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Coordinate Induction of Glutathione S-Transferase α, μ, and π Expression in Murine Liver after a Single Administration of Oltipraz

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SUMMARY

The antischistosomal agent oltipraz displays a unique ability to inhibit chemically induced carcinogenesis in a variety of animal models. Its apparent lack of carcinogen specificity and low toxicity make it an attractive candidate for further development as a chemopreventive agent. The mechanism by which oltipraz affords cellular protection is thought to involve the modulation of phase II detoxication enzymes. The present study examines the regulation of each class of glutathione S-transferase (EC 2.5.1.18) in mice after a single oral administration of oltipraz. Glutathione S-transferase activity in the liver increased in a dose-dependent manner after drug exposure. Oltipraz administration (1 g/kg, by gavage) elevated glutathione S-transferase activity to a maximum (4.5-fold) on day 4 after treatment. Western blot analyses demonstrated the induction of all three classes of glutathione S-transferase (α, μ, and π) by oltipraz. Our murine studies suggest that the chemopreventive activity of oltipraz may be due in part to its ability to elevate glutathione S-transferase-μ activity. Consistent with this possibility, associations between the glutathione S-transferase-μ-null phenotype and increased risk for lung, larynx, and bladder cancer have been recently demonstrated in humans. Coordinate elevations in enzymatic activity were preceded by significant elevations in glutathione S-transferase α, μ, and π RNA on day 2 after treatment. Although nuclear run-on assays confirmed the transcriptional induction of all three classes, the maintenance of elevations in enzymatic activity after RNA levels returned to base-line suggests that additional mechanisms are required to regulate glutathione S-transferase expression. Preclinical findings are presented that characterize the response of each class of glutathione S-transferase to oltipraz exposure and support the use of these enzymes as intermediate markers of the chemopreventive activity of oltipraz.

Experimentation continues to document the effectiveness of oltipraz in inhibiting tumor formation. Oltipraz is a synthetic dithiolthione [5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione] that was marketed by Rhône-Poulenc (Vitry-sur-Seine, France) for the treatment of schistosomiasis and was found recently to inhibit human immunodeficiency virus type 1 replication (1). Characterization of the chemopreventive activity of oltipraz using animal models of chemically induced carcinogenesis has demonstrated the ability of this agent to protect the liver (2, 3) and several other tissues from a number of structurally diverse carcinogens, including aflatoxin B_1 (3). This broad specificity of oltipraz, in combination with its low toxicity (LD_{50}, >20 g/kg in rats), supports its further development as a chemopreventive agent for clinical usage.

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The GSTs are a family of detoxication enzymes that catalyze the conjugation of both endogenous and foreign electrophilic compounds with the thiol glutathione. The resulting conjugate is more water soluble and more readily excreted. In general, this metabolic derivative is less cytotoxic than the parent compound. The cytosolic GSTs have been categorized into three classes (α, μ, and π) based upon their immunoreactivity and sequence homology. In addition, GST μ (*GSTM*), α (*GSTA*), and π (*GSTP*) are encoded by genes on separate chromosomes.

Although the molecular target and mechanism of action of oltipraz remain to be elucidated, previous reports suggested that its chemopreventive activity may be related to its ability to increase the levels of a battery of phase II detoxication enzymes, in particular the glutathione-associated enzymes (4). Contribution of the GSTs to the chemopreventive activity of oltipraz has been suggested by experimentation in mice and rats. Significant elevations in the total GST activity of mouse liver accompanied the reduced hepatotoxicity of acetaminophen

ABBREVIATIONS: GST, glutathione S-transferase; SSC, standard saline citrate (1× is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0); SDS, sodium dodecyl sulfate.

and carbon tetrachloride after oltipraz administration (2). Kensler *et al.* (5) observed that the inhibition of aflatoxin B_1 DNA adducts by oltipraz directly correlated with the induction of hepatic GST (correlation coefficient, 0.95). No significant association was present between the prevalence of DNA adducts and the activities of other enzymes (i.e., glutathione reductase and cytochrome P-450). Further investigation of the protective activity of oltipraz against the hepatotoxicity of aflatoxin B_1 revealed the ability of oltipraz to alter the steady state mRNA levels and rate of transcription of the *rGSTA1* gene (6). The involvement of GST α in the detoxication of aflatoxin B_1 has been described recently (7, 8).

Although the effect of oltipraz on the expression of rat GST-α has been established, the ability of oltipraz to induce other classes of GST remains less certain. The identification of chemopreventive agents that modulate GST μ expression is of particular interest, because several studies have suggested that the μ class of GST may play a significant role in determining the susceptibility of individuals to cancer. Phenotypic (9) and genotypic studies (10–12) have identified a polymorphism in the expression of the *GSTM1* isozyme in humans. It is estimated that 40–60% of the population fail to express *GSTM1* because of a deletion in this GST μ gene (13). Large interindividual variability in GST μ activity among expressors (100–200-fold) has also been reported (9). A significant decrease in the expression of GST μ has been observed in patients with lung (14), bladder (15, 16), or larynx (15) cancer, compared with healthy controls matched for age and smoking history. In contrast, two recent studies by Zhong *et al.* (17) and Brockmöller *et al.* (18) have been unable to detect a significant association between the GST μ-null phenotype and increased risk for lung cancer. This difference in results appears to be unrelated to the variable methods of detection used (18).

Although GST activity appears to be a biomarker of chemopreventive activity, a thorough characterization of the regulation of this enzyme by oltipraz has not been conducted and is required for the optimal design of future chemoprevention trials. The present study describes the time course of enzymatic induction and decline at both the protein and RNA levels after a single administration of oltipraz. The effect of oltipraz exposure on the expression of each class of GST is reported.

Materials and Methods

Animal treatment. Female ICR (H_a) mice were selected for this study, based upon the ability of oltipraz to significantly inhibit tumors of the lung and forestomach in these animals (19). Additional support for the use of ICR (H_a) mice was provided by the established role of GST μ in the detoxication of one of the carcinogens used in the previous study, benzo(*a*)pyrene (20), and the potential association of GST μ expression with decreased susceptibility to lung cancer (15). Mice (20 g) were obtained from an in-house laboratory animal colony and maintained at four per cage, receiving food and water *ad libitum*. For the dose-response study, oltipraz was administered to mice at 250, 500, 750, and 1000 mg/kg, by gavage. The vehicle was 1% carboxymethylcellulose/25% glycerol. Animals were sacrificed by cervical dislocation at 4 days after treatment, and livers were collected. Tissues were rinsed in cold phosphate-buffered saline, pH 7.4, immediately frozen on dry ice, and stored at −80° until the time of analysis.

In subsequent studies, mice received a single dose of either oltipraz (1000 mg/kg) or vehicle. Body weights were recorded for 10 days after treatment and were used to monitor toxicity. Both vehicle- and oltipraz-treated animals (at least four per treatment group) were sacrificed by cervical dislocation at 0.3–16 days after treatment. Liver tissue was excised, rinsed in cold phosphate-buffered saline, pH 7.4, and either frozen on dry ice for enzymatic activity assays or snap frozen in liquid nitrogen for RNA and nuclear isolations.

Enzyme activity assays. At the time of analysis, tissues were thawed on ice and dispersed in 10 mM Tris·HCl, pH 7.8, using an Omni 1000 tissue homogenizer (Thomas Scientific, Swedesboro, NJ). Extracts were centrifuged for 15 min at 10,000 × *g* at 4°, and the protein concentration of the resulting supernatant was determined using the Bradford assay (Bio-Rad, Richmond, CA), with bovine IgG as a standard.

The total GST activity of the crude cytosolic preparation was measured spectrophotometrically using 1-chloro-2,4-dinitrobenzene (Sigma Chemical Co., St. Louis, MO) as substrate (21). Activity specific for the μ isozyme was quantified in a similar manner using 3,4-dichloronitrobenzene (Sigma). Activity measurements were standardized to protein and expressed as nmol/min/mg.

Statistical analyses. Statistical comparisons were made using the Wilcoxon two-sample test with normal approximation and a continuity correction of 0.5 (22). In all cases, drug-treated animals were compared with vehicle-treated animals sacrificed at the same time.

Western blot analyses. Tissue homogenates were applied to 12% polyacrylamide slab gels and separated according to the method of Laemmli (23). Maximal separation of GST isozymes was achieved by running a prestained 18-kDa molecular weight marker (Diversified Biotech, Newton Center, MA) to the bottom of the slab gel. Proteins were electrophoretically transferred to nitrocellulose in 25 mM Tris, 192 mM glycine, pH 8.3, containing 20% methanol (150 mA overnight) (24). Membranes were rinsed three times (10 min each) with 50 mM Tris, 400 mM NaCl, pH 7.5 (Tris-buffered saline), containing 0.05% Tween 20, and were incubated sequentially for 1 hr at room temperature with Tris-buffered saline containing 3% bovine serum albumin and either rabbit anti-rat GST α, rabbit anti-rat GST μ, or rabbit anti-mouse GST π antibodies diluted 1/1000 (Biotrin International, Dublin, Ireland). These antibodies have been characterized and cross-react only with other members of the same GST class. Membranes were rinsed, incubated for 1 hr with peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad), and developed using 4-chloro-1-naphthol as substrate (Bio-Rad).

Northern blot analyses. Total RNA was isolated from liver tissues according to the single-step guanidinium thiocyanate method of Chomczynski and Sacchi (25). The resulting RNA was electrophoretically separated on a 1% agarose gel containing 2.2% formaldehyde and was transferred to a nylon membrane (Gene Screen Plus; NEN Research Products, Boston, MA) by capillary action. Membranes were UV-cross-linked, baked at 80° for 2 hr, and probed with cDNAs specific for rat liver GST-μ (pGTA/C48) and GST α (pGTB42) (provided by Dr. Cecil Pickett, Merck Frosst Canada, Inc., Dorval, Quebec, Canada), GST π (pGp5) (provided by Dr. Masami Muramatsu, University of Tokyo, Tokyo, Japan), and mouse fibroblast GSTM1 (pmGT10) and GSTM2 (pmGT2) (26). Membranes were hybridized and washed as described (Gene Screen Plus Protocols; NEN Research Products) and were exposed to X-ray film. Membranes were stripped and reprobed with human β-actin (27) to standardize RNA loading.

Nuclear run-on assay. Nuclei were isolated from adult mouse liver (2–3g) according to the method of Tata (28). Nuclear integrity and abundance were monitored by staining an aliquot of the preparation with methyl green and pyronin and examining the nuclei under the light microscope. Before transcription, isolated nuclei were suspended in storage buffer containing 50 mM Tris·HCl, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA, and 40% glycerol and were stored at −80°. *In vitro* transcription assays were performed essentially as described by Groudine *et al.* (29). Briefly, 2×10^8 nuclei were incubated for 30 min at 30° in a reaction mixture containing 10 mM Tris·HCl, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 16% glycerol, 0.4 mM levels each of ATP, GTP, and CTP, and 250 μCi of [α-^{32}P]UTP (3000 Ci/mmol; NEN Research Products). The reaction was terminated with the addition of RNase-free DNase I (20 μg/ml, for 5 min at 30°). After treatment with proteinase K (200 μg/

ml), the labeled RNA was isolated by phenol/chloroform extraction and precipitated with trichloroacetic acid, in the presence of tRNA (100 μg/ml), onto a 0.45-μm glass fiber filter. The RNA was eluted from the filter and precipitable counts were equilibrated in hybridization buffer containing 50% formamide, 4× SSC, 50 mM sodium phosphate, pH 6.5, 2× Denhardt's solution, 0.1% SDS, and 20 μg/ml tRNA. Nylon membranes containing immobilized cDNA were hybridized with equal counts at 42° for 48 hr. Membranes were washed two times at room temperature with 2× SSC/0.1% SDS and once at 65° with 0.2× SSC/0.1% SDS and were then autoradiographed. Radiographic signals were quantified by Ambis Imaging and the results were normalized by comparison with β-actin levels.

Results

Treatment response. All animals continued to gain weight after oltipraz exposure (0.1 ± 0.05 g/day), at a rate that was not significantly different from that of vehicle-treated controls (0.05 ± 0.1 g/day), and failed to exhibit any signs of toxicity at all drug dosages. Localization of oltipraz (a bright red compound) in lipids was evident from yellowing of the skin and abdominal fat of the mice by 8 hr after treatment. The color of the urine was also intensified in drug-treated animals at this time.

GST activity and protein analyses. The effect of oltipraz exposure on hepatic GST activity was evaluated after the administration of oltipraz at dosages ranging from 250 to 1000 mg/kg. Hepatic GST activity was elevated 1.5–3.2-fold over controls (for the lowest to highest dosages, respectively) (Fig. 1). The high viscosity of the drug suspension for oral administration prevented analysis of dosages greater than 1000 mg/kg. Comparison of the response of detoxication enzymes in the liver to oltipraz exposure at the highest dosage with the responses in lung, bladder, and colon tissues indicated that the expression of liver GST was most affected by drug treatment (Table 1).

The time course of the biochemical response to a single administration of oltipraz was characterized next, to establish the dosing schedule required to achieve maximal induction of enzymatic activity. The total GST activity of livers from oltipraz-treated animals was significantly elevated (1.7-fold) on day 1 after treatment ($p = 0.037$) and peaked at 4.5-fold that of controls on day 4 ($p = 0.0001$) (Fig. 2). Activity remained significantly elevated over controls through day 10 after treatment ($p = 0.030$). The availability of a substrate with high specificity for murine GST μ, i.e., 3,4-dichloronitrobenzene (30), facilitated an independent evaluation of the effect of oltipraz on GST μ activity. The time course of GST μ induction was similar to that observed for total GST (Fig. 2). GST-μ activity was elevated 5.5-fold on day 4 after treatment ($p = 0.0001$) and remained significantly elevated, compared with control animals, through day 12 ($p = 0.030$). Western blot analyses using antibodies specific for the μ class of isozymes identified GST μ as the predominant form of GST in mouse liver and provided additional evidence for the ability of oltipraz to induce its expression (Fig. 3). Increased levels of μ protein correlated directly with the observed increases in the conjugating activity of GST μ on days 1–10 after treatment. GST μ protein levels eventually returned to base-line on day 14 after treatment (data not shown).

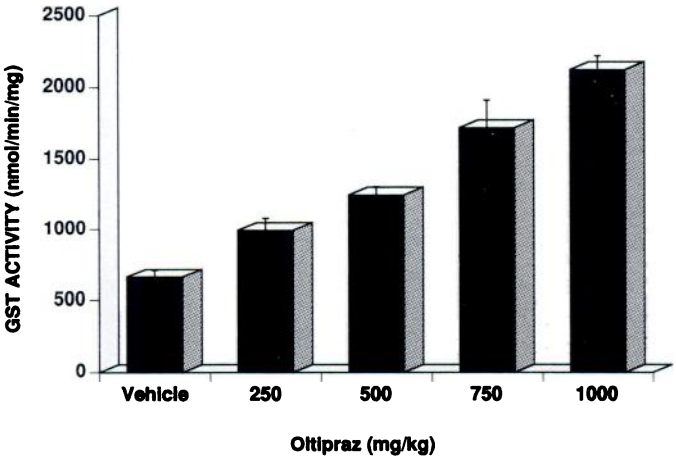

Fig. 1. Hepatic GST activity 4 days after a single dosage of oltipraz by gavage. Total GST activity was determined using 1-chloro-2,4-dinitrobenzene as substrate and is expressed as a percentage of the activity of vehicle-treated controls sacrificed at the same time (mean ± standard error, $n = 4$/group).

Immunoreactivity of hepatic extracts with antibodies against the GST α and π classes indicated that the expression of these isozymes was also induced by oltipraz. Both GST α and GST π were elevated on day 1, with the level of GST-α returning to approximately that of controls on day 10, whereas GST π expression continued to be elevated. A comparison of the extent of GST π elevation with that of GST μ and α was difficult to make, because the level of GST π in control liver was significantly lower than that of the other GST isozymes.

GST RNA analyses. Northern blot analyses demonstrated that the observed increases in GST protein and activity were preceded by significant elevations in RNA expression (Fig. 4). RNA levels for GST α, μ, and π were increased by day 1 and reached a maximum on day 2 after treatment. Elevated expression of all three classes of GST isozymes was confirmed by nuclear run-on assays. The ratios of the rates of transcription of nuclei isolated from vehicle- and drug-treated livers at 2 days after treatment (treated/control) were 4.8 for GST α, 2.1 for GST μ, and 2.3 for GST π.

