·*Original Article*·

Novel functional association of rat testicular membrane-associated cytosolic glutathione S transferases and cyclooxygenase *in vitro*

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Abstract

Aim: To analyze the role of cytosolic glutathione S-transferases (cGSTs) and membrane-associated cytosolic GSTs (macGSTs) in prostaglandin biosynthesis and to evaluate the possible interaction between glutathione S-transferases (GSTs) and cyclooxygenase (COX) *in vitro*. **Methods:** SDS-PAGE analysis was undertaken for characterization of GSTs, thin layer chromatography (TLC) to monitor the effect of GSTs on prostaglandin biosynthesis from arachidonic acid (AA) and spectrophotometric assays were done for measuring activity levels of COX and GSTs. **Results:** SDS-PAGE analysis indicates that macGSTs have molecular weights in the range of 25–28 kDa. In a coupled assay involving GSTs, arachidonic acid and cyclooxygenase-1, rat testicular macGSTs produced prostaglandin E_2 and $F_{2\alpha}$, while the cGSTs caused the generation of prostaglandin D_2, E_2 and $F_{2\alpha}$. *In vitro* interaction studies on GSTs and COX at the protein level have shown dose-dependent inhibition of COX activity by macGSTs and vice versa. This effect, however, is not seen with cGSTs. The inhibitory effect of COX on macGST activity was relieved with increasing concentrations of reduced glutathione (GSH) but not with 1-chloro 2,4-dinitrobenzene (CDNB). The inhibition of COX by macGSTs, on the other hand, was potentiated by glutathione. **Conclusion:** We isolated and purified macGSTs and cGSTs from rat testis and analyzed their involvement in prostaglandin biosynthesis. These studies reveal a reversible functional interaction between macGSTs and COX *in vitro*, with possible interactions between them at the GSH binding site of macGSTs. *(Asian J Androl 2005 Jun; 7: 171–178)*

1　Introduction

Glutathione S-transferases (GSTs EC 2.5.1.18) are a group of multigene, multifunctional proteins that catalyze glutathione (GSH)-dependent reactions like

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conjugation, isomerization and reduction as part of the cellular detoxification mechanism of extracellular xenobiotics and biotransformation of intracellular toxicants like the lipid peroxide. In addition they have non-catalytic binding functions by virtue of which they play an important role in intracellular binding and transport of bilirubin, steroid hormones and numerous drugs [1].

GSTs are grouped broadly into cytosolic GSTs (cGSTs) (Alpha, Mu, Pi, Sigma, Theta, Zeta and Omega classes with molecular masses of 22–27 kDa), mitochondrial GSTs (mGSTs) (Kappa class with a molecular mass

of about 25 kDa), membrane-associated cytosolic GSTs (macGSTs) (that are genetically identical to the cytosolic transferases); and mGSTs (now called membrane-associated proteins in eicosanoid and glutathione metabolism [MAPEGs], of which there are six isoenzymes with molecular masses of 14–17 kDa that have been divided into three classes). GSTs exist as homo- or hetero-dimers with each subunit having a molecular mass of 14–29 kDa. Each monomer has two domains: the smaller G-site or the GSH binding site and the larger H-site for binding the electrophilic substrate [2].

GSTs play an important role in arachidonic acid (AA) metabolism by virtue of their peroxidase activity, commonly referred to as non-selenium glutathione peroxidase activity. The initial process in AA metabolism is the release of AA from membrane phospholipids in a reaction catalyzed by phospholipases. Subsequently, free AA can be processed via the lipoxygenase pathway leading to the formation of leukotrienes (LTs) or the cyclooxygenase pathway leading to the production of prostaglandins (PGs). The initial step in PG production is the formation of an unstable PGH_2 intermediate from AA by the action of an enzyme, PG endoperoxide synthase, also called cyclooxygenase (COX). COX-1 and COX-2, the two distinct COX isoenzymes, with differential regulation are reported to be expressed in various tissues including rat testes [3]. Various GST isoenzymes like Alpha, Mu and Pi classes have been implicated in the conversion of PGH_2 to a mixture of PGD_2, PGE_2 and $PGF_{2\alpha}$. Earlier reports have indicated an interaction between microsomal GSTs and leukotriene C_4 (LTC_4) synthase, a microsomal enzyme involved in peptido leukotriene biosynthesis, both *in vitro* and *in vivo* and that such interactions reduced the activity of both enzymes [4, 5].

As GSTs play an important role in the production of PGs via the COX pathway, we conceived that there might be a possible functional interaction between COX and GSTs. The present study is designed to analyze the role of affinity purified rat testicular cGSTs and macGSTs in PG production in testes and to study the putative interaction between GSTs and COX.

2 Materials and methods

2.1 Chemicals and animals

Phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), triton X-100, 1-chloro 2,4 dinitrobenzene (CDNB), GSH, diethyldithiocarbamate (DTC), N,N,N', N'-tetramethyl-p-phenylenediamine (TMPD), nordihydroguaretic acid (NDGA) and hematin were purchased from Sigma Chemicals (St Louis, USA). DE-52 material is from Whatman and prostaglandin standards are from Cayman Chemicals (Ann Arbor, USA). Tris, sucrose and other chemicals were purchased from Sisco Research Laboratories (Mumbai, India). Rats, one month old, were purchased from the animal house facility of the National Institute of Nutrition (NIN), Hyderabad, India.

2.2 Processing of testicular tissue for GSTs

Testicular tissue from six 1-month-old Wistar strain male rats were dissected, thoroughly washed in saline, minced and a 20 % homogenate was made in 10 mmol/L potassium phosphate buffer (pH 7.0) containing 1 mmol/L EDTA, 1 mmol/L PMSF, 1 mmol/L DTT and 250 mmol/L sucrose in a glass homogenizer. All the steps in the processing of the tissue after dissection were done at 4 °C. The homogenate was centrifuged at $10\,000 \times g$ for 15 min and the resulting supernatant was subjected to ultra centrifugation at $105\,000 \times g$ for 1 h. The resultant supernatant was used as the cytosolic source of the enzyme. The pellet was then thoroughly washed and treated with trypsin (0.1 % final concentration) for 10 min and trypsinization was stopped with soybean trypsin inhibitor (0.1 % final concentration) and again centrifuged at $105\,000 \times g$ for 1 h. The pellet was then dissolved in the homogenizing buffer containing a final concentration of 1 % triton X-100 and used as the microsomal source of the enzyme. GSH affinity matrix was prepared as described earlier [6]. The cytosolic and microsomal fractions of the rat testes were dialyzed extensively against 10 mmol/L potassium phosphate buffer overnight. The dialyzed samples were spun at $10\,000 \times g$ for 10 min and were then loaded on to the affinity column pre-equilibrated with 10 mmol/L phosphate buffer (pH 7.0). The column was washed thoroughly with the same buffer containing 0.15 mol/L KCl (pH 7.0) till the absorbance at 280 nm dropped to zero. The affinity bound GSTs were eluted with 50 mmol/L potassium phosphate buffer pH 7.5, containing 10 mmol/L GSH and 1 mL fractions were collected. Active fractions were pooled and dialyzed against 10 mmol/L phosphate buffer overnight with three buffer changes to remove GSH and then concentrated by lyophilization.

2.3 Protein determination

Protein content in the crude preparations was measured by folin-phenol method [7] and in chromatographic fractions was determined spectrophotometrically by measuring the absorbance at 280 nm and 260 nm.

2.4 SDS-PAGE

Protein samples were mixed at a ratio of 1:1 with sample buffer (0.2 mmol/L Tris, 8 % SDS [w/v], 40 % glycerol, 20 % 2-mercaptoethanol [v/v] and 0.2 % bromo phenol blue [w/v]), boiled for 3 min, loaded and separated on a 10 % SDS gel, fixed and stained with silver nitrate [8].

2.5 Assay for GST activity

GST activity was assayed by the conventional method [9] in which the typical reaction mixture in a volume of 1 mL of 100 mmol/L phosphate buffer pH 6.5, contained 1 mmol/L CDNB and 1 mmol reduced glutathione. The reaction was initiated by the addition of enzyme. The thioether formation was determined by reading the absorbance at 340 nm and quantification was done using the molar extinction coefficient of CDNB (9.6 mmol/L per cm). One Unit of enzyme activity was defined as one micromole of thioether formed per min and the specific activity was expressed as units per mg protein. For the determination of the effect of COX on GST activity, various concentrations of COX (0, 10, 100, 150 µg/mL) were incubated with cGSTs/macGSTs (100 µg/mL) at 4 ℃ for 3 min prior to the initiation of the reaction. In order to test the effect of GSH and CDNB on the interaction of macGSTs and COX, various concentrations of GSH (1, 1.5, 2, 2.5, 3 µmol/L) and CDNB (1, 1.5, 2, 2.5, 3 µmol/L) were incubated with GST assay mixture containing 100 µg of macGST and 150 µg of COX.

