2O_2 and CuOOH serves as an overall measure of the GST contribution to GPX activity ($GPX_{GST} = GPX_{CuOOH} - GPX_{H_2O_2}$) [18].

GST activity toward 4HNE (a substrate for hGSTA4-4 [19]) was determined using GSH affinity-purified liver cytosolic fractions due to high background absorbance at the wavelength used in the assay (224 nm) by interfering proteins. GST–4HNE activities were determined by the spectrophotometric method of Singhal *et al.* [20] with minor modifications. In brief, the reaction mixture (final volume 1 mL) contained 100 mM KPO_4, pH 6.5, 0.5 mM GSH, purified GST, and 0.1 mM 4HNE. The reactions were initiated by the addition of 4HNE, and its utilization was monitored at 224 nm in a Cary dual beam spectrophotometer against a blank that contained all of the reactants

except 4HNE. All catalytic activity assays were conducted at 30° and were corrected for non-enzymatic activity.

2.7. Comparative tissue-specific expression of hGSTA1/2 and hGSTA4 mRNA

The relative mRNA expression levels of hGSTA1/2 and hGSTA4 mRNAs were examined using human multiple tissue expression arrays (Clonetech) containing pooled human poly A^+ mRNAs. The human multiple tissue array contained poly A^+ RNA from 76 different human tissues (61 adult tissues, 7 prenatal tissues, and tissues from 8 human cancer cell lines) and 8 different control RNAs and DNAs. Negative controls on the blot include *Escherichia coli* RNA, *E. coli* DNA, synthetic poly R(A), yeast total RNA, and yeast tRNA. Accordingly, hybridization of the GST-specific cDNA probes to any of the aforementioned DNA from the negative controls would reflect non-specific binding. The poly A^+ mRNAs were prepared by the manufacturer from individual human tissues and pooled prior to loading. All mRNA pools were pre-normalized to nine different housekeeping genes (β-actin, G3PDH, ubiquitin, 23 kDa highly basic protein, α-tubulin, phospholipase A_2, ribosomal protein S9, transferrin receptor, and HPRT to minimize tissue-specific variations associated with the expression of any single housekeeping gene. Because the blots are normalized for several housekeeping genes and contained pooled mRNA from many human donors, the relative level of tissue-specific gene expression within each blot can be compared. The hGSTA1 and hGSTA4 cDNA probes were labeled as described above and hybridized according to the instructions of the manufacturer. Autoradiography was performed at −70° as described above, and the tissue-specific mRNA expression of hGSTA1/2 and hGSTA4 was quantitated by densitometric scanning of the autoradiographs with Adobe Photoshop.

2.8. Data analysis

GST catalytic activity values reported represent the mean of triplicate reactions with the exception of GST–4HNE activities, which were carried out in duplicate. Significant differences in GST activities among the adult and prenatal groups were determined using an unpaired Student's *t*-test and a probability value of $P \leq 0.05$ (Statview 4.5, Abacus Concepts).

3. Results

3.1. hGSTA1/2 and hGSTA4 steady-state mRNA expression in individual human prenatal liver tissues

Northern blotting of a panel of seven prenatal livers was used to determine the presence of hGSTA1/2 and hGSTA4 mRNAs in each of a panel of seven donors. Long exposure times were necessary for optimal visualization of blots hybridized with an hGSTA4 probe (1 week) relative to blots hybridized with an hGSTA1/2 probe (24 hr) on the northern blot autoradiographs. As observed in Fig. 1, hGSTA1/2 and hGSTA4 mRNAs were present in liver RNA preparations from all seven prenatal samples.

3.2. Cytosolic and mitochrondrial localization of hGSTA1/2 and hGSTA4-4 proteins

A recent study from our laboratory indicated that the mitochondria may represent an important source for alpha GST in adult liver [14]. Therefore, we analyzed alpha GST expression in second trimester prenatal liver to determine if a similar distribution occurred during this stage of development. Western blotting of prenatal liver cytosolic and mitochondrial fractions against human LDH (a cytosolic protein involved in intermediary metabolism) cross-

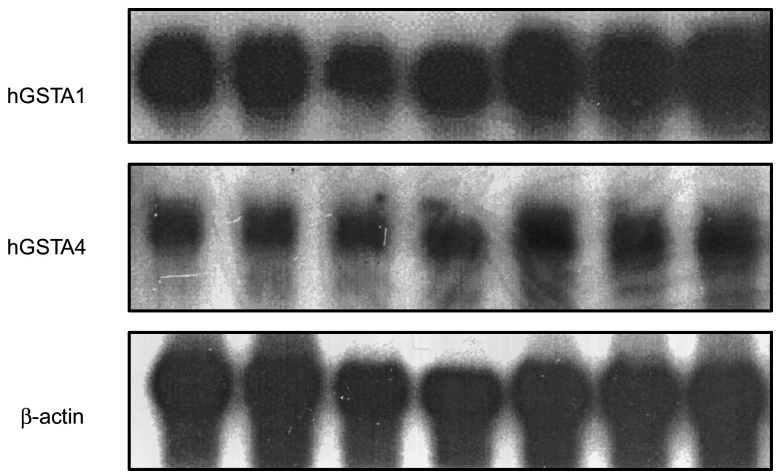


Fig. 1. Northern blot of hGSTA1/2, hGSTA4, and β-actin mRNA expression in liver tissues isolated from seven prenatal liver samples. Total RNA (10 μg) from seven second trimester prenatal livers was resolved on denaturing agarose gels and subjected to northern blot hybridization using cDNA probes for hGSTA1 or hGSTA4. The blots were stripped and rehybridized with a β-actin cDNA probe.

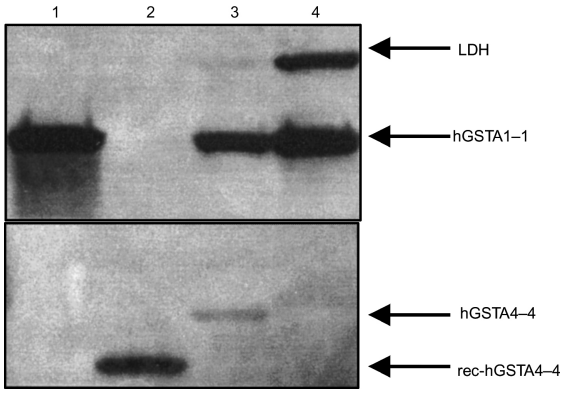


Fig. 2. Western blot of hGSTA1-1 and hGSTA4-4 reactive protein expression in prenatal liver cytosolic and mitochondrial fractions. Lane 1: recombinant hGSTA1-1, lane 2: recombinant hGSTA4-4, lane 3: prenatal liver mitochondria, and lane 4: prenatal liver cytosol. Mitochondrial and cytosolic proteins (62 μg each) from a second trimester prenatal liver were subjected to western blotting using hGSTA1-1 and hGSTA4-4 polyclonal antibodies. The blot in the upper panel was probed with anti-hGSTA1-1 and anti-human LDH (a cytosolic marker). The bottom panel was probed with anti-hGSTA4-4. The positions of hGSTA1-1, native hGSTA4-4, recombinant (rec-) hGSTA4-4, and LDH are indicated by the arrows.

reacted with a 35 kDa protein in cytosolic fractions, but not in mitochondrial fractions (Fig. 2). These results indicated minimal cytosolic contamination of prenatal mitochondrial fractions. As observed in Fig. 2, western blotting of prenatal liver fractions with an hGSTA1-1 antibody revealed the presence of hGSTA1/2-reactive proteins of similar molecular weights in both cytosolic and mitochondrial fractions. In contrast, western analysis using an antibody specific to hGSTA4-4 demonstrated a 30 kDa band in mitochondria, but not in cytosol. This band ran considerably more slowly than the rec-hGSTA4-4 (27 kDa, Fig. 2). We have shown previously that the N-terminal sequence of an immunopurified 30 kDa band from adult liver mitochondria is homologous to hGSTA4-4, and migrates more slowly than the recombinant protein in SDS–PAGE gels [14].

3.3. Prenatal and adult liver GST catalytic activities

Upon establishing the presence of two alpha class GST mRNAs in individual second trimester prenatal livers, we determined the capacity of prenatal cytosolic fractions to carry out GST-mediated enzymatic reactions. GST enzymatic activities were carried out in cytosolic fractions prepared from adult liver donors for comparative purposes. In all experiments, activities were analyzed on a minimum of 6 donors from the two developmental age groups (prenatal and adult). However, because the present study necessitated carrying out a number of catalytic assays as well as affinity purifications on limited amounts of human tissue, it was sometimes necessary to conduct catalytic activity assays on different donors within the two age groups.

As observed in Fig. 3, human second trimester prenatal liver cytosolic GSTs were generally highly active toward the broad-specificity GST substrates CDNB (Fig. 3A), δ^5-ADI (Fig. 3B), and 4HNE (Fig. 3C). A 6-fold variation was observed in prenatal liver GST–CDNB activities, with a range of 176 nmol/min/mg (sample FL2) to 1046 nmol/min/mg (sample FL5) and a mean ± SEM of 683 ± 93 nmol/min/mg (Fig. 3A). Mean GST–CDNB activities in adult liver samples (1010 ± 119 nmol/min/mg) were significantly higher than those observed in prenatal donor samples. In general, adult GST–CDNB activities showed less interindividual variation than observed in prenatal tissues. A 5-fold variation was observed in prenatal liver GST–ADI activities, with a low of 9 nmol/min/mg (sample FL2) to a high of 45 nmol/min/mg (sample FL1, Fig. 3B). As observed in Fig. 3B, adult δ^5-ADI activities ranged from a low of 23 nmol/min/mg (donor 30396) to a high of 66 nmol/min/mg (donor HL1). Mean GST–ADI activities for prenatal liver donors (30 ± 5 nmol/min/mg) were not significantly different from those observed for adults (39 ± 8 nmol/min/mg). Because of high assay interference associated with the detection of GST–4HNE conjugation at 224 nm, analysis of prenatal liver GST activity toward 4HNE was conducted on GST affinity-purified fractions (practical detection limit of 5 nmol/min/mg in cytosol). As observed in Fig. 3C, affinity-purified prenatal liver cytosolic GST fractions were highly active toward 4HNE. GST–4HNE activities ranged from 1.5 to 2.8 μmol/min/mg, with a mean ± SEM of 2.2 ± 0.2 μmol/min/mg). The prenatal GST–4HNE activities were statistically similar to those observed in adult liver donors (range of 0.7 to 6.4 μmol/min/mg, mean ± SEM of 3.0 ± 1.6 μmol/min/mg), although more variation was observed among the adults.

Fig. 4 compares the total cytosolic GPX activities to the Se-dependent and GST-dependent components of total GPX activity in adult and prenatal liver tissues. Total GPX (CuOOH reduction, Fig. 4A) in adult liver samples was 5-fold higher than the Se-GPX component (H_2O_2 reduction) in adult liver (Fig. 4B). These data suggest that GST has a strong contribution to total peroxide metabolism in adult liver. In contrast, total GPX activity in prenatal liver samples was less than 2-fold that of the Se-GPX component, suggesting a relatively stronger contribution of Se-GPX. The mean levels of total GPX, Se-GPX, and GST-dependent GPX were significantly higher in adult liver than in prenatal liver samples. The adult liver fractions had 18-fold higher mean GST-dependent peroxidase activities than prenatal livers (269 ± 39 nmol/min/mg in adult liver vs 15 ± 5 nmol/min/mg in prenatal liver, Fig. 4C). Furthermore, two of the ten prenatal tissues analyzed did not exhibit detectable GST-dependent peroxidase activity (practical limit of detection of 2 nmol/min/mg).

3.4. Human adult and prenatal tissue-specific expression of hGSTA1/2 and hGSTA4

To gain further insight into the nature of alpha GST expression during development, we characterized

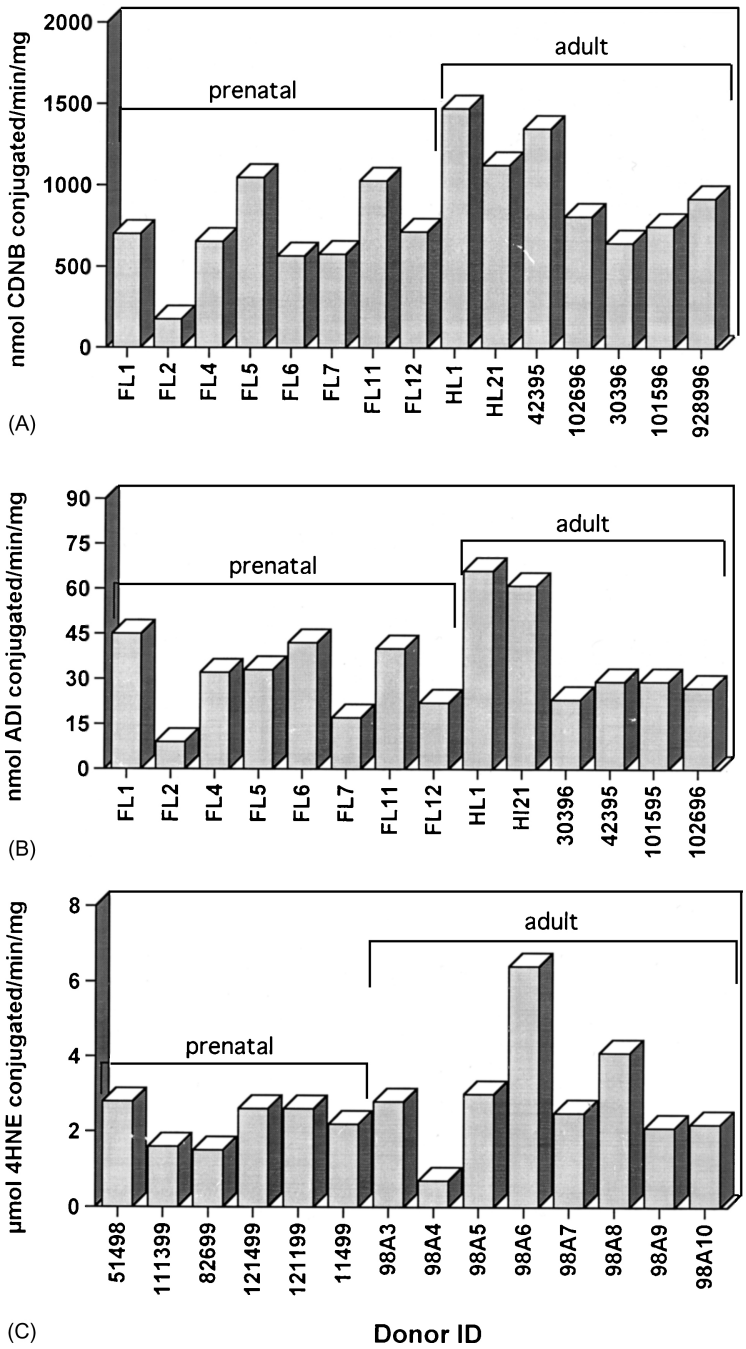


Fig. 3. Comparison of initial rate cytosolic GST activities in human adult and prenatal liver donors. GST activity toward (A) CDNB and (B) ADI is presented as nmol substrate conjugated/min/mg protein. GST conjugation of (C) 4HNE was carried out using GSH affinity-purified cytosolic GST and is presented as µmol 4HNE conjugated/min/mg of affinity-purified cytosolic GST. All assays were carried out in triplicate except for GST-4HNE activities, which were carried out in duplicate, and corrected for non-enzymatic activity.

hGSTA1/2 and hGSTA4 mRNA expression using multiple human tissue array analysis. As in the northern blotting experiments, relatively long exposure times were necessary for optimal visualization of hGSTA4-hybridized arrays relative to hGSTA1/2-hybridized arrays, and, thus, the relative intensities of the hGSTA1/2 and hGSTA4 signals cannot be compared directly. Accordingly, the blots were developed over time periods that optimized for detecting differences between the weakest and the strongest signals for each array as a function of tissue expression. This approach allowed for a quantitative assessment of differences in GST mRNA expression level across the pooled tissues. As observed in Fig. 5A, the hGSTA1/2 cDNA probe did not cross-react with the four negative control RNAs [*E. coli* RNA, synthetic poly R(A), yeast total RNA, and yeast tRNA], or with *E. coli* DNA (Fig. 5A, coord. D12). No hybridization of the four negative control RNAs by the hGSTA4 cDNA probe was observed. However, there was some cross-reactivity of the hGSTA4 probe with *E. coli* DNA (Fig. 5B, coord.

