Transcriptional Control of Glutathione S-Transferase Gene Expression by the Chemoprotective Agent 5-(2-Pyrazinyl)-4-methyl-1,2-dithiole-3-thione (Oltipraz) in Rat Liver

Nancy E. Davidson, Patricia A. Egner, and Thomas W. Kensler

Oncology Center [N. E. D.] and Departments of Environmental Health Sciences [P. A. E., T. W. K.] and Pharmacology and Molecular Sciences [T. W. K.], Johns Hopkins Medical Institutions, Baltimore, Maryland 21205

ABSTRACT

The substituted 1,2-dithiole-3-thione oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione] protects against the acute and chronic toxicities of many xenobiotics, including aflatoxin B₁, in rodents. These protective effects are mediated, in part, through elevation of glutathione S-transferase (GST) activities. Because studies by Coles et al. [Carcinogenesis (Lond.), 6: 693-697, 1985] suggested that the detoxication of aflatoxin through conjugation with glutathione is principally catalyzed by GST homodimer YaYa, we have investigated the regulation of the gene coding for the Ya subunit in the liver of F344 rats following dietary administration of oltipraz. Overall GST activity, as measured by conjugation with 1,2-dichloro-4-nitrobenzene or 1-chloro-2,4-dinitrobenzene, as well as the levels of GST Ya protein, was elevated 1.5-fold by 24 h and maximally (2.7- to 3.5-fold) and persistently after 5 days on a purified diet supplemented with 0.075% oltipraz. Steady state mRNA levels for GST subunit Ya, as quantified by slot blot analysis using rat liver GST complementary DNA clone pGTB38, were also elevated by 24 h, with a maximal elevation of 3-fold observed at 3 days. However, mRNA levels decreased thereafter, despite continued feeding of oltipraz. Northern blot analyses demonstrated that oltipraz did not alter the size of GST mRNA. Transcriptional activity of the GST Ya gene, as determined by nuclear run-off analysis, was increased 2-fold after 24 h of feeding of oltipraz, was maximally induced 2.4-fold at 3 days, and returned to near control levels at 7 days, despite sustained feeding of oltipraz. Modulation of GST activity by oltipraz was not accompanied by changes in the methylation pattern at internal sites of the GST Ya gene. These results show that the initial induction of hepatic GST activity during oltipraz exposure correlates with changes in steady state levels of GST mRNA and rates of GST gene transcription; however, the continued elevation of GST enzymatic activities and GST Ya protein levels in the face of declining GST Ya mRNA levels and transcription rates suggests that additional mechanisms may be involved in regulating GST Ya expression by oltipraz.

INTRODUCTION

1,2-Dithiole-3-thiones are five-membered cyclic sulfur-containing compounds with antioxidant, chemotherapeutic, and chemoprotective activities. Several substituted 1,2-dithiole-3-thiones are used as antioxidant additives in rubber, metals, and commercial oils and greases, while others are used medicinally as antischistosomal agents, choleretics, and stimulants of salivary secretion (1-3). One substituted 1,2-dithiole-3-thione, oltipraz (Fig. 1), has recently been demonstrated to protect against the acute and chronic toxicities of structurally diverse agents in multiple target organs and may be a useful compound for inhibition of human neoplasia. Pretreatment of mice or rats with oltipraz protects against the acute hepatotoxicities of carbon tetrachloride (4), acetaminophen (5), allyl alcohol (5), and AFB₁ (6). Wattenberg and Bueding (7) have observed inhibitory effects of oltipraz on carcinogenesis induced by benzo[a]pyrene, diethylnitrosamine, and uracil mustard in the lung and forestomach of mice. Our laboratory has recently reported on the protective effects of dietary oltipraz on DNA adduct formation and hepatic tumorigenesis induced by AFB₁ in rats (8).

The ability to induce the activities of electrophile-detoxification systems, such as the GSTs, is a property common to many chemoprotective agents (9), and the elevation of GST activities has been observed in tissues of rats and mice treated with oltipraz (8, 10). The rat GSTs are a family of abundant and widely distributed catalytic and binding proteins that promote the conjugation of glutathione with electrophilic groups of a variety of molecules, including AFB₁. At least 10 cytosolic forms of GST have been isolated from rat liver. These isozymes exhibit broad and overlapping specificities for substrates and are composed of binary combinations of at least six subunits designated as Ya, Yb₁, Yb₂, Yc, and Yn (11). The protective actions of oltipraz and other antioxidants against AFB₁-induced neoplasia appear to be mediated, at least in part, through the induction of hepatic GST activity. We have observed an excellent correlation between the degree of inhibition of AFB₁-DNA adduct formation and induction of GSTs by four different dietary antioxidants in rat liver (12). Furthermore, rats fed either oltipraz or ethoxyquin prior to AFB₁ administration show 3- to 5-fold increases in the rate of elimination of 8,9-dihydro-8-(S-glutathionyl)-9-hydroxyaflatoxin B₁ in the bile following carcinogen exposure (13, 14). Coles et al. (15) have shown that aflatoxin conjugation to glutathione is catalyzed by the Ya and Yc homo- and heterodimers of rat liver GST, with the YaYa dimer exhibiting the highest catalytic rate. However, the molecular mechanism(s) by which oltipraz elevates AFB₁ glutathione conