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# INDUCTION OF GLUTATHIONE *S*-TRANSFERASE ISOENZYMES IN MOUSE LIVER BY 5-(2-PYRAZYNL)-4-METHYL-1,2-DITHIOLE-3-THIONE (OLTIPRAZ)

BAKELA NARE, JAMES M. SMITH and ROGER K. PRICHARD*
Institute of Parasitology of McGill University, Macdonald Campus, Ste-Anne de Bellevue, Quebec, Canada H9X 1C0

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**Abstract**—Treatment of mice with a single dose of oltipraz (OPZ) at 200 mg/kg led to a significant ($P < 0.05$) increase in hepatic cytosolic glutathione *S*-transferase (GST) activity and content. GST activity monitored with 1,2-dichloro-4-nitrobenzene was increased 3.8-fold 3 days after treatment, suggesting the induction of $\mu$ class isoenzymes. Ethacrynic acid, a marker for $\pi$ class isoforms, showed only a slight increase in GST activity while no induction was observed with cumene hydroperoxide, an indicator for the $\alpha$ class. The increase in $\mu$ class isoenzymes was further confirmed by separation of the mouse liver affinity purified GST by chromatofocusing and also by resolving the GST subunits by reverse-phase high performance liquid chromatographic procedures. Therefore, OPZ induces mainly the $\mu$ class isoenzymes in mouse hepatic tissues.

Glutathione *S*-transferases (GSTs†) are a family of widely distributed multifunctional proteins that catalyse the conjugation of reduced glutathione (GSH) with various electrophilic substrates. GSTs can also bind a wide range of hydrophobic non-substrate ligands in a non-covalent fashion and facilitate their intracellular transportation [1]. The catalytic and transport properties of GST enable the protein to neutralize both endogenous and exogenous toxic substances including secondary products of lipid peroxidation [2]. The multiplicity of GSTs (isoenzymes) is essential to cover the detoxification of a wide range of molecular targets via both catalytic and non-catalytic activities. GSTs occur as either cytosolic or membrane bound (microsomal) forms. The cytosolic forms, however, are more abundant and have been studied to a greater extent. Cytosolic GSTs are heterodimers or homodimers comprised of at least seven subunits. The GSTs from humans, rats and mice have been divided into three classes; $\alpha$, $\mu$ and $\pi$, based on similar structural and catalytic properties. GST isoenzymes can be induced by a wide variety of xenobiotics including polycyclic aromatic compounds [3] as well as dithiolthiones [4]. The mechanism of GST induction is thought to be associated with a xenobiotic responsive element that is responsible for transcriptional activation of GST genes [5] and increases in the levels of relevant mRNA [6]. This process is usually specific for particular GST genes and may result in differential increases in the various GST forms.

Oltipraz [5-(2-pyrazynl)-4-methyl-1,2-dithiol-3-thione] (OPZ) is a thiono-sulfur compound that has been used as a chemoprotective agent [7] as well as an antiparasitic drug in the treatment of human schistosomiasis [8]. The effect of OPZ as an anticancer agent is thought to be associated with its ability to increase tissue GSH levels and the activity of GST. This facilitates the inactivation of carcinogens via increased conjugation reactions [4]. The antiparasitic action of OPZ, on the other hand, is thought to be due to its ability to decrease levels of GSH and some GSH-dependent enzymes, e.g. GST [9]; this reduces the ability of the parasite to defend itself against toxic metabolites and eventually leads to death. The expression of antischistosomal activity by OPZ and its analogues has some rather stringent structural requirements. For example, replacement of the thione sulfur with oxygen leads to abolition of the antischistosomal action [8]. The differential effects of OPZ on the host's (mammalian) and parasite's (schistosome) glutathione metabolism have offered avenues for selective inhibition of parasite defense systems as a chemotherapeutic strategy. It is, therefore, important to understand the basis and extent of such host–parasite differences in response to chemotherapeutic agents. The effects of OPZ on host (mouse) GST activity are the subject of this investigation.

The induction of liver GST activity by OPZ has been reported for rats [4, 6, 7] and for mice [10, 11]. Substrate specificity studies with cytosolic fractions from male CF-1 mice suggests that GST isoenzymes in the mouse liver are induced to differing extents [11]. However, due to overlapping substrate specificity among the GST isoenzymes, the exact isoforms that are induced have not been identified clearly. Also, decreases in some forms of GST may be masked by increases in others and hence difficult

* Corresponding author: Dr. Roger K. Prichard, Institute of Parasitology of McGill University, Macdonald Campus, 21, 111 Lakeshore Road, Ste-Anne de Bellevue, Quebec, Canada H9X 1C0. Tel. (514) 398-3991; FAX (514) 398-7857.

† Abbreviations: GST, glutathione *S*-transferase; OPZ, oltipraz; GSH, reduced glutathione; CNDB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; pNBC, *p*-nitrobenzylchloride; ECA, ethacrynic acid; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy) propane; and Cu·OOH, cumene hydroperoxide.

to identify in non-fractionated samples. In addition, there are strain, sex and tissue specific differences in GST content and isoform patterns in various organisms and these make any generalizations impossible. In this study we have identified three major isoenzymes [12] in the hepatic tissues of female CD-1 mice. A combination of substrate specificities, purification of GST, isoenzyme fractionation by chromatofocusing and subunit analysis by HPLC indicate that the class $\mu$ isoforms (MIII) account for most of the induction of GST activity by OPZ in these mice. The oxy-analogue of OPZ, RP 36 642 (which lacks antischistosomal activity), also induces GST activity in mouse hepatic tissues.

## MATERIALS AND METHODS

*Chemicals and reagents.* All chemicals and reagents used in this study were of analytical grade. OPZ and RP 36 642 were gifts from Rhône-Poulenc Sante, Paris, France. GSH, GSH-agarose, 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP), *p*-nitrobenzylchloride (pNBC), ethacrynic acid (ECA) and cumene hydroperoxide (Cu·OOH) were purchased from the Sigma Chemical Co., St. Louis, MO. 1,2-Dichloro-4-nitrobenzene (DCNB) was obtained from the Aldrich Chemical Co., Milwaukee, WI. Polybuffer Exchanger 118 and Pharmalyte were from Pharmacia, Canada.

*Treatment of mice and tissue preparation.* Female CD-1 mice (18–20 g) were purchased from Charles River, Canada. OPZ and RP 36 642 were administered to the mice by oral intubation in an aqueous solution containing 25% (v/v) glycerol and 1% (v/v) cremophor El. Control animals received the drug vehicle alone. Groups of four mice each were killed daily up to day 5 after OPZ treatment. For comparison of OPZ with RP 36 642 and also for subunit analysis by HPLC, mice were killed 3 days after administration of the drugs. The animals were killed by cervical dislocation, and livers were perfused with cold saline and excised immediately.

The livers were homogenized individually in Tris–HCl buffer, pH 7.0. Homogenates were centrifuged at 15,000 *g* for 20 min and the resulting supernatant was further centrifuged at 105,000 *g* for 1 hr to obtain the cytosolic fraction. The centrifugation and all further purification steps were carried out at 4°.

*Enzyme activity.* GST activity was measured in the cytosolic fractions by the method of Habig *et al.* [13] with GSH and different substrates including CDNB, DCNB, EPNP, pNBC, ECA and Cu·OOH. GSH peroxidase activity was measured by the method of Paglia and Valentine [14].

*GSH-affinity chromatography.* Cytosolic fractions prepared from the pooled livers of three to four mice were applied to a GSH-affinity column according to the procedure of Simons and Vander Jagt [15]. A 22 mM potassium phosphate buffer was used to equilibrate the column and to wash through unbound proteins. GST activity was eluted from the column by the addition of 50 mM Tris–HCl buffer, pH 9.6, containing 1 mM EDTA, 0.2 mM dithiothreitol, 10% (v/v) glycerol and 5 mM GSH. Fractions containing GST activity were pooled and concentrated and the protein concentration was measured by the method of Bradford [16].

*Isoenzyme separation by chromatofocusing.* Mouse liver GST isoenzymes were resolved by applying affinity-purified fractions onto a chromatofocusing column at pH 11–8. The column procedure was carried out according to the recommendations of the manufacturers (Pharmacia, Uppsala, Sweden). A 1 × 15 cm Polybuffer Exchanger 118 column was employed at a flow rate of 20 mL/hr. The column was equilibrated with triethylamine–HCl, pH 11. GST isoenzymes were eluted by a pH gradient established with Pharmalyte 8 to 10.5, pH 8 (80-fold dilution). The column was washed with 1 M sodium chloride to recover any unresolved proteins. Fractions (2.5 mL) were collected and monitored for GST activity and pH. Active fractions were pooled and concentrated.

*Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).* This was carried out using both cytosolic and purified GST fractions on 13% slab gels as described by Laemmli [17]. The gels were stained with Coomassie Brilliant Blue to visualize the protein bands.

*Analysis of GST subunits by HPLC.* Liver samples were obtained from mice, homogenized individually, and purified via a GSH-affinity column. The samples were concentrated and GST subunit composition of untreated and OPZ-treated mice was analysed by reverse-phase HPLC as described by Ostlund Farrants *et al.* [18]. Analysis was carried out with a Bondex 10 $\mu$m $C_{18}$ reverse-phase column (Phenomenex, CA, U.S.A.) on a model 2152 LKB Bromma (Bromma, Sweden), LKB 2150 solvent delivery pumps and an LKB spectral variable-wavelength absorbance detector (model 2140). The solvents employed were water (A) and acetonitrile (B) both containing 0.06% (v/v) trifluoroacetic acid. GST samples from the affinity column were applied at 35% (v/v) solvent B and a linear gradient was run from 35 to 55% (v/v) solvent B over 30 min with a flow rate of 1.5 mL/min; and detection of polypeptides was performed at 214 nm. Peak area integration was achieved by using Nelson Analytical model 2600, version 3.1 chromatography software (Nelson Analytical Inc., CA, U.S.A.) on an IBM-XT computer. When higher amounts of sample were loaded and long analytical runs were performed, blank runs were introduced to avoid the effect of memory. Identification was confirmed by SDS–PAGE. The relative amounts of the various subunits were calculated from the integrated peak of the HPLC chromatograms using purified isoenzymes for calibration.

*Statistical analysis.* All statistical comparisons are with the vehicle control group using a one-way analysis of variance and Dunnett's procedure.

## RESULTS

*Substrate specificity changes in OPZ-induced GST.* Treatment of mice with a single dose of OPZ (200 mg/kg) resulted in a significant increase in hepatic cytosolic GST activity over a 5-day period. Differential expression of GST isoenzymes was

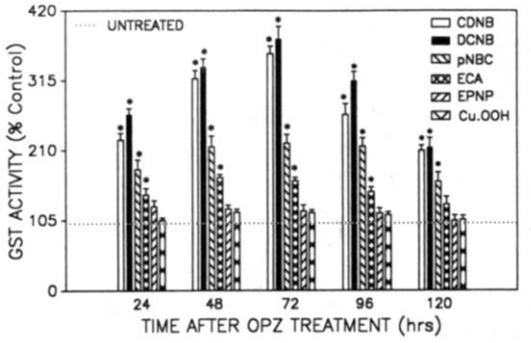


Fig. 1. Effects of OPZ on GST enzyme activity with various substrates. Female CD-1 mice received 200 mg/kg of OPZ (p.o.) or drug vehicle alone. A group of four mice was killed daily following treatment up to 5 days post-drug treatment. Liver cytosolic fractions were prepared from untreated and OPZ-treated mice as described in Materials and Methods. Control values were established each day and the means of these measurements are considered to be 100% for each substrate. The average control specific activities (nmol/min/mg) were as follows: CDNB, 793.3 ± 36.8; DCNB, 20.6 ± 2.4; pNBC, 31.4 ± 3.2; ECA, 36.7 ± 2.9; EPNP, 22.5 ± 2.7; and Cu·OOH, 375.2 ± 18.6. Enzyme specific activities for each sampling time are expressed as a percentage of the means for the controls (100%) ± SEM. Key: (*) $P < 0.05$ vs control.

Table 1. Effects of OPZ and RP 36 642 on the levels of glutathione S-transferase

| Treatment | GST activity (nmol/min/mg protein) | |
|---|---|---|
| | CDNB | DCNB |
| Untreated | 932.5 ± 28.4 (100) | 22.7 ± 5.4 (100) |
| RP 36 642 | 1435.1 ± 73.2* (156) | 39.6 ± 5.6* (174) |
| OPZ | 1514.9 ± 84.5* (162) | 44.3 ± 6.4* (195) |

Cytosolic proteins were prepared from livers recovered from untreated mice (control) and mice treated with 100 mg/kg of OPZ or RP 36 642 3 days previously. GST activity was determined as described in the text with CDNB and DCNB as substrates. Values are means ± SEM; N = 4 animals. Numbers in parentheses represent percent of control.

* $P < 0.05$ vs control.

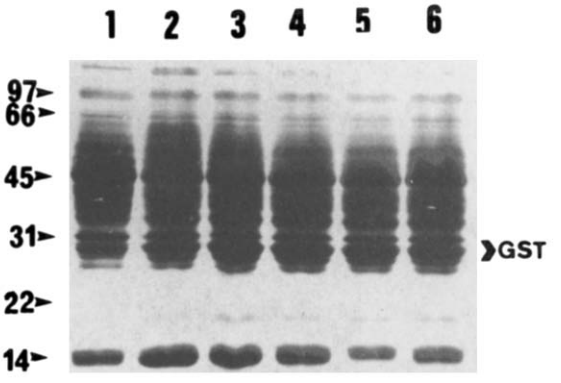


Fig. 2. SDS–PAGE of cytosolic fractions from livers of untreated and OPZ-treated mice. Hepatic cytosolic fractions (35 μg each) were separated on polyacrylamide gels and stained with Coomassie Blue to visualize the various protein bands. Lane 1, cytosol from untreated mice; and lanes 2–6 liver cytosol from OPZ-treated mice obtained on days 1–5 post-treatment, respectively.

monitored with different substrates (Fig. 1). In OPZ-treated animals, activities with CDNB, DCNB and pNBC were significantly higher than in control livers up to day 5 after treatment with OPZ. GST activity with ECA was also significantly higher in OPZ-treated mice than in control animals. However, the induction observed with ECA was much lower than that with CDNB, DCNB or pNBC. The highest induction was obtained with DCNB (3.8-fold) on day 3 following OPZ treatment. Maximum GST induction with all substrates was observed on day 3 after dosing of mice with OPZ; the activity declined thereafter towards control levels. GST activity with EPNP and Cu·OOH (non-selenium dependent GSH peroxidase) remained essentially unaffected by OPZ treatment. The selenium-dependent GSH peroxidase activity (hydrogen peroxide as substrate) was not influenced by administration of the drugs, whereas glutathione reductase activity was increased markedly (data not shown). The oxy-derivative of OPZ induced GST activity with both CDNB and DCNB as substrates (Table 1). However, OPZ was slightly more effective as an inducer of GST activity than the oxy-analogue. Both drugs, however, showed higher induction with DCNB than with CDNB. Analysis of cytosolic proteins from OPZ-treated animals by SDS–PAGE demonstrated an increase in proteins corresponding to the GSTs (25–27 kDa region (Fig. 2). Purification of GST by affinity chromatography followed by SDS–PAGE analysis revealed three distinct protein bands (Fig. 3) named MI, MII and MIII as before [19]. MIII, in particular, appeared to co-migrate with proteins that showed an increase in intensity (Fig. 2). The increase in GST activity induced by OPZ was accompanied by a corresponding increase in GSH-affinity purified proteins (GST) (Fig. 4).

*Effects of OPZ on GST isoenzyme pattern in mouse liver.* Results of chromatofocusing of mouse liver cytosolic GSTs from control and OPZ-treated animals (3 days post-treatment) are shown in Fig. 5. This procedure resolved three basic cytosolic GST peaks, MI, MII and MIII, according to the elution order from the chromatographic column with a pH range 8–11. These have been identified before as belonging to the $\alpha$, $\pi$ and $\mu$ classes, respectively [12]. While the activity of MI remained essentially unchanged, the activities of MII and MIII were 1.2- and 4.3-fold greater than their corresponding controls.

*Effects of OPZ on GST subunit composition.* The resolved GST forms (MI, MII and MIII) were run individually on HPLC in order to establish their retention times. Each form yielded a single major peak on the reverse-phase column and the retention

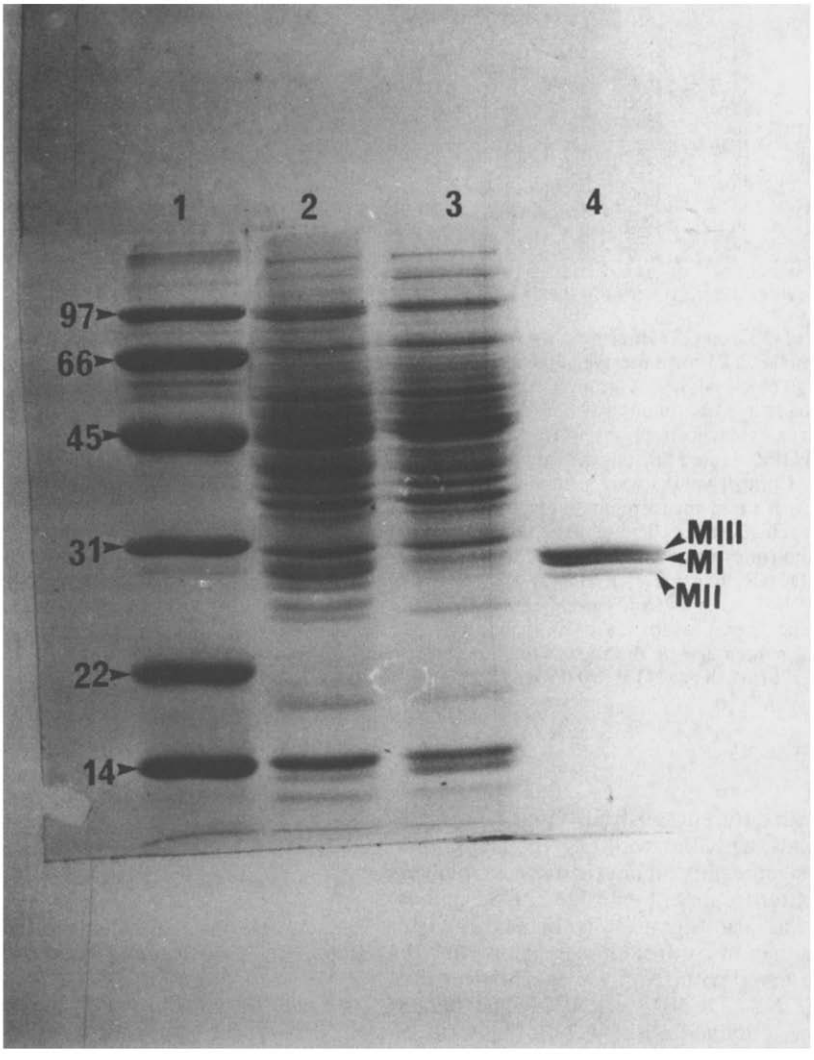


Fig. 3. SDS–PAGE of cytosolic fractions and GSH-affinity purified GST. Lane 1, molecular weight standards; lane 2, cytosolic fractions; lane 3, unbound cytosolic proteins; and lane 4, GSH-bound proteins (GST).

times were 13.8, 15.2 and 9.8 min for MI, MII and MII, respectively. Calibration curves were established by chromatographing six different concentrations of the MIII isoenzyme (2–15 $\mu$g). The relationship between peak area and protein content was linear and the regression value was 0.99. The HPLC analysis of GSH-affinity purified GST from livers of untreated and OPZ-treated mice showed some qualitative changes in isoenzyme composition (Fig. 6). In untreated mice the MI subunits (class $\alpha$) represented the major GST form, while MIII (class $\mu$) became the major form following OPZ treatment. The MII GST form (class $\pi$) remained essentially unaffected. The relative proportions of GST subunits from control mice were approximately MI 60%, MII 14% and MIII 26% while in OPZ-treated samples the proportions changed to MI 33%, MII 15% and MIII 52%. Clearly there was induction of MIII (class $\mu$) GST subunits while MI (class $\alpha$) was probably unaffected. The MII (class $\pi$) isoenzymes may have been increased slightly.

## DISCUSSION

The major cytosolic GSTs have been identified in mice [12], and in this study changes in the relative concentrations of GST isoenzymes in mouse liver after OPZ treatment were observed with various substrates. The highest increase in activity was obtained with DCNB; however, CDNB and pNBC also gave significantly higher activities in OPZ-treated than in untreated animals. This is indicative of elevation in the levels of MIII (class $\mu$). However, CDNB is a universal substrate for most GST isoenzymes. ECA is diagnostic for MII (class $\pi$) subunits and the present results indicate a slight