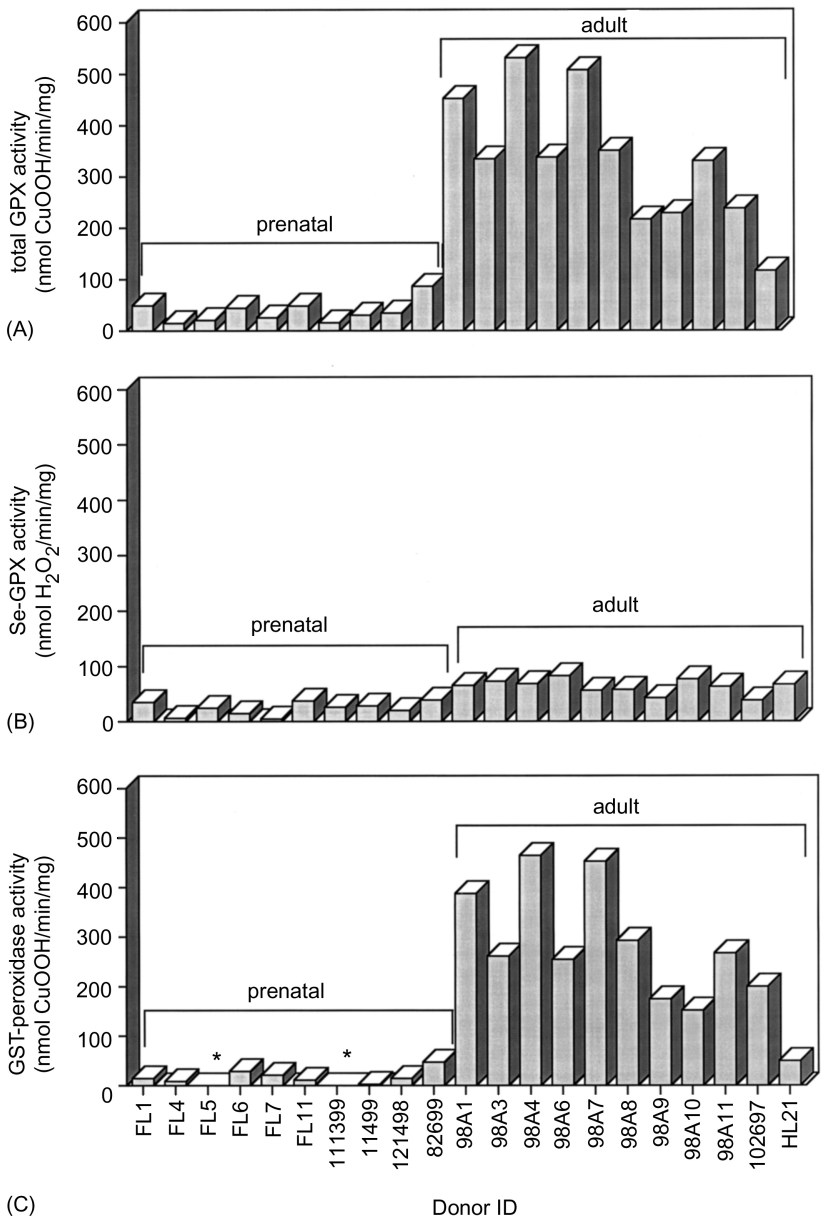
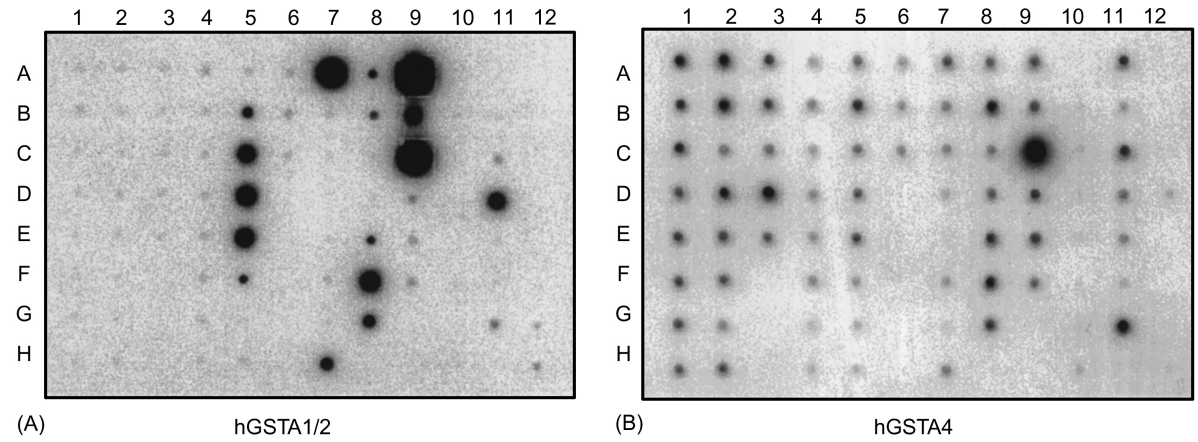


Fig. 4. Comparative initial rate cytosolic GPX activities in prenatal and adult hepatic cytosolic fractions. (A) Total GPX specific activity (nmol CuOOH reduced/min/mg protein) in ten prental liver samples and eleven adult liver samples. (B) Selenium-dependent GPX activity (nmol H_2O_2 reduced/min/mg protein) in the same samples assayed in (A), and (C) subtracted values of (B) from (A) yielding an estimate of GST-dependent peroxidase activities for these samples. Asterisks (∗) denote activity values below the practical limit of detection (2 nmol/min/mg).

D12). The signal for *E. coli* DNA but not the four RNA negative controls by hGSTA4 cDNA probe suggests the presence of an *E. coli* gene sequence(s) sharing homology with hGSTA4. The locations of tissue poly A^+ mRNAs and associated nucleic acid controls are presented in Fig. 5C.

As observed in Fig. 5A, human hGSTA1/2 mRNA expression was particularly high in adult liver (coord. A9) and adult adrenal gland (coord. C9). hGSTA1/2 mRNA expression was also relatively high in adult kidney (coord. A7), but relatively low in the central nervous system (coords. A1–H3), as well as in cardiac tissues (coords. A4–H4). Among the prenatal tissues analyzed, hGSTA1/2 mRNA expression was highest in prenatal liver (coord. D11). hGSTA1/2 mRNA expression in prenatal lung was 11% that of liver, whereas hGSTA1/2 mRNA expression in prenatal heart, brain, spleen, and thymus were all less than 10% that observed in liver (Fig. 6A). As observed in Fig. 5B, hGSTA4 mRNA was expressed in all prenatal tissues examined (Fig. 5B, column 11) and was very highly expressed in the adult adrenal gland (Fig. 5B, coord. C9). In general, hGSTA4 mRNA expression showed less tissue-specific variation than did hGSTA1/2. Quantitative analysis of hGSTA4 mRNA expression in prenatal tissues revealed the highest expression in prenatal lung, followed by kidney > brain > liver > heart = spleen > thymus (Fig. 6B). A qualitative comparison of fetal and adult hGSTA1/2 mRNA abundance suggests a lower level of expression in fetal kidney (Fig. 5A, coord. C11) and fetal liver (coord. D11)



	1	2	3	4	5	6	7	8	9	10	11	12
A	Whole brain	Cerebellum, left	Substantia nigra	Heart	Esophagus	Colon, transverse	Kidney	Lung	Liver	Leukemia, HL-60	Fetal brain	Yeast total RNA
B	Cerebral cortex	Cerebellum, right	Accumbens nucleus	Aorta	Stomach	Colon, descending	Skeletal muscle	Placenta	Pancreas	HeLa S3	Fetal heart	Yeast tRNA
C	Frontal lobe	Corpus callosum	Thalamus	Atrium, left	Duodenum	Rectum	Spleen	Bladder	Adrenal gland	Leukemia, K-562	Fetal kidney	*E. coli* rRNA
D	Parietal lobe	Amygdala	Pituitary gland	Atrium, right	Jejunum		Thymus	Uterus	Thyroid gland	Leukemia, MOLT-4	Fetal liver	*E. coli* DNA
E	Occipital lobe	Caudate nucleus	Spinal cord	Ventricle, left	Ileum		Peripheral blood leukocyte	Prostate	Salivary gland	Burkitt's lymphoma, Raji	Fetal Spleen	Poly r(A)
F	Temporal lobe	Hippo-campus		Ventricle, right	Ilocecum		Lymph node	Testis	Mammary gland	Burkitt's lymphoma, Daudi	Fetal Thymus	Human C_0t-1 DNA
G	Paracentral gyrus	Medulla oblongata		Inter-ventricular septum	Appendix		Bone marrow	Ovary		Colorectal adeno-carcinoma, SW480	Fetal lung	Human DNA 100 ng
H	Pons	Putamen		Apex of the heart	Colon, ascending		Trachea			Lung carcinoma, A549		Human DNA 500 ng

(C)

Fig. 5. Human tissue-specific expression of (A) hGSTA1/2 and (B) hGSTA4 mRNA. The human tissue arrays contained poly A^+ mRNAs prepared by the manufacturer from many individual human tissues that were pooled prior to loading. (C) Diagram of the location of tissue poly A^+ mRNAs and associated control nucleic acids on the human multiple tissue array.

relative to the corresponding adult kidney (coord. A7) and liver (coord. A9) tissues. In the case of hGSTA4, levels of expression in fetal tissues were similar to those of adult tissues, except for fetal kidney (Fig. 5B, coord. C11) and fetal lung (coord. G11), in which hGSTA4 mRNA expression was slightly more abundant than in adult kidney (coord. A7) and adult lung (coord. A8).

4. Discussion

In utero exposure to certain drugs and chemicals can accelerate the production of toxic aldehydes and other reactive species in prenatal tissues [21–23]. Examples of maternally transferred toxicants include drugs such as phenytoin and cyclophosphamide, and environmental chemicals such as polycyclic aromatic hydrocarbons, aflatoxins, and nitrosamines (reviewed in Ref. [21]). If not repaired, *in utero* cell injury may initiate a process that can cause prenatal tissue damage, teratogenesis, or mortality [22,23]. The ontogenic onset of prenatal susceptibility to maternally transferred toxicants has been correlated to the time of appearance of oxidative enzymes [23]. If specific phase II or antioxidant protective enzymes are not expressed at the site of electrophile formation, the reactive intermediates are free to covalently bind embryonic cellular DNA and proteins [11]. Despite the importance of GST isoenzymes in protecting against the damaging effects of electrophilic agents, however, relatively little is known regarding their expression in the developing human conceptus. Moreover, much of teratology is based upon investigations that have utilized rodents or other animal models.

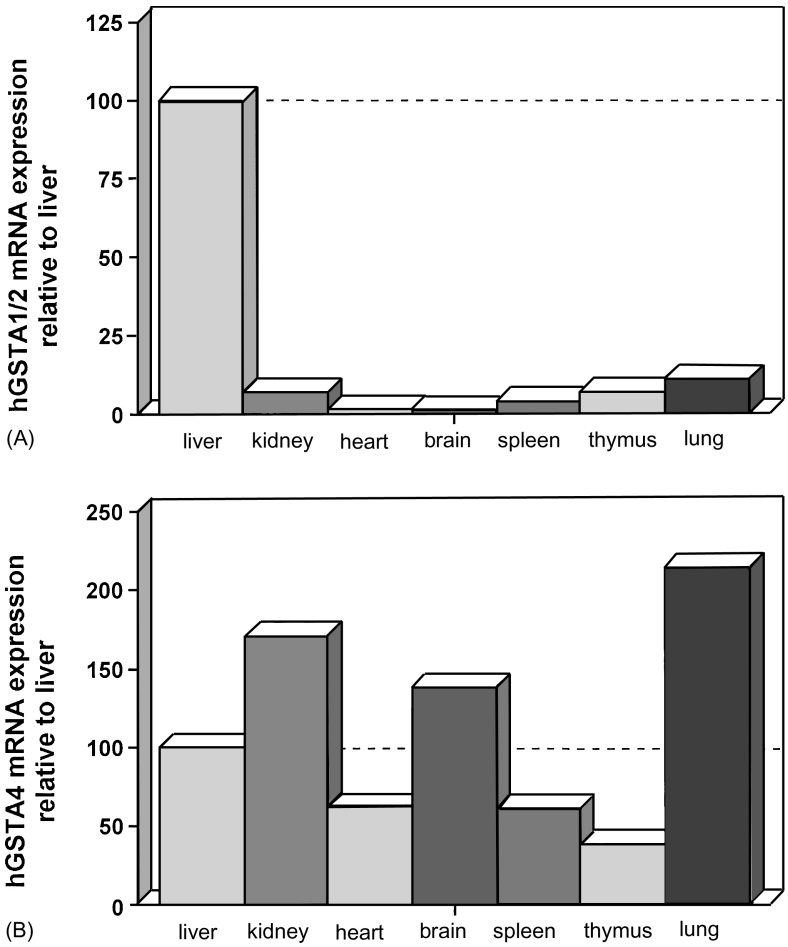


Fig. 6. Comparative expression level of hGSTA1/2 mRNA and hGSTA4 mRNAs in prenatal extrahepatic tissues relative to prenatal liver. Data are from Fig. 5 after densitometric analysis; the tissue-specific mRNA expression of (A) hGSTA1/2 and (B) hGSTA4 was quantitated by densitometric scanning of the autoradiographs. Values are presented as a percentage of tissue expression relative to prenatal liver.

Even though there are practical and ethical considerations associated with the use of human tissues, extrapolation from animal developmental models to humans should be used with caution in lieu of known differences in gene structure and substrate specificity between the human biotransformation enzymes and their rodent orthologues [24].

4.1. Hepatic subcellular hGSTA1 and hGSTA4 expression and activity

Our northern blotting analysis extends the findings from other groups [7,25,26], indicating that at least two of the three currently identified human GST alpha isoforms, including hGSTA1 and/or hGSTA2 as well as hGSTA4, are expressed in second trimester human liver. As discussed, the majority (>90%) of the hGSTA1/2 expression appears to be accounted for by the hGSTA1 subunits. The detection of hGSTA4-4- and hGSTA1-related proteins in the mitochondria of prenatal liver cells is a particularly interesting finding. We recently demonstrated a similar distribution for hGSTA4-4 in adult human liver cells [14], but have not investigated the subcellular localization of hGSTA1-1. Although the overwhelming majority of soluble GST isozymes are cytosolic, it may be that the mitochondria represent an important source for cellular GSTs in mammals [27–29]. In this regard, it is possible that GSTA4-4-related enzymes have evolved specifically to detoxify products of oxidative injury generated in mitochondria.

Second trimester prenatal liver tissues exhibited GST catalytic activities toward the general GST substrate CDNB, as well as toward substrates preferentially metabolized by alpha class GST isozymes. In general, the initial rates of prenatal liver GST activities toward CDNB, δ^5-ADI, and 4HNE were similar to those observed in adult liver tissues. Collectively, our mRNA, protein, and catalytic activity data indicate that these protective pathways are active in the liver at this stage of human ontogeny. Relative to adults, however, the prenatal liver GSTs clearly exhibited lower NADPH-dependent metabolism of the peroxides CuOOH and H_2O_2, reflecting comparatively low prenatal Se-GPX activities and GST-peroxidase activities. Accordingly, this condition may underlie a decreased ability for prenatal liver to detoxify peroxides, and a greater sensitivity to oxidative injury relative to adults. Such an hypothesis is supported by *in vivo* studies demonstrating oxidative injury in rat fetal liver after maternal

ethanol consumption and an increased susceptibility to *t*-butylhydroperoxide-induced mitochondrial oxidative injury [30].