2.6 Processing of tissue for Cyclooxygenase-1

Ram seminal vesicles (60 g), a rich source of COX-1, were used as the enzyme source for cyclooxygenase. The tissue was homogenized in 100 mmol/L Tris HCl (pH 8.0) containing 0.5 mmol/L EDTA, 300 µmol/L DTC and 100 µmol/L NDGA and centrifuged at $7000 \times g$ for 15 min at 4 ℃. The supernatant was further centrifuged at $105\,000 \times g$ for 1 h at 4 ℃. The pellet was solubilized in homogenization buffer containing 1 % triton X-100 and then centrifuged at $105\,000 \times g$ for 1 h as described above and the supernatant used as the source of enzyme. The solubilized microsomal fraction was loaded onto anion exchange (DE-52) column equilibrated with 50 mmol/L Tris and 5 mmol/L EDTA at 4 ℃ and the flow through was collected, dialyzed overnight extensively and used as the source of enzyme [10]. The COX, thus obtained was more than 90 % pure, as evidenced by SDS-PAGE.

2.7 Assay for the activity of cyclooxygenase

Cyclooxygenase activity was measured spectrophotometrically using TMPD [11]. The activity was expressed as change in absorbance/min and the specific activity as change in absorbance/min $\times$ mg protein. For the determination of the effect of GST on COX activity, various concentrations of GSTs (0, 5, 10, 20 µg/mL) were incubated with COX (100 µg/mL) at 4 ℃ for 3 min prior to the initiation of the reaction.

2.8 Assay for GSTs-catalyzed prostaglandin formation

GSTs-catalyzed prostaglandin biosynthesis was measured in a coupled assay involving COX-1 and AA. The prostaglandin H$_2$ formed *in situ* will form the substrate for GSTs. The reaction was carried out in a buffer containing 100 mmol/L Tris HCl (pH 8.0), 5 mmol/L GSH, 1 µmol/L hematin and 5 µmol/L tryptophan and 50 µg GST and 150 µg of COX enzyme. The reaction was initiated by the addition of AA with a final concentration of 133 µmol/L and allowed to proceed for 5 min at room temperature and the reaction was terminated by the addition of 6 mol/L HCl. The products were extracted twice into ethyl acetate and petroleum ether (1:1) precooled to –20 ℃, evaporated and redissolved in ethyl acetate and separated on TLC along with the standards, on a mobile phase of water: saturated ethyl acetate: acetic acid: 2,2, 4-trimethyl pentane (51:110:25:50) at 4 ℃ for 1 h and the color was developed with iodine vapors. Individual PGs formed were identified in comparison with PG standards on TLC plates and quantified by measuring the density of signal per pixel of the scanned TLC plates.

2.9 Statistical analysis

Statistical analysis was done using the paired Student's *t*-test and the significance was set at $P < 0.05$.

3 Results

GSTs were isolated and purified from rat testicular cytosolic and microsomal fractions by employing GSH affinity column. The GSH affinity purified rat testicular cGSTs had a specific activity of 67.4 Units/mg protein.
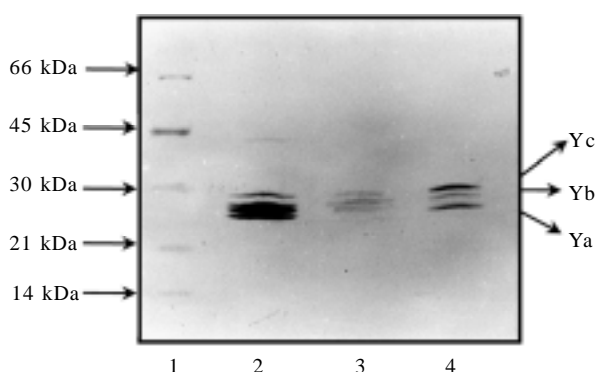
Figure 1. SDS-PAGE analysis of rat testicular GSTs. GSH-affinity-purifiedrat testicular cGSTs and macGSTs were separated on 12 % gel. Lane 1: molecular weight markers; Lane 2: affinity-purified GSTs from testis cytosol (20 µg protein); Lane 3: affinity-purified GSTs from testis microsomes (6 µg protein); Lane 4: Affinity-purified GSTs from rat liver cytosol (10 µg protein). Ya, Yb, Yc are different subunits of the GSTs.

When separated on SDS-PAGE cGSTs resolved into three bands with molecular weights ranging from 25–28 kDa (Figure 1, lane 2). The affinity purified GSTs from rat testicular microsomes had a specific activity of 41.1 Units/ mg protein, with molecular weights very similar to those of cGSTs (Figure 1, lane 3). Also the GSTs purified from microsomes cross-reacted with polyclonal antibodies raised against rat liver cGSTs (data not shown), showing their close similarity with cGSTs. In view of their close similarity with cGSTs in terms of molecular weights and immunological cross reactivity, these affinity purified rat testicular mGSTs were termed as macGSTs (macGSTs) as per the recent nomenclature [2].

GST isozymes are known to exhibit distinct differences in their catalytic rates in the formation of classical PGs [12, 13]. In the present study *in vitro* coupled assays were carried for the generation of PGs by the incubation of affinity purified GSTs with COX-1 from ram seminal vesicles and AA as the substrate. The PGs formed were extracted and separated by thin layer chromatography (TLC) as described in methodology. While the reaction mixture with cGSTs generated PGD_2, PGE_2 and $PGF_{2\alpha}$, the macGSTs preferentially caused the production of PGE_2 and $PGF_{2\alpha}$ (Figure 2A). The relative concentration of PGD_2 was much higher in the presence of cGSTs, followed by PGE_2 and $PGF_{2\alpha}$. No detectable PGD_2 was formed in the presence of macGSTs. The PGE_2 and $PGF_{2\alpha}$ formed in the presence of macGSTs were in
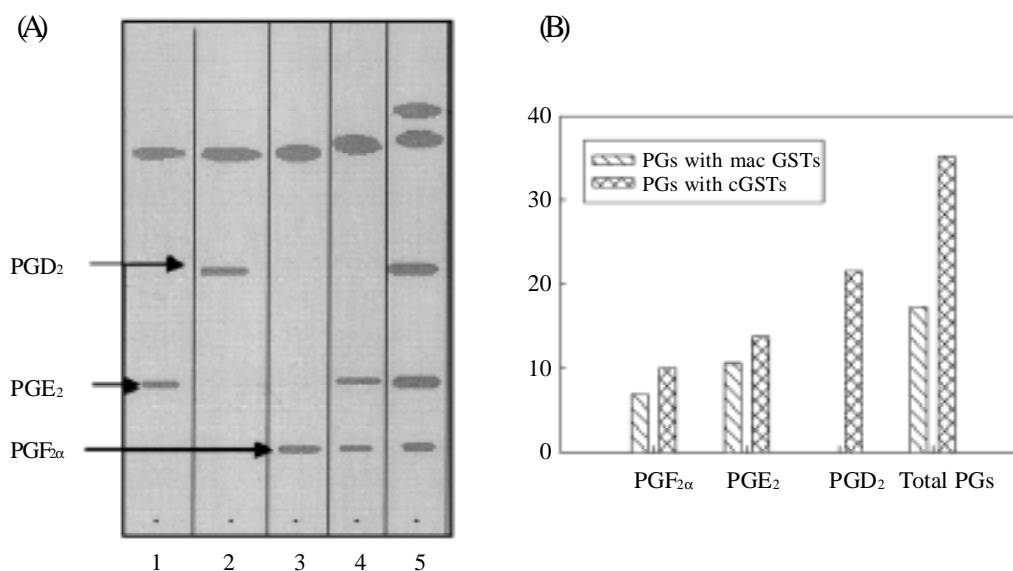

Figure 2. TLC separation of prostaglandins formed from cyclooxygenase in the presence of GSTs from rat testis. (A): TLC separation of various PGs formed by GSTs in a coupled reaction with COX-1 (150 µg) using arachidonic acid as the substrate. Lane 1: PGE_2 standard; Lane 2: PGD_2 standard; Lane 3: $PGF_{2\alpha}$ standard; Lane 4: PGs formed with macGSTs (50 µg) from rat testis; Lane 5: PGs formed with cGSTs from rat testis. (B): Bar diagram showing the relative intensities of the TLC bands shown in (A) (lanes 4 and 5). Each value is the mean of at least six different observations.

equal concentrations. The total PGs generated with macGSTs, however, were much lower in comparison to those of cGSTs (Figure 2B).

The affinity-purified GSTs were employed for further studies on interactions with COX. Incubation of COX with macGSTs resulted in a dose dependent inhibition of COX activity with 50 % inhibition at a concentration of 10 μg of macGSTs/mL (Figure 3A). The cytosolic GSTs, however, did not show any significant effect on COX activity (Figure 3B). Incubation of COX with GSH at 5 μmol/L concentration showed no significant effect on COX activity, but a significant inhibition was observed at higher concentration (10 μmol/L)

(Figure 3C). The combination of mac GSTs (10 μg/mL) and GSH (5 μmol/L), however, had synergistic effect with nearly 70 % inhibition of COX activity (Figure 3D).

Similarly the enzymatic activity of GSTs, upon incubation with COX was determined. More than 50 % inhibition of macGSTs activity was observed with 100 μg of COX (Figure 4A). Hematin, a cofactor required for COX activity, had no effect on the activity of macGSTs (Figure 4B). No inhibitory effect of COX was observed on cGSTs at all the concentrations studied (Figure 5).

We further analyzed the inhibition of macGSTs activity by COX in the presence of increasing concentra-
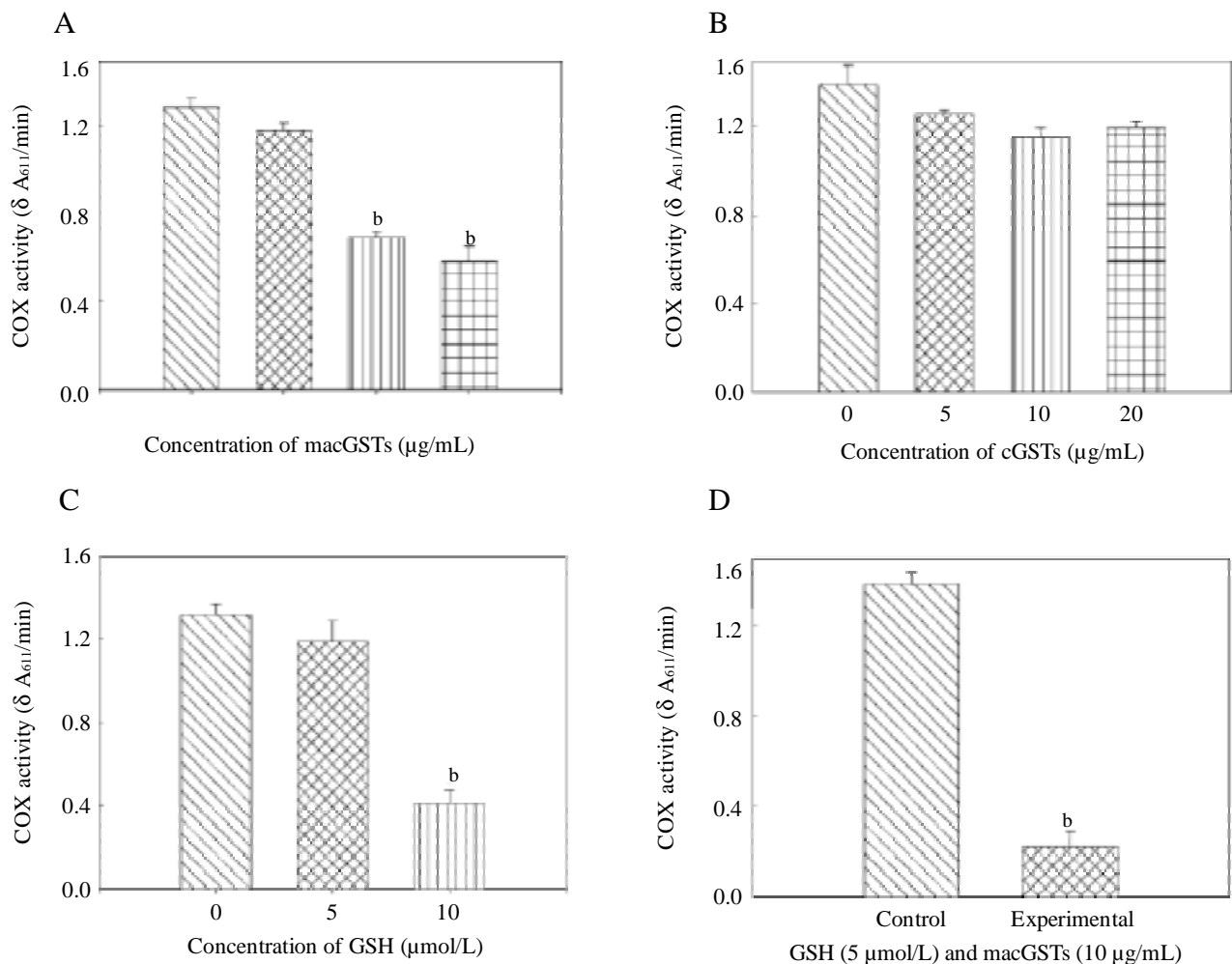

Figure 3. Effect of GSTs and GSH on cyclooxygenase activity. The effect of GSTs and GSH on the activity of COX (100 μg) was studied by incubating COX enzyme with different concentrations of (A) macGSTs, (B) cGSTs, (C) GSH, and (D) 5 μmol/L GSH and 10 μg/mL macGSTs. The activity of COX was measured spectrophotometrically using arachidonic acid as the substrate. COX activity was expressed as δA_{611}/min. Each value is the mean ± SD of at least six different observations. $^{b}P<0.05$, compared with the control.
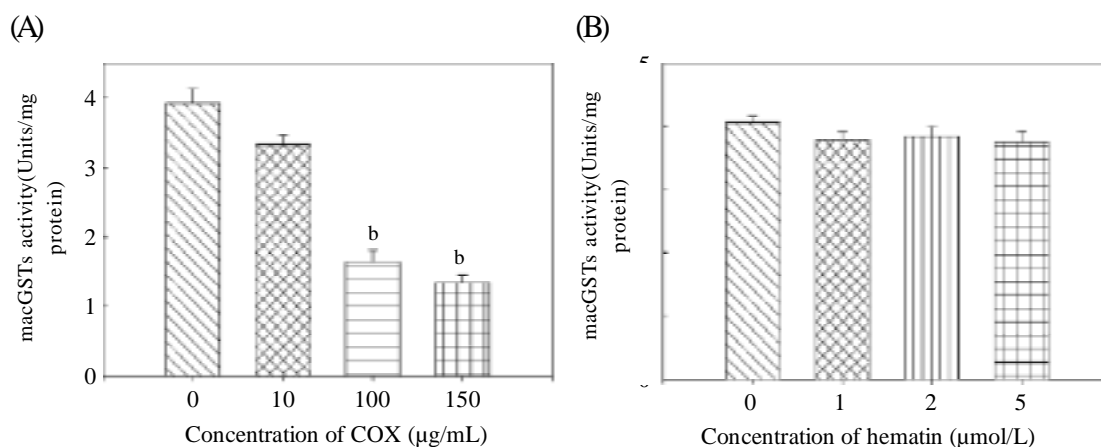
(A)

(B)

Figure 4. Effect of cyclooxygenase and hematin on macGST's activity. Effect of (A) COX and (B) hematin on macGSTs was studied by incubating macGSTs (100 µg) with different concentrations of COX and hematin. Activity of GSTs was measured spectrophotometrically by using CDNB as substrate and expressed in Units/mg protein, where one Unit is defined as one micromole of thioether formed per minute. Each value is the mean ± SD of at least six different observations. [b]$P < 0.05$, compared with the control.
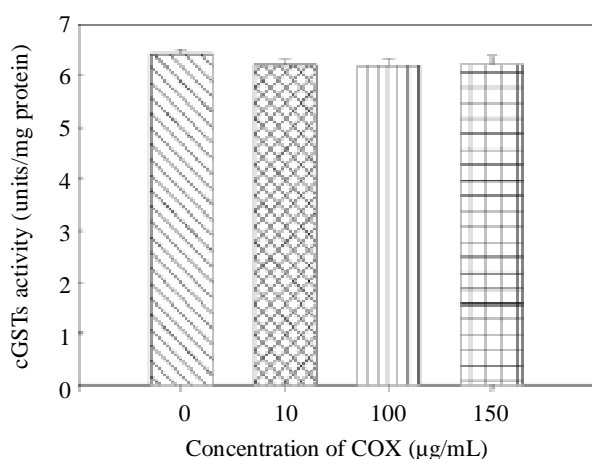

Figure 5. Effect of cyclooxygenase on cGST activity. Effect of COX on cGSTs was studied by incubating cGSTs (100 µg) with different concentrations of COX. Activity of GSTs was measured spectrophotometrically by using CDNB as substrate and expressed in Units/mg protein, where one Unit is defined as one micromole of thioether formed per minute. Each value is the mean ± SD of at least six different observations.

tions of GSH and CDNB. The increasing concentrations of GSH reduced the inhibitory effect of COX on macGSTs activity (Figure 6A) but no effect was observed with CDNB at all the concentrations studied (Figure 6B).

4 Discussion

In the present study, the molecular mass of the GSTs

purified from rat testicular microsomes is closer to the cGSTs (25–28 kDa), but distinct from those of the microsomal GSTs, now called MAPEGs that do not bind to the GSH affinity column [14]. However, the different pI values, indicate that the cGSTs and mGSTs were indeed different and that the presence of GSTs similar to cGSTs in the microsomes is not due to any contamination (data not shown). As these GSTs are closely associated with microsomal membranes but distinct from MAPEG family members, they were designated as macGSTs [2]. The precise role of these macGSTs in testes is not clear. A recent report has shown that sheep liver microsomes have GSTs similar to cGSTs and exhibit glutathione peroxidase activity [15].

Since various GST isozymes are known to influence the type of PGs formed from the unstable PGH_2 intermediate, it is conceivable that the distribution of GSTs in different compartments of testis can influence the type of PGs formed. In the present study also cGSTs and macGSTs showed differential pattern of PGs formed with the overall yield of PGs being lower with macGSTs. This decreased level of PGs formed with macGSTs suggests their possible regulation on PG biosynthesis. The inhibition in the COX activity by macGSTs observed in the present study supports such a possibility.

The regulation of COX activity appears to be unique for macGSTs as cGSTs showed no such effect. Also the interaction is dependent on GSH as the inhibition of COX by macGSTs was potentiated by 5 µmol/L GSH. However, the precise role of GSH in potentiating the in-
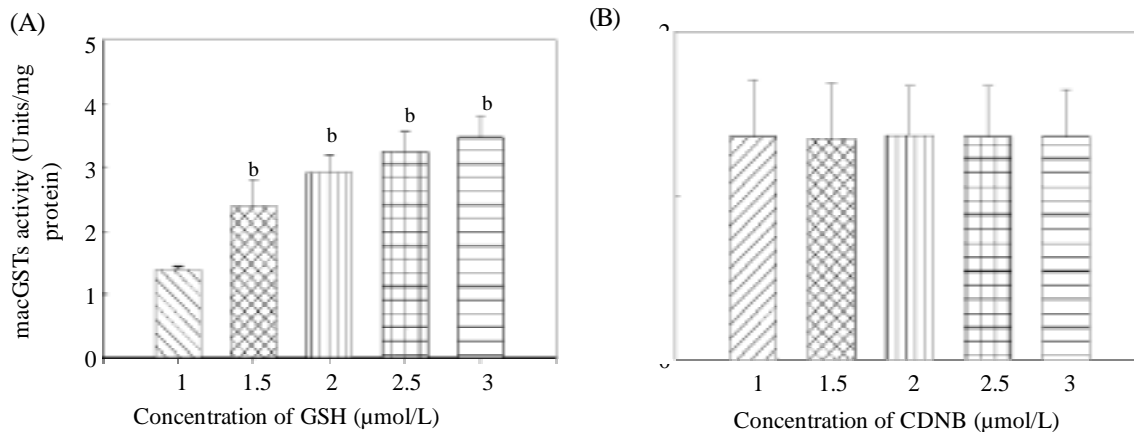
Figure 6. Effect of increasing concentrations of GSH and CDNB on inhibition of macGSTs by cyclooxygenase. Effect of GSH and CDNB on the inhibition of macGSTs by COX was studied by incubating macGSTs (100 µg) with increasing concentrations of (A) GSH and (B) CDNB along with fixed concentration of COX enzyme (150 µg). The activity of GSTs was measured spectrophotometrically using CDNB as substrate. Each value is the mean ± SD of at least six different observations. [b]$P<0.05$, compared with 1 µmol/L GSH.

hibitory effects of macGSTs on COX is not clear. One possibility is the reduction of fatty acid hydroperoxides, which are essential for COX activity, by the reported peroxidase activity of macGSTs [15]. There appears to be a competition between GSH and COX for GSH binding site on macGSTs, with higher affinity probably for GSH.

Similar protein-protein interaction of enzymes associated with eicosanoid and glutathione metabolism, better defined as MAPEG, has been reported [4]. The interaction reported in the present study, however, appears to be different from those of MAPEGs as it is associated with the regulation of prostanoid biosynthesis unlike the MAPEGs which regulate leukotriene biosynthesis. Also GSTs of the MAPEG family have subunits of about 18 kDa, whereas macGSTs in the present study have subunit molecular weights in the range of 25–28 kDa.

Inhibition of macGSTs, but not that of cGSTs, by COX indicates the possible interaction between the two at their respective active sites. Since macGSTs and COX are microsomal proteins and hydrophobic in nature, there could be hydrophobic interaction between the two proteins. The cyclooxygenase reaction occurs within a hydrophobic channel that extends from the membrane-binding domain of the enzyme into the core of the globular domain. The fatty acid substrate is positioned within this site in an extended L-shaped conformation [16]. If this domain is blocked by macGSTs, the fatty acid may not be able to reach the active site of COX, leading to

inhibition. The molar concentrations employed in the present study (100 µg of COX [~72 kDa]] and 20 µg of macGSTs [~26 kDa]] for COX inhibition and 100 µg of macGSTs and 150 µg of COX for macGSTs inhibition)) indicates approximately one to one molar interaction between two proteins.

Hematin had no effect on the activity of macGSTs, indicating that the heme-binding site has no effect on GST activity. Earlier studies reported that the binding of hematin by GSTs was non-competitive with transferase activity and did not involve the bilirubin-binding site, suggesting the existence of a unique heme-binding site on these proteins [17]. However, the GSH binding site of macGSTs appears to be involved in the interaction with COX, as the presence of GSH overcomes these inhibitory effects. Thus GSH is involved in relieving the inhibition on macGSTs exerted by COX, while potentiating the inhibitory effects of macGSTs on COX. This effect of GSH may contribute to enhanced detoxification systems through macGSTs, while reducing the formation of harmful PGs. The importance of glutathione redox system in offering protection against oxidative stress in male reproduction has been recently reviewed [18].

Both isoforms of cyclooxygenase, COX-1 and COX-2, are expressed constitutively in rat testis [3] and the prostaglandin products of these enzymes are implicated in steroidogenesis [19] and spermatogenesis [20], the primary functions of testis. Also a wide range of GST isozymes are expressed in testis and in an earlier study

we have reported the different forms of cGSTs in rat testes and their role in the prostaglandin formation *in vitro* [12]. The present study demonstrates the negative interaction between macGSTs of rat testis and cyclooxygenase *in vitro*. It will be interesting to investigate the occurrence and significance of such interactions *in vivo* as these interactions could be local mechanisms regulating eicosanoid biosynthesis, specifically the prostanoid pathway. This is in contrast to the MAPEG family of proteins, where membrane associated GSTs are involved in the regulation of leukotriene biosynthesis. In general, these studies suggest a greater and important physiological role for macGSTs, but not for cGSTs, in regulating prostanoid biosynthesis in rat testes. Further, a reversible functional interaction was observed between macGSTs and COX *in vitro*, with possible interaction between the two at the GSH binding site.

Acknowledgment

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