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Comparative study on glutathione transferases of rat brain and testis under the stress of phenobarbitol and β-methylcholanthrene*

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Abstract: A comparative study was made on the tissue specific expression of glutathione transferases (GST) in brain and testis after exposure of rat to phenobarbitol (PB) and β-methylcholanthrene (MC). Glutathione transferases, a family of multifunctional proteins are involved in intracellular transport processes and in detoxication of electrophilic xenobiotics by catalyzing reactions such as conjugation, isomerization, reduction and thiolysis. On purification, the yield of GST proteins by affinity chromatography was 39% in testis and 32% in brain. The affinity purified testis GSTs were resolved by chromatofocusing into six anionic and four cationic isozymes, and in brain glutathione transferases were resolved into four anionic and three cationic isozymes, suggesting the presence of multiple isozymes with Yc, Yb, Yβ and Yδ in both of them. In testis and brain, these isozymes at identical pI values showed variable functions with a battery of substrates and the cationic isozymes of brain and testis showed identical properties in CHP (cumene hydroperoxide) at pH values of above 7.0. Substrate specificity studies and immunoblot analysis of testis and brain proteins revealed that they play a predominant role in the detoxication of phenobarbitol or β-methylcholanthrene. Expression of the isozymes in testis and brain on exposure to PB and MC indicated elevated subunit variation. In both testis and brain, Yδ of π class was expressed on PB treatment and Yc of α class and Yβ of μ class was expressed in MC treated testis and only Yc was predominantly expressed in MC treated brain. Thus these subunits expression is considered as markers for carcinogenesis and specific to chemical toxicity under phenobarbitol and β-methylcholanthrene stress.

Key words: Glutathione transferases, Testis, Brain, Phenobarbitol, β-Methylcholanthrene
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INTRODUCTION

Glutathione transferases (GSTs; EC, 2.5.1.18) are phase II enzymes of defense that catalyze the conjugation of reduced glutathione to a wide range of electrophiles, carcinogens and other xenobiotics with genotoxic and cytotoxic activities (Mannervik and Danielson, 1988). There are eight classes of mammalian soluble GST (alpha, mu, pi, theta, omega, zeta, sigma and kappa) and at least three membrane bound GST (MGST1, MGST2 and MGST3) have been described (Board *et al*., 2000; Mannervik *et al*., 1992; Rowet *et al*., 1998). GST isoforms are expressed specifically in almost all the tissues, including the reproductive tissues like testes and ovaries in a discrete tissue-specific pattern (Rabahi *et al*., 1999).

Subtypes of GSTs have been grouped into classes on the basis of isoelectric point, substrate and inhibitor properties, antibody recognition, and N-terminal amino acid sequence; the main cytosolic classes are α, μ and π, previously known as basic, near-neutral and acidic, respectively on the basis of isoelectric point (Mannervik *et al*., 1992). The GST enzyme family includes the cytosolic isoforms GST-α, μ (GSTM), π (GSTP), θ (GSTT) and σ (GSTS). GSTT1, GSTA1 and 2, GSTO1, GSTM3, GSTP1 and GSTM1 are polymorphic and altered polymorphic frequency of genes encoding these proteins has been

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suggested as a potential risk factor for the development of hematopoietic malignancies. Over expression of glutathione transferases has also been implicated in chemotherapeutic drug resistance. The α glutathione transferases are highly expressed in the testis, adrenal glands and proximal tubule of the kidney. The μ class glutathione transferases has been found in high concentrations in brain, muscle, liver, kidney and lung (Hayes and Mantle, 1986; Hayes *et al*., 1987). The π class glutathione transferase is highly acidic enzyme that is widely distributed except in adult liver, where it is localized to the biliary epithelium (Sato *et al*., 1984; Satoh *et al*., 1985). The θ class enzymes have only recently been described enzymatically in liver (Meyer *et al*., 1991; Hiratsuka *et al*., 1990).

The glutathione transferases achieve detoxication by catalyzing the conjugation of reduced glutathione to various electrophilic substrates. Glutathione (GSH), as the chief intracellular non-protein thiol compound, functions as a cellular storage pool of reduced thiols. GSH conjugation is the first step in mercapturic acid synthesis, which helps in the protection of the cell by enhancing the excretion of toxic metabolites from both animals and humans. Therefore, the levels of glutathione transferases have been suggested as important determinant of the susceptibility of organisms or tissues to pharmacological or physiological changes. In particular, the over expression of GSTs in tumours appear to be a factor in the development of acquired resistance towards anti-cancer drugs and hence GSTs are a therapeutic target for rational drug design (Coles and Ketterer, 1990).

Phenobarbitol (PB), a barbiturate, and β-methylcholanthrene (MC), a polycyclic aromatic hydrocarbon have been extensively studied as the prototype promoting agents (inducing phase II enzyme system in rats). Tumor promotion by PB and MC is organ specific and dose dependent (Dragan *et al*., 1996; Devi *et al*., 2002a; Reynaud *et al*., 2002). The present work was aimed at studying the effects of PB and MC on GSTs in rat testis and brain and their characterization. Therefore, measurement of the concentrations and activities of GSTs, and their modulation in tissues that are subjected to physiological or environmental stimuli may provide researchers with an important tool in monitoring the detoxication potential of cellular systems.

MATERIALS AND METHODS

The GST affinity matrix, polybuffer exchangers (PBE) 94 and 118, polybuffers-74 and 96, Ampholytes, pH 3~10.5, GST substrates, SDS-PAGE (sodium dodecyl polyacrylamide gel electrophoresis) chemicals, phenobarbitol (PB) and β-methylcholanthrene (MC) were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Phenobarbitol (PB) treatment

Male Wistar rats (six for every treatment) procured from Venkateswara Enterprises, Bangalore, weighing 200~250 g were treated (intraperitoneal administration) with PB in water. Control animals were received water. The rats were given 8 mg of PB per 100 g body mass per day three times a week, at 48 h interval, for a total dose of 24 mg. The animals were sacrificed 24 h after the last injection.

Methylcholanthrene (MC) treatment

Male wistar rats weighing (200~250 g) were injected intraperitoneally with 4 mg of MC in coconut oil every 24 h per each injection to a total of 24 mg. Control animals received coconut oil only.

Isolation and purification of GST

The buffers used for purification of GSTs were: Buffer A: 25 mmol/L Tris(hydroxy methyl)amino methane hydrochloric acid (Tris-HCl), pH 8; Buffer B: 0.3 mol/L phosphate buffer, pH 6.5; Buffer C: 25 mmol/L imidazole HCl, pH 7.0; Buffer D: 25 mmol/L triethanolamine-HCl, pH 10.5; Buffer E: polybuffer 74 (1:8), pH 4.0; Buffer F: polybuffer 96 (1:13): ampholine (1:45), 1:1 *V*/*V*, pH 7.0.

The tissues, testes and brain collected from rats killed by decapitation were washed with saline to remove blood and fat debris and a 20% tissue homogenate was prepared in Buffer A containing 0.2 mol/L sucrose in a Potter Elvejhem homogenizer with a teflon pestle. This homogenate was centrifuged at 78000×g for 1 h at 4 °C. The supernatant of this preparation was collected and dialyzed for 24 h against 10 volumes of Buffer A with five changes to remove endogenous GSH. The dialysate was considered as cytosolic extract.

Affinity column chromatography

The swollen S-hexylglutathione (S-hexylglut-

athione) linked Agarose-4B affinity matrix was packed into a column (2 cm×7 cm) and equilibrated with 100 ml of Buffer A to pH 8.0 and the flow rate was adjusted to 60 ml/h.

The dialysate was loaded into a pre-equilibrated affinity chromatography column and washed with Buffer A containing 0.2 mol/L KCl until the absorbance of protein in the eluate was less 0.005 OD at 280 nm. Bound protein was eluted with Buffer A containing 5 mmol/L S-hexyl GSH, 2.5 mmol/L GSH and 0.2 mol/L KCl. The eluent was collected in 3 ml fractions. Active fractions with high enzyme activity with CDNB (1-Chloro-2,4-dinitrobenzene) were pooled and dialyzed against Buffer A and concentrated by freeze-drying. Isoenzymes of GSTs were separated on chromatofocussing columns, PBE 94 for anionic proteins and PBE 118 for cationic proteins, according to the instructions of the manufacturer.

Enzyme activity and substrate specificity assays

Glutathione transferases activity was measured spectrophotometrically at 340 nm by the method of Habig *et al*.(1974) with CDNB (1-Chloro-2,4-dinitro benzene) as substrate. One unit of enzyme activity was defined as the amount of enzyme that catalyses the formation of one mole of 2,4-dinitrophenol GSH product per minute. Specific activity was expressed as moles of GSH conjugate formed per minute per milligram of protein.

Glutathione transferases activity with several substrates was determined with purified proteins (Habig and Jakoby, 1981). The reactions were carried out using different substrates, buffers and initiators in a total volume of 3 ml, the blank reaction without enzyme was substracted from the test values and the activities were calculated using their respective molar extinction coefficients. All enzyme assays were carried out at 25 °C. Glutathione peroxidase (GPx) assay was carried out by monitoring the oxidation of Nicotinamide adenine dinucleotide phosphate (NADPH) in a recycling assay (Wendel, 1981). Total GPx activity was determined using cumene hydroperoxide (CHP) as substrate. Selenium-dependent GPx activity was measured using H_2O_2 as substrate.

Protein estimation

Protein contents were determined by the method of Lowry *et al*.(1951) using bovine serum albumin (BSA) as the standard and also by measuring absorbance at 260 nm and 280 nm (Warburg and Christian, 1941). Polyacrylamide gel electrophoresis (PAGE) was conducted according to the method of Laemmli (1970).

Immunological studies

Antisera were raised against affinity purified GSTs of rat testis, brain and liver isozymes by following the published procedure of Tu and Reddy (1985). Purified protein from affinity eluant was used for immunization of rabbits (New Zealand white male, 3 months old) prior to the injection of sample. Rabbits were obtained from poultry department, Acharya N.G. Ranga Agricultural University, Tirupati. Freund's complete and incomplete adjuvant were purchased from Sigma Chemical Company, ST Louis USA.

Brain and testicular affinity purified glutathione transferases separated on SDS-PAGE were screened with polyclonal antibodies raised against rat liver, brain and testis cytosolic glutathione transferases. Affinity purified GST protein 200 mg per milliliter was emulsified with an equal volume of Freund's complete adjuvant. The emulsified mixture was injected subcutaneously to the rabbit at 4 to 6 sites. The booster doses were given at interval of a week for about four to five times. The titre of antibodies was tested before the fifth dose. The last dose was given with an incomplete adjuvant. One week after the last injection, the rabbits were bled and the serum was prepared by centrifuging at 10000 r/min in a Himac refrigerated centrifuge for half an hour at 4 °C and the supernatant obtained was considered as antisera. Antiserum was raised against affinity purified glutathione transferases of rat liver, testis and brain.

Immuno diffusion

The Ouchterlony (1996) double immuno diffusion method was followed for the cross reactivity determination of antibody and antigen. The slides were prepared with 0.8% agarose in normal saline and kept in a humid chamber. The wells were punched on gels. The central well was loaded with antisera and the four encircling wells were with the specific affinity purified GST proteins as antigens. The precipitin bands were visualized by staining with 0.1% commassie brilliant blue R. 250 and upon destaining with methanol and acetic acid.

Western blotting (immunoblotting)

Immunoblot analysis was carried out on nitrocellulose membranes according to the published procedures of Towbin *et al*.(1979). The purified enzyme and cytosolic fractions were resolved by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The gels were initially soaked in 25 mmol/L Tris, 192 mmol/L Glycine and 20% Methanol. The separated peptides were transferred with a current of 0.8 mA/cm^2 for four hours.

After the transfer process, the membrane was air dried for a few seconds. Immediately the membrane was wetted in Tris buffer Saline (TBS) and thoroughly rinsed, then transferred into TBS containing 5% non-fat milk for 30 min or more to block the nonspecific binding sites. The membrane was immersed in TBST (Trisbuffer Saline with 0.05% Tween 20) with non-fat milk containing the primary antibody and incubated for 30 min. The unbound primary antibody was removed by washing with TBST, 3 times (5~10 min each). The membrane was incubated for 30 min in TBST with 5% non-fat milk containing secondary antibody linked to alkaline phosphatase then washed with TBST 5 times (5 min each) and then subjected to colour development with alkaline phosphatase kit, developed with 5-bromo-4-chloro-3-indolyl phosphate nitro blue tetrazolium (BCIP NBT) reagent.

RESULTS

Purification and characterization of GSTs of rat testis and brain

1. Affinity chromatography and chromatofocusing

The cytosolic extracts of rat testis and brain were loaded onto S-hexyl GSH linked agarose-4B affinity column and on elution the bound protein was eluted as indicated by a single sharp peak. The pooled active fractions were assayed for GST activity using CDNB. The specific activity was found to be 6.5 and 8.6 μmol/(min·mg protein) for testis and brain, respectively (Table 1 and Fig.1). The yield of GST protein was 39% in testis and 32% in brain. The affinity purified testis GST proteins were resolved into six anionic peaks (Fig.2 and Fig.3), while the brain samples were resolved into four anionic peaks on PBE-94 (Fig.4) chromatofocusing column at pH gradient 7 to 4. They were designated as anionic GSTs, t-1 to t-6 and b-1 to b-4, respectively, based on their elution order. The unbound protein peak eluted from PBE-94 was resolved into four cationic peaks in testis (Fig.5 and Fig.6) and three cationic peaks in brain samples (Fig.7) on chromatofocusing on PBE-118 column at pH gradient of 10.5 to 7. They were designated as cationic GSTs, t-7 to t-10 and b-5 to b-7, based on

Table 1 Isozyme profile of GSTs of rat testis and brain

Testis				Brain			
Sample	pI	Specific activity*	Subunit composition	Sample	pI	Specific activity*	Subunit composition
Crude	–	0.222	–	Crude	–	0.29	–
Affinity purified GSTs	–	6.50	Yc, Yb, Yβ, Yδ	Affinity purified GSTs	–	8.60	Yc, Yb, Yβ, Yδ
PBE-94 anionic isozymes				PBE-94 anionic isozymes			
GST t-1	6.20	5.17	Yc, Yb	GST b-1	7.00	0.20	Yδ
GST t-2	6.14	7.54	Yc, **Yb,** Yβ	GST b-2	6.80	0.46	**Y**δ**,** Yβ
GST t-3	6.00	0.96	**Yb**, Yβ	GST b-3	6.70	0.17	Yδ, **Y**β
GST t-4	5.80	0.14	Yb, Yβ	GST b-4	6.50	0.16	Yb, **Y**β
GST t-5	5.60	0.04	Yδ, Yβ				
GST t-6	5.40	0.90	Yβ				
PBE-118 cationic isozymes				PBE-118 cationic isozymes			
GST t-7	9.20	10.50	**Yc,** Yb	GST b-5	9.40	0.34	Yc
GST t-8	9.00	5.9	Yb, Yδ	GST b-6	8.50	0.81	Yc, Yb, Yβ, Yδ
GST t-9	8.60	15.9	Yc, Yb	GST b-7	8.40	0.18	Yb, Yβ, Yδ
GST t-10	7.90	29.85	Yb				

*One unit is defined as one μmol of GSH conjugate formed per minute per mg protein; Bold letters indicate the dominant subunit in the gel

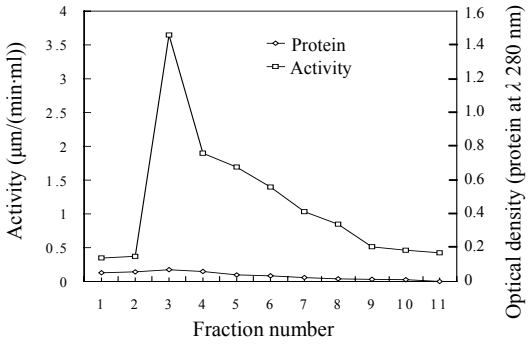

Fig.1 Typical elution profile of testis Glutathione transferases

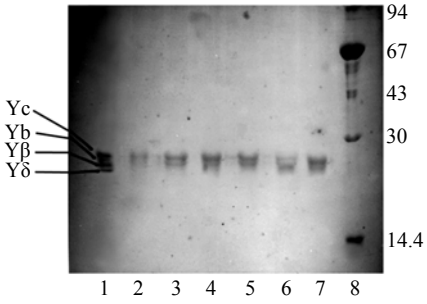

Fig.2 SDS-PAGE pattern of anionic GST isozymes of rat testis chromatofocused on PBE-94 column
Lane 1: Rat testis affinity purified GSTs; Lanes 2−7: Anionic GST isozymes t1−t6; Lane 8: Marker proteins

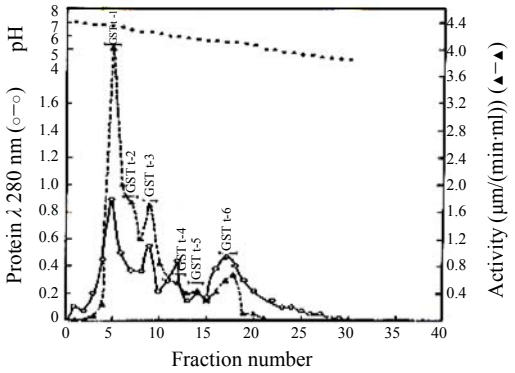

Fig.3 Polybuffer exchanger (PBE-94) chromatogram of rat testis GST anionic isozymes

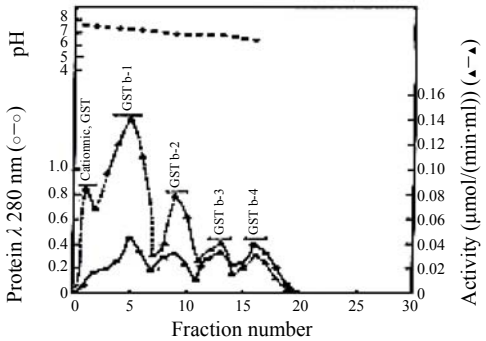

Fig.4 Polybuffer exchanger (PBE-94) chromatogram of rat brain GST anionic isozymes

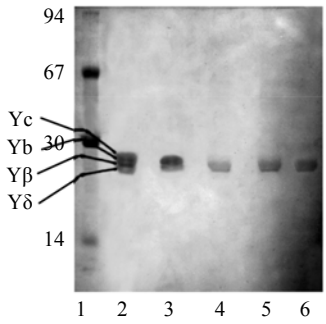

Fig.5 SDS-PAGE pattern of cationic GST isozymes of rat testis chromatofocused on PBE-118 column
Lane 1: Marker proteins; Lane 2: Rat testis affinity purified GSTs; Lanes 3−6: Cationic GST isozymers t7−t10

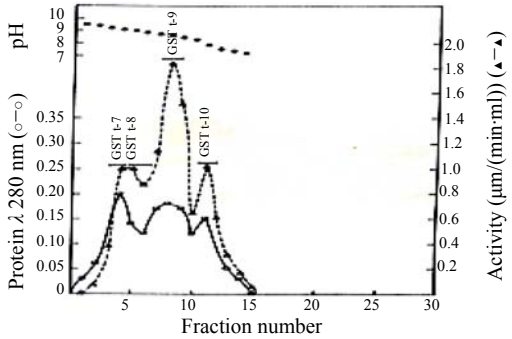

Fig.6 Polybuffer exchanger (PBE-118) chromatogram of rat testis GST cationic isozymes

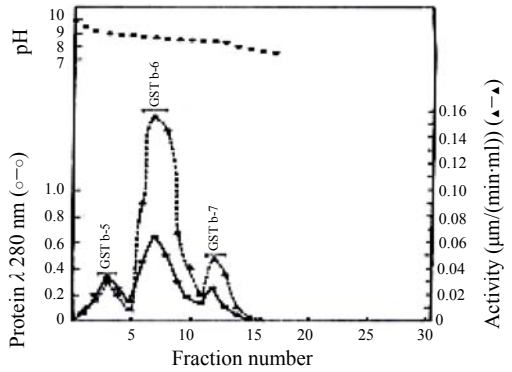

Fig.7 Polybuffer exchanger (PBE-118) chromatogram of rat brain GST cationic isozymes

their order of elution. The specific activities, total protein and pI values are given in Table 1.

2. Electrophoresis

GST isozymes were analysed by SDS-PAGE (Table 1). Affinity purified GSTs of rat testis and brain contained four subunits, Yc: 27500, Yb: 26300, Yβ: 26000 and Yδ: 24800 (Fig.8).

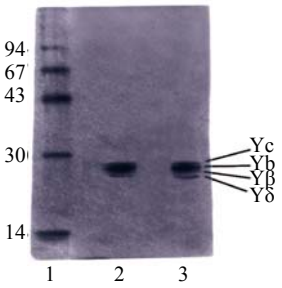

Fig.8 Testis and Brain affinity purified GSTs
Lane 1: Marker proteins; Lane 2: Testis GSTs; Lane 3: Brain GSTs

3. Substrate specificity

The specific activities of testis and brain GST isozymes with CDNB, *p*-NPA (*p*-Nitrophenyl acetate) and CHP as substrates are tabulated in Tables 2 and 3. Among all testis anionic isozymes maximum specific activity and yield was observed in GST t-2 and least in GST t-5. In brain, maximum specific activity was observed in cationic isozyme b-6 and least in b-4. GST t-4 and t-6 containing Yβ subunits with Yb as minor component, respectively, showed identical activity towards *p*-NPA. The GST t-1, t-7 and t-9 containing Yc and Yb subunits showed activities towards CHP. Neither anionic nor cationic GSTs of rat testis showed activity towards *p*-NBC (*p*-Nitrobenzyl chloride), BSP (Bromosulfo phthalein) and EPNP (1,2-Epoxy-3(*p*-nitro phenoxy propane)). In brain, GST b-5 and b-6 showed activity towards CHP containing Yc, Yb, Yβ and Yδ. GST b-1 containing Yδ and GST b-5 containing Yc showed activity towards EPNP. GST b-1, b-2 and b-3 showed activity towards *p*-NBC showing maximum Yδ subunits. GST b-4 containing Yb and Yβ showed activities towards *p*-NPA.

The pI values of rat testis and brain GSTs were determined by separating individual isozymes on chromatofocusing columns, PBE-94 and 118. Testis GSTs had pI between 9.2 and 5.4 and brain protein pIs ranged from 9.4 to 6.5 (Table 1).

Effect of phenobarbitol and β-methylcholanthrene on GSTs

Administration of PB and MC as single dose to rats resulted in increase in GST activity in testis by 1.32 and 1.16 fold and in brain by 1.48 and 1.41 fold as compared to control rats (n=6). Also, the GPx activities in testis were increased by 1.4 and 1.59 fold, respectively. In brain, they were increased by 1.23 and 1.9 fold respectively, as compared to controls. Multiple dose treatment with PB and MC showed that GST activity increased by 3.5 and 1.82-fold in testis and 1.89 and 1.39-fold in brain respectively compared to controls (Table 4). GPx levels were elevated by 5.35 and 4.17-fold in testis, and 4.49 and 5.11-fold in brain, respectively.

The specific activity of GSTs of rat testis and brain with the substrates CDNB, *p*-NPA, *p*-NBC, EPNP and BSP and GPx with CHP and H_2O_2 was determined (Table 4). In PB and MC treated samples, higher activity was observed with CDNB and *p*-NPA over *p*-NBC, BSP and EPNP. GPx levels were decreased with CHP and H_2O_2 in PB treated testis and brain samples. On exposure to MC, the largest activity variation was found towards BSP and EPNP. The testis GST activities with substrates CDNB, *p*-NPA, *p*-NBC, EPNP and BSP were found to be increased by 1.36, 1.9, 1.2, 2.0 and 1.5 fold of control, respectively, and in brain by 1.03, 1.42, 1.07, 1.54 and 1.86 fold of control, respectively. The GPx levels with substrates CHP and H_2O_2 increased by 2.18 and 2.13-fold in testis and by 1.27 and 1.83 in brain with respect to controls.

Immunology

Antisera against affinity purified GSTs of testis and liver of rats (raised in our laboratory) and brain Yδ GSTs, showed immunoprecipitin bands with affinity purified GST proteins of the respective tissues of rat. Cross-reactivities of control (i), PB (ii) and MC (iii) induced GST proteins of testis (A) and brain (B) as antigens with the antisera of affinity purified GSTs of testis and brain are given in Table 4. On immunoprecipitation with rat liver GST antisera, the control rat liver GST protein showed 3 bands, Yc, Yb and Ya (Fig.9) and control testis GST showed 3 bands Yc, Yb and Yβ. In treated proteins differential expression of subunits was observed in both brain and testis (Table 5). With testis GST antiserum (Table 5), the control testis, PB treated testis and brain and MC treated brain proteins showed four bands, and thus indicating induced expression of Yc, Yb, Yβ and Yδ subunits, respectively. However, induction of Yδ was larger than for the other three. Both dot and protein transblot immunoprecipitation analysis showed identical results for MC treated rats and in controls, Yc and Yβ bands were prominent.

Table 2 Substrate Specificities of GST isozyme of rat testis

Sample	Specific activity (μmol/(min·mg protein))		
	1-Chloro 2,4-dinitro benzene	*p*-Nitrophenyl acetate	Cumene hydroperoxide
Affinity purified GSTs	7.60	0.013	0.010
PBE-94 anionic isozymes			
GST t-1	5.17	ND	0.005
GST t-2	7.54	ND	ND
GST t-3	0.96	ND	ND
GST t-4	0.14	0.018	ND
GST t-5	0.04	ND	ND
GST t-6	0.90	0.019	ND
PBE-118 cationic isozymes			
GST t-7	10.50	ND	0.026
GST t-8	5.9	ND	ND
GST t-9	15.90	ND	0.048
GST t-10	29.85	ND	ND

ND: Not detectable activity (<0.001)

Table 3 Substrate Specificities of GST isozyme of rat brain

Sample	Specific activity (μmol/(min·mg protein))				
	1-Chloro 2,4-dinitro benzene	*p*-Nitrophenyl acetate	*p*-Nitrobenzyl chloride	1,2-Epoxy-3(*p*-nitro phenoxy propane)	Cumene hydroperoxide
Affinity purified GSTs	8.60	0.027	0.127	0.0960	0.144
PBE-94 anionic isozymes					
GST b-1	0.20	ND	0.025	0.0016	ND
GST b-2	0.46	ND	0.019	ND	ND
GST b-3	0.17	ND	0.033	ND	ND
GST b-4	0.16	0.02	ND	ND	ND
PBE-118 cationic isozymes					
GST b-5	0.34	ND	ND	0.0012	0.016
GST b-6	0.81	ND	ND	ND	0.019
GST b-7	0.18	ND	0.012	ND	ND

ND: Not detectable activity (<0.001)

Table 4 Effect of PB and MC treatment on the levels of GSTs and GPx of rat testis and brain with different substrates

Substrate	Control		PB treated		MC treated	
	Testis	Brain	Testis	Brain	Testis	Brain
1-Chloro 2,4-dinitro benzene*	253±5	20.63±0.5	335^{NS}±0.005	64^{b}±0.004	343^{b}±26	21.34±0.3
p-Nitrophenyl acetate*	139±40	34.5±4.0	380^{c}±0.03	47^{a}±0.002	265^{b}±20	49.14^{b}±2.0
p-Nitrobenzyl chloride*	551±10	10.52±0.5	63^{NS}±0.001	35^{d}±0.008	661^{b}±20	11.27±0.11
Bromosulfo phthalein*	18±9.0	22±1.6	70^{NS}±0.006	47^{c}±0.01	36^{a}±3.0	41^{c}±6.7
1,2-Epoxy-3(*p*-nitrophenoxy)propane*	12±5.0	4.05±0.22	35^{a}±0.0024	8.8^{b}±2.0	18.6±2.0	6.42^{a}±0.41
Hydrogen peroxide**	4.93±1.0	3.749±0.9	5.60^{d}±0.03	2.19^{b}±2.2	10.52^{c}±2.2	6.88^{c}±1.1
Cumene hydroperoxide**	15.43±2.8	18.94±3.5	10.8^{a}±0.05	4.3^{a}±3.0	33.75^{b}±3.0	24.11^{c}±1.5

*One unit is defined as nanomoles of GSH conjugate formed per minute per mg protein; **One unit is defined as nanomoles of NADPH oxidized per minute per mg protein; Values are average of three separate experiments of three samples (mean±*SD*). [a]: $P<0.001$, [b]: $P<0.01$, [c]: $P<0.02$; [d]: $P<0.05$; [NS]: Not significant (*T*-test). Doubling the dose per day per 100 gm body weight

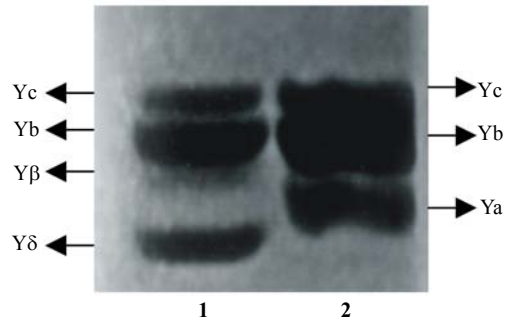

Fig.9 Testis and liver affinity purified GSTs
Lane 1: Testis GSTs; Lane 2: Liver GSTs

Table 5 Effect of PB and MC on subunit composition of GSTs of brain and testis Cytosols as evidenced by immunoprecipitation with rat liver and testis GST antibodies

Sample	Subunit composition precipitated with anti-GST of	
	Rat liver	Rat testis
Control liver	Yc, Yb, Ya	Yc, Yb
Control testis	Yc, Yb, Yβ	Yc, Yb, Yβ, Yδ
Control brain	Yc, Yb, Yβ	Yc, Yb, Yβ, Yδ
PB testis 24 mg	Yc, Yβ	Yc, Yb, Yβ, **Yδ**
PB brain 24 mg	Yc	Yc, Yb, Yβ, **Yδ**
MC testis 24 mg	**Yc**, Yb, **Yβ**	**Yc**, Yb, **Yβ**
MC brain 24 mg	**Yc,** Yb, Yβ	**Yc**, Yb, Yβ, **Yδ**
GST b4-isozyme	Yc, Yβ	Yc, Yβ

Bold letters indicate the dominant precipitated bands in the gel

DISSCUSSION

Rat GSTs are encoded by a multigene family and are differentially regulated in a tissue specific manner (Abramovitz *et al*., 1988) to meet the special detoxication needs of various organs (Awasthi and Singh, 1985). The isoforms may be distinguished on the basis of differences in binding specificities and catalytic properties toward various molecules (Tahir and Mannervik, 1986). The existence of various GSTs and their specific activities vary tissue specifically from one to another, hence the present study was conducted to observe the GSTs variability of rat brain and testis tissues. The testis and brain cytosols have high GST activity but their isozyme pattern differs from each other with respect to the species as well as organ (Guthenberg *et al*., 1983).

Lung cationic and anionic isozyme patterns in sheep were observed (Thyagaraju *et al*., 1994) to be separated in the same way as the affinity purified rat testis GSTs were separated (into six anionic and four cationic isozymes) and as the affinity purified rat brain GSTs were separated (into four anionic and three cationic isozymes). Maximum (68%) GST activity was associated with anionic isozymes and less (32%) with cationic isozymes in the brain. Different from brain GST, the testis GST was 65% cationic isozymes and 35% anionic isozymes. Our results on rats indicated that the cationic isozymes in testis and anionic isozymes in brain are abundant.

The basic GSTs in rat brain and testis showed very high activity towards CHP. Two brain isozymes with pI values of 9.4 (GST b-5) and 8.5 (b-6) and three rat testis isozymes 9.2 (t-7), 8.6 (t-9) and 6.2 (t-1), which are hetero dimers of Yc subunits showed more not-selenium dependent GPx activity. The GPx activity associated with cationic GSTs in brain and testis should protect the brain and testis tissues from oxidative stress (Chang *et al*., 1987; Thyagaraju *et al*., 1996).

Rat brain isozymes with pI values of 7 (GST b-1), 6.8 (b-2) and 6.7 (b-3) showed activity towards *p*-NBC; on the other hand rat testis isozymes showed little or no activity towards this substrate. The earlier reports of Singh *et al*.(1987) showed that rat brain GST 6.6, 6.1 and 5.7, which are dimers of Yb showed maximum activity towards *p*-NBC. This study showed that the isozymes of testis and brain are different at these pI values. The rat brain and testis isozymes having the pI values of 6.4 (b-4), 5.8 (t-4) and 5.4 (t-6), showed activity towards *p*-NPA, studies of which that some of the isozymes at these pI values have identical proteins. The brain isozymes 9.4 (GST b-5) and 7.0 (b-1) showed activity towards the substrate 1,2-Epoxy-3(*p*-nitrophenoxy) propane (EPNP). These results accord with earlier reports of Thyagaraju *et al*.(1996) that the brain cationic isozymes showed activity towards EPNP. EPNP under nuclophilic attack on a strained oxirane ring are preferentially catalyzed by μ and θ class GSTs, respectively (Ketterer *et al*., 1987). However, in testis microsomal GSTs may show activity towards EPNP.

The existence of cytosolic GSTs as homo, hetero, di and trimers was reported in rat tissues by Thyagaraju *et al*.(1996), and Mannervik and Danielson (1988). This type of combination of subunits in GST may occur due to the exchange of gene product at the level of transcription and translation and may offer

protection from various xenobiotics (Kaplowitz *et al*., 1975) and even with slightly modified xenobiotics. Gene conversion may be an important mechanism for generating sequence diversity in the GST multigene family. The generation of sequence divergence between members of related genes allows the expression of protein with broad overlapping substrate specificities (Morton *et al*., 1990). These mechanisms should influence the GST proteins to have either different or identical functions with testis and brain GST proteins at a single pI value.

The Ya subunit is absent in rat brain and testis (Li *et al*., 1986; Devi *et al*., 2002b) (Fig.8). The brain GSTs contained more of Yb subunits, which are separated at near neutral points on chromatofocusing. Both brain and testis GSTs comprised more of Yb and Yβ subunits, which may indeed play a pivotal role in the detoxication process. The testis cationic GSTs have more GPx activity than the brain isozymes. The existence of extra Yβ and Yδ subunits in testis accords with earlier reports of Li *et al*.(1986).

Many detoxication enzymes exist as isozymes with multiple genes and exhibit differential expression with a wide variety of xenobiotics to prevent chemical lesions such as mutagenesis, carcinogenesis and tissue necrosis (Guthenberg *et al*., 1980). GSTs exhibit differential expression to a wide variety of xenobiotics. In this study the activity levels of GST and the GSH-mediated peroxidases, were increased significantly in both brain and testis on exposure to PB and MC in multiple doses. This result accords with earlier reports that GST levels were induced in liver, small intestine and kidney by intraperitoneal administration of Polynuclear Aromatic Hydrocarbons (PAH) and PB (Clifton and Kaplowitz, 1978). Therefore the induction of GST serves to overcome the deleterious effects caused by PB and MC. This was further confirmed by immunological studies.

Variation in activity levels of GST in testis and brain accords with earlier studies of Kaplowitz *et al*.(1975), and further showed that the GST activity was variable towards CDNB after PB and MC treatment. The classes of GST isozymes differed in their specificity toward xenobiotic or endogenous substrates such as PB or MC. CDNB is the main substrate, which undergoes nucleophilic displacement of the chloro moiety by GSH. All classes (α, μ, π) of GSTs except θ catalyze this reaction. The most characteristic feature of testicular GSTs is their association with approximately 40% of total cytosolic activity with proteins having isoelectric points below pH 7 (Guthenberg *et al*., 1980). These results were further confirmed by Western blot analysis. Cytosol of PB and MC treated testis showed cross reactivity with Yc and Yβ subunits. However, it is differed on immunoblot analysis of testicular and brain GST with testis GST antibodies. The MC treated testis samples showed the induction of Yc and Yβ subunits prominently while brain samples showed only Yc subunit prominently. In PB treated testis Yc, Yb, Yβ and Yδ were induced with maximum induction of Yδ (Thyagaraju *et al*., 2004). The induction of specific isozymes in rat testis and brain was also confirmed by dot blot analysis using a GST subunit specific antiserum. These results indicated that Yc, Yb and Yβ subunits of rat testis and brain GST have sequence homology with rat liver Yc and Yb GSTs respectively, because these proteins showed immunoprecipitation with rat liver antisera. Whereas testis GST antisera showed immunoprecipitation of Yδ subunit in control, treated rat testis and brain (but not liver) to indicate that Yδ is not related to any one of the subunits of rat liver GSTs (Fig.9).

In PB treated testis and brain of rats four fold increase in GST activity elicitation of π (Yδ) class was observed. GST π is an acidic isozyme and is expressed in high concentrations in chemically induced preneoplastic rat hepatocyte nodules (Kitahara *et al*., 1984; Rushmore *et al*., 1987) and in rat primary hepatomas (Meyer *et al*., 1985). The elevated levels of π class GSTs (Yδ) on PB treatment of testis indicates increased carcinomas in testis. The π form is not expressed in normal spermatogonia but is over expressed in germ cell neoplasia and can be used as a marker of germ cell cancer (Klys *et al*., 1992). The GST π class is absent in normal liver of rats (Fig.9) and is induced during carcinogenesis (Howie *et al*., 1989). It is present in brain and testis as Yδ, and in placenta as Yp (Fig.8). This protein is induced by phenobarbitol in testis, brain and liver. Hence Yδ belonging to π-class GST may be used as a marker protein for detoxication of specific tissues.

The over expression of α (Yc) and μ (Yβ) class GSTs in MC treated testis in rapidly multiplying cells indicates that they can act as markers of neoplasia and thereby play a role as chemo protector. Additionally,

over expression of α GSTs in brain cells enhances their protection from necrotic toxicity produced by MC. Elevated α GSTs have been found in plasma of patients with hepatocellular damage caused by hypoglycemia (Aliya *et al*., 2003), birth asphyxia (Beckett *et al*., 1989), or autoimmune chronic hepatitis (Hayes *et al*., 1988). These study results have been related to acute or chronic liver disease and renal damage (Beckett and Hayes, 1993; Feinfeld *et al*., 1981). The α GSTs can also be used for successful intervention to alleviate rejection following transplantation, thereby demonstrating the important clinical use of monitoring GST levels in biological tissues (Trull *et al*., 1994). The α (Yc) and μ (Yb, Yβ) classes induced by PB and MC should inhibit the initiation of chemical carcinogenesis by detoxication process and thereby act as chemo protector. Elevated levels of α, μ and π GSTs have been associated with protection of tissues from cytotoxicity produced by PB and MC. Therefore, measurement of GST α, μ and π class expression have clinical benefit for monitoring therapeutic progression of cancerous disease or identification of populations susceptible to chemotherapeutic interventions.

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