Probes specific for the 3′ untranslated regions of *mGSTM1* and *mGSTM2* (26) were used to examine the effect of oltipraz on the expression of individual members of the GST μ class. Basal expression of *GSTM1* in murine liver was higher than that of *GSTM2*, with *GSTM2* transcript levels remaining undetectable after 8 days of autoradiography (Fig. 5). Drug exposure produced significant elevations in the levels of *GSTM1*-encoding transcripts on days 1, 2, and 4 after treatment. Similar to the profile of GST μ elevation observed in Fig. 4, RNA levels peaked on day 2 and decreased substantially by day 4. *GSTM2* expression was increased to detectable levels on both days 1 and 2 after oltipraz exposure.

Discussion

Recent progression of oltipraz experimentation from animal modeling to phase I clinical trials has indicated the need for additional preclinical characterization of the oltipraz dosage and schedule that produce maximal elevation of detoxication enzyme activity. The use of various dietary concentrations of oltipraz and experimental conditions in previous studies has made it difficult to compare existing results. The ideal chemopreventive agent would be effective when given chronically at a low nontoxic dosage. Elevation of the enzymatic activity in liver tissue for 12 days after a single drug treatment suggests

TABLE 1

GST activity in mouse tissues after oltipraz administration

Values represent the ratio of the enzymatic activity in tissues from oltipraz-treated mice (1 g/kg) to that of vehicle-treated controls sacrificed at the same time. Mean GST activities (nmol/min/mg) of vehicle-treated controls (n = 8) were as follows: liver, 550.75; lung, 156.41; bladder, 1026.31; and colon, 178.08. Mean GST μ activities (nmol/min/mg) of vehicle-treated controls (n = 8) were as follows: liver, 15.49; lung, 6.58; bladder, 63.25; and colon, 4.57.

	GST ratio				GST μ ratio			
	Liver	Lung	Bladder	Colon	Liver	Lung	Bladder	Colon
Day 2 after treatment	2.55 ± 0.15[a]	1.40 ± 0.06[a]	1.48 ± 0.10[a]	1.57 ± 0.15	3.83 ± 0.29[a]	1.59 ± 0.06[a]	1.75 ± 0.20[a]	1.50 ± 0.20
Day 4 after treatment	4.50 ± 0.45[a]	1.36 ± 0.08[a]	1.64 ± 0.20[a]	1.79 ± 0.70	5.79 ± 0.41[a]	1.20 ± 0.05	1.22 ± 0.10	0.86 ± 0.50

[a] Significantly different from controls, by Wilcoxon test ($p < 0.05$).

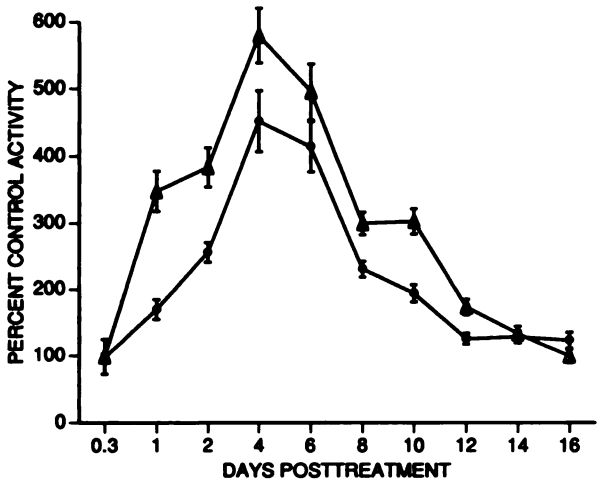

Fig. 2. Total GST (●) activity and GST μ (△) activity of liver tissue at various time intervals after oltipraz administration (1000 mg/kg, by gavage). Enzymatic activities of GST and GST μ were determined spectrophotometrically, using 1-chloro-2,4-dinitrobenzene and 3,4-dichloronitrobenzene, respectively, as substrates. Values represent a percentage of the activity of vehicle-treated controls sacrificed at the same time (mean ± standard error, n = 4/group).

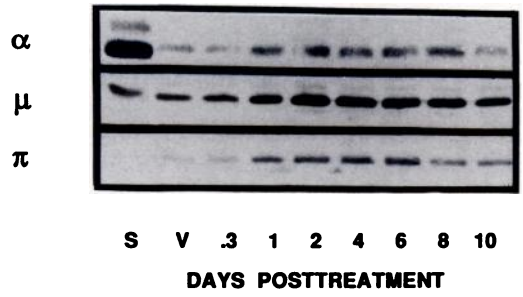

Fig. 3. Western blot analyses of liver extracts (75 μg of the S-10 fraction) from mice treated with either vehicle (V) or oltipraz (1000 mg/kg) and sacrificed on days 0.3–10 after treatment. Rat liver GST (5 μg) was included as a reference standard (S). Separated proteins were transferred to nitrocellulose membranes and sequentially incubated with antibodies against each class (α, μ, and π) of GST. Membranes were developed using the peroxidase method.

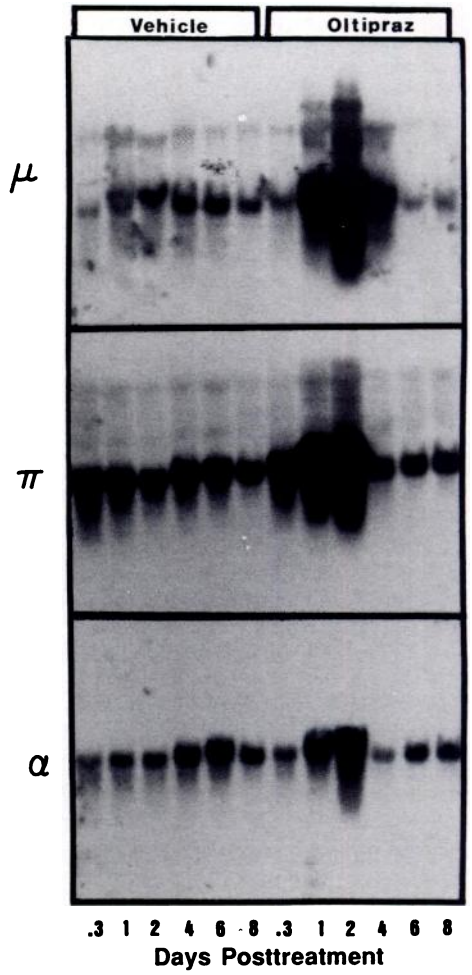

Fig. 4. Northern blot analyses of RNA hybridized to cDNAs specific for each class of GST (α, μ, and π). Mice were treated with either vehicle or oltipraz and were sacrificed on various days after treatment. Total RNA was isolated from liver, fractionated by electrophoresis, and transferred to nylon membranes. Blots were standardized by hybridization with β-actin.

that administration of oltipraz once each week may be sufficient to maintain cellular protection from carcinogen exposure. Although significant increases in enzymatic activity were observed at dosages as low as 250 mg/kg, the absolute level of detoxication enzyme activity required to inhibit carcinogenesis remains to be elucidated.

Examination of the response of extrahepatic tissues to oltipraz administration 0.3–10 days after treatment indicated that the time course of enzymatic induction was similar in all tissues, with activity peaking between days 2 and 4. In many instances, elevations in total GST activity were accompanied by increases in GST μ (Table 1). Although the enzymatic response to oltipraz was decreased in all extrahepatic tissues examined, compared with liver, inhibition of chemically induced tumors by oltipraz has been reported for lung (19), bladder (31), and colon (32).

Induction of GST α, μ, and π expression was observed after oltipraz exposure. Although the large number and structural diversity of the GST isozymes facilitate their interaction with numerous substrates, cellular protection will be afforded by GST inducers only if the carcinogen to which the individual

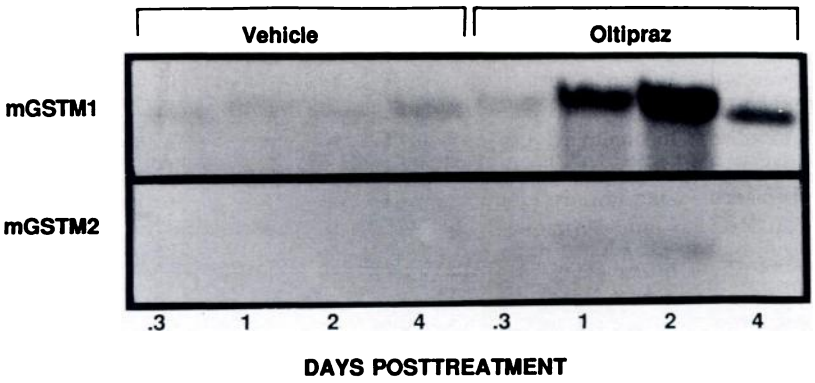

Fig. 5. Expression of the GST μ class of subunits in liver. Total hepatic RNA was isolated from mice that had received either vehicle or oltipraz and were sacrificed on 0.3–4 days after treatment. Northern blots were hybridized with cDNA probes specific for the 3′ untranslated regions of *mGSTM1* and *mGSTM2* and were exposed to X-ray film for 2 days. The RNA content of each lane was standardized by hybridization with β-actin.

has been exposed is inactivated via conjugation with glutathione. Treatment of cultured human hepatocytes with oltipraz in a previous study produced a consistent elevation of GST α expression only (33).

To date, only GST μ expression has been linked with cancer prevention in humans. This association is consistent with the high specific activity of GST μ with mutagenic and carcinogenic epoxides (34). Increased susceptibility to cancer has been correlated with the GST μ-null genotype in several studies, whereas the contribution of interindividual variability in GST μ activity among expressors (9) has not been investigated. One can assume that a minimum threshold level of GST μ activity is required to confer protection from carcinogens. It then follows that the additional increases in GST μ activity produced by oltipraz exposure may represent a significant contribution to the success of individuals in preventing cancer. It should be noted that the detrimental effects of GST μ overproduction have not been examined and may include alterations in steroid metabolism and transport (35).

One explanation for the consistent delay in peak RNA levels and enzymatic activity until 2 and 4 days after treatment, respectively, is that a metabolite of oltipraz and not the parent compound is the ultimate inducer of GST α, μ, and π expression. Likewise, the schistosomicidal activity of oltipraz is dependent upon its metabolic activation (36). This mechanism of induction is in agreement with that proposed by others, who have postulated that the redox-labile species generated by cytochrome P-450 serve as the induction signal for phase II enzymes (37). Extensive examination of urine from mice, rats, monkeys, and humans has led to the identification of 13 metabolites (pyrrolo[1,2-*a*]pyrazine derivatives) of oltipraz (38). Of these metabolites, $C_{10}H_{12}N_2O_2S_2$ (two diastereoisomers) and $C_{15}H_{18}N_2O_6S_2$ are the major derivatives found in mouse urine. Additional experimentation is required to determine whether the metabolism of oltipraz is required to regulate the expression of the GSTs.

The identity of the time course profiles of GST α, μ (M1 and M2), and π at both the protein and RNA levels suggests that these genes are coordinately regulated by oltipraz. A comparison of this finding with the specific induction of *GSTA2* in drug-resistant mammary carcinoma cells by the alkylator chlorambucil (39) suggests that several control mechanisms exist to regulate the expression of this multigene family of GSTs. Peaks in RNA expression correlated with subsequent elevations in GST protein and were attributed to the transcriptional activation of all three classes of GST isozymes. Significant elevations of GST protein levels beyond day 6 after treatment, when transcript levels returned to base-line, indicated that transcriptional induction was not the only molecular mechanism involved in the coordinate regulation of the GST isozymes by oltipraz. Alterations in the post-translational modification and stability of these proteins may contribute to their prolonged elevation after drug exposure. The ability of oltipraz to increase the activity of several other glutathione-associated enzymes has been reported, but the time course of their responses has not been characterized.

Although the expression of a number of enzymes is altered by oltipraz, data from the present study indicate that oltipraz exposure does not produce a nonspecific cellular stress response. No changes in the levels of either the inducible heat shock protein (HSP70) or transcripts encoding the multidrug resistance proteins (*mdr1*, *mdr2*, and *mdr3*) have been observed after oltipraz administration (data not shown). In order for oltipraz to specifically induce phase II detoxication enzymes, it must regulate gene expression at several chromosomal locations by interacting with a common regulatory sequence or by acting through a common signal transduction pathway.

In conclusion, the unique ability of oltipraz to simultaneously regulate a battery of detoxication enzymes makes this drug an effective inhibitor of the cellular damage induced by a broad range of carcinogens. Further understanding of the mechanism by which oltipraz coordinately regulates the expression of GST α, μ, and π will facilitate the identification of the common molecular regulator of chemopreventive activity. The combined function of oltipraz as a monofunctional enzyme inducer and an inhibitor of several types of tumors makes it a model compound for the development of future chemopreventive strategies.

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