Our data indicate that prenatal liver cytosolic GSTs efficiently catalyze 4HNE conjugation with GSH. 4HNE accumulates comparatively rapidly in rat prenatal liver mitochondria after *in utero* ethanol exposure [31]. Given the lower ability of fetal cytosolic GSTs to reduce peroxides relative to adults, the presence of GST pathways for 4HNE removal may be particularly important in protecting against *in utero* exposure to pro-oxidant compounds such as ethanol. In addition to hGSTA4-4, a second distinct 4HNE-metabolizing GST isozyme, hGST5.8, has been reported in human tissues [32]. Although the partial amino acid sequence of hGST5.8 shows a high degree of homology with rodent GSTA4 proteins, hGSTA4-4 and hGST5.8 exhibit markedly different isoelectric points and antibody cross-reactivities. Also, these two functionally similar enzymes appear to have differing tissue distribution in humans. Differences in subcellular distribution may also exist for these enzymes, as our group recently demonstrated the presence of hGSTA4-4-like enzyme in hepatic mitochondria, to the apparent exclusion of the cytoplasm, nucleus, and microsomal fractions [14].

The high interindividual variations observed in GST catalytic activities toward CDNB, δ^5-ADI, and CuOOH observed among the prenatal samples in the present study are consistent with extensive interindividual variation in certain hepatic cytosolic GST activities among adults [33]. Such individual differences in biotransformation enzyme activities among adults may be accounted for by several factors, including genetics, age, or environmental factors (e.g. drug and chemical exposure and diet). In contrast, interindividual variation in the rates of GST conjugation among the prenatal tissues are more likely the result of genetic factors that result in polymorphic expression of GST enzymes (reviewed in Ref. [34]), including hGSTA1 [13]. Some GST polymorphisms have been associated with interindividual variability in xenobiotic metabolism [34] and an increased susceptibility to developing certain cancers in adults [35–38].

4.2. Extrahepatic GST expression

There is evidence to suggest that extrahepatic organs are significant sources of GST-pi during development [39]. Our results using the multiple tissue expression array clearly demonstrate the presence of alpha class hGSTA1/2 and hGSTA4 mRNAs in other prenatal tissues, including brain, liver, lung, kidney, spleen, and heart. The multiple tissue expression array used in the present study provides a rapid method to obtain information about genes of interest across a large number of human tissues. This method is particularly useful for determining the developmental stage of gene expression and limited information about mRNA abundance. Our multiple tissue array experiments indicate a markedly different expression profile for hGSTA1/2 and hGSTA4, with hGSTA4 being expressed at comparatively low levels in all tissues. In this regard, the presence of tissue-specific regulatory elements in the promoters of certain GST genes can underlie marked differences in tissue GST expression [1]. The low ubiquitous tissue expression of hGSTA4 mRNA is consistent with a study by Desmots *et al*. [8], who observed that the 5′ flanking region of the hGSTA4 gene lacks a TATA box and a CAAT sequence at the usual positions. TATA-less promoters typically produce continuous transcripts at a relatively low level, which may explain the modest expression of hGSTA4 in many of the tissues. The presence of hGSTA4 in all human tissues analyzed further supports the idea of an important role for this enzyme as a housekeeping gene functioning in removing peroxidative products produced during oxidative stress. The level of expression of hGSTA1/2 appeared to be significantly lower in prenatal kidney and liver relative to adults. This may underlie the diminished level of GST–GPX activity in prenatal hepatic cytosolic fractions, as hGSTA2-2 is the primary contributer to this activity [5].

In conclusion, we have demonstrated the expression of two major alpha class GST isozymes in a panel of second trimester prenatal samples, and we have conducted comparative expression studies in other tissues. These studies indicate that in the second trimester, the prenatal liver has a high capacity to detoxify a number of electrophilic GST substrates as well as the potent mutagen 4HNE. However, there does appear to be a strongly diminished capacity for fetal liver to metabolize peroxidative substrates via glutathione-dependent reduction, relative to adults. It cannot yet be predicted whether there is a developmental window of susceptibility resulting from a reduced ability of the human fetus to detoxify certain maternally transferred toxicants through GST pathways. However, our observations support hypotheses set forth by other investigators that a diminished capacity to protect against reactive oxygen species and peroxidative intermediates may contribute to teratogenic actions of certain drugs and environmental chemicals. In this regard, we are continuing to study the alpha class GSTs and their relationship to drug and chemical exposure in human prenatal liver. Of particular interest is the possibility that variations in levels of GST activity underlie susceptibility to *in utero* cell injury. Ultimately, information collected on human developmental GST isoenzyme expression may improve the risk assessment of transplacental drug and chemical exposures.

Acknowledgments

The technical assistance of Karen Pastos is greatly appreciated. This work was supported, in part, by grants from NIH (R03-ES09360 and R01-ES09427) and from the U.S. Environmental Protection Agency (R827441).

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