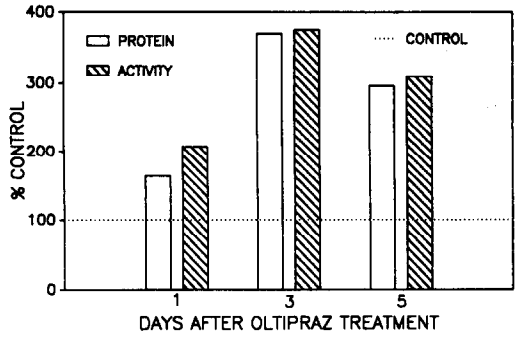


Fig. 4. Levels of GST induced by OPZ in mouse liver. GST was purified as described in the text using equivalent cytosolic proteins (13 mg) from control and OPZ-treated mice. The control values were 379 $\mu$g GST with a specific activity of 27.53 $\mu$mol/min/mg. Total recovered activity and protein content from OPZ-treated mice are expressed as percent of control.

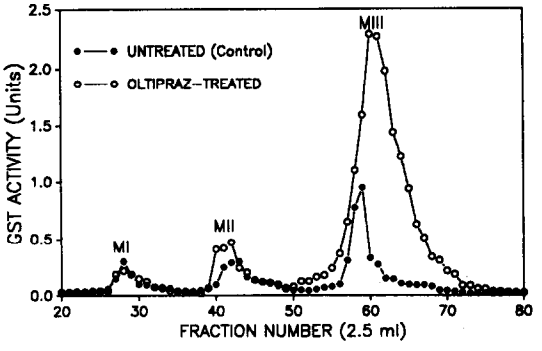


Fig. 5. Separation of mouse hepatic GST isoenzymes by chromatofocusing. Affinity-purified GSTs from equivalent cytosolic proteins (5 mg) were applied to a polybuffer exchanger column previously equilibrated with triethylamine/HCl, pH 11. Proteins were eluted with an 80-fold diluted Pharmalyte 8 to 10.5, pH 8. Fractions (2.5 mL) were collected and monitored for protein and activity. Active fractions for each peak (MI, MII or MIII) were pooled.

induction. Activity with Cu·OOH suggests that MI (class $\alpha$) remains essentially unchanged after OPZ treatment. The overwhelming induction of MIII may account in part for the induction observed with ECA because the $\pi$ isoenzymes also show activity towards this substrate [20].

Chromatofocusing of liver GST activity is in agreement with the substrate specific data by demonstrating an overwhelming induction of the class $\mu$ isoenzymes. HPLC analysis of the individual subunits also indicated a differential effect of OPZ on the individual subunits. The homodimeric nature of the mouse isoenzymes was suggested by the individual peaks obtained by HPLC when each form was analysed. Previous studies have suggested that most if not all mouse liver GSTs are homodimers.

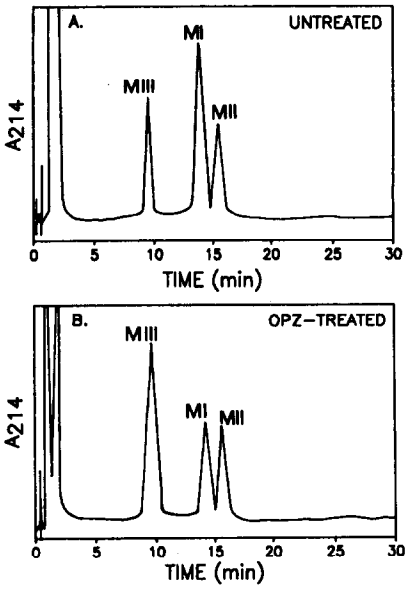


Fig. 6. Glutathione *S*-transferase isoenzyme pattern from control (A) and OPZ-treated (B) female CD-1 mice. GSH-affinity purified GST isoenzymes (20 $\mu$g) from mouse liver were separated by HPLC on a Bondex $C_{18}$ reverse-phase column and eluted with 0.06% (v/v) trifluoroacetic acid in acetonitrile as described in the text.

Although the present study demonstrated that $\alpha$ isoenzymes are not induced by OPZ, a novel isoenzyme of this class that is not normally expressed in mouse liver has been found to be induced markedly (20-fold) by the anti-carcinogenic compound 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) [21]. Previously, it was demonstrated that BHA induces $\mu$ but not $\alpha$ subunits, whereas Benson *et al.* [22] reported an increase in $\alpha$ isoenzymes following BHA treatment. However, the latter study demonstrated that the inducible $\alpha$ class isoenzyme did not bind to the GSH-affinity column. The present study focused only on bound GSTs and no obvious differences were observed between the activities of the unbound fractions from control versus OPZ-treated liver samples. Benson *et al.* [22] found that BHA and bisenthylxanthogen (a thiono-sulfur compound) have slightly different inducer specificities of the GST subunits in male and female CD-1 mice; however, the class $\mu$ isoenzymes were the primary targets of these compounds. It appears that a wide variety of structurally dissimilar compounds have similar inducing effects on murine GSTs with the class $\mu$ subunits being particularly affected [20–23]. Although different inducers have been shown to elevate essentially the same isoenzymes, disparities may arise due to experimental procedures, treatment regimens and other unexplained mechanisms.

The activity of cytosolic GSTs has been shown to be induced by dietary OPZ [7, 4] and by orally administered drug [10, 11]. OPZ was the most effective of the dithiolethiones investigated in inducing GST activity and all the organs examined

showed an increase in GST activity; however, the liver showed the most induction of GST activity after OPZ treatment [4].

The mechanisms by which xenobiotics induce GST are becoming clearer as recent research efforts have focused on this area. Prochaska *et al.* [23] proposed a common mechanism by which several compounds induce both phase I and phase II detoxication enzymes. Numerous studies have since demonstrated transcriptional activation of specific GST genes as responsible for the induction of GST activity [5, 24, 25]. OPZ has been shown to increase GST activity in rat liver by mechanisms that are accompanied by changes in GST gene transcription and steady-state levels of mRNA [6]. Friling *et al.* [5] identified a regulatory DNA sequence on rat GST Ya gene that is considered an electrophilic responsive element (EpRE) and is activated exclusively by inducers containing an electrophilic center. Metabolism of OPZ in mouse liver could generate various metabolites with electrophilic centers and this would provide the right signals to the EpRE or EpRE-like regulatory elements. A similar mechanism would explain the inducing effect of the oxy-analogue. The ability of RP 36 642 to induce GST activity suggests that this analogue may be an effective anticarcinogenic compound just like OPZ. This suggests that the antischistosomal action of OPZ requires a mechanism of action completely unrelated to its actions in the host, since RP 36 642 has no antiparasitic action.

Some mutagenic and carcinogenic substances, such as epoxides, show relatively high activity with class $\mu$ GST [26], giving this class of GST a special physiological role in protection against this class of carcinogens. It is estimated that only 60% of the human population express class $\mu$ GST [27] and, in fact, smokers who lack this isoenzyme class were shown to have significantly higher lung cancer incidents than those with $\mu$ GST. Thus, the suggested use of OPZ as a chemoprotective agent may be limited by the absence of the class $\mu$ GST in 40% of the human population. However, it is not clear whether the induction studies reported here, in which the increase in GST content after OPZ treatment was due mainly to increased synthesis of class $\mu$ subunits, holds true for humans. The multiplicity of GST genes and thus isoenzymes makes generalizations across the mammalian species impossible.

In our laboratory we have been investigating the effects of OPZ on schistosome glutathione metabolism. This study and others have demonstrated that following treatment of *Schistosoma mansoni* infected mice with OPZ, there is a decrease in the levels of parasite GSH [8, 9], the activities of glutathione reductase [9, 28] and GST [9] over a 2- to 14-day period. However, a slight induction of GST activity (5–10%) has been observed in the worms within the first 24 hr following OPZ treatment. This is followed by an irreversible decline in GST activity [9] until parasite death. The present study indicates that in mouse liver, a laboratory host for *S. mansoni*, OPZ induces GST activity in an isoenzyme specific manner. In the parasite on the other hand, OPZ decreases the activities of GST isoenzymes over the long term. This decrease does not appear to be isoenzyme selective [29]. In addition, the oxy-analogue (which lacks antischistosomal action) has no effect on parasite GST activity [30] but induces enzyme activity in mouse liver. Thus, there appears to be fundamental differences in the regulation of GST by OPZ in the parasite versus the host (mouse). The influence of inducers on GST gene expression in parasites, however, has not been studied yet. Understanding the molecular basis and the extent of the differences in GST responses to certain xenobiotics, e.g. OPZ, may prove to be a landmark in advances towards defining rational chemotherapeutic targets in parasites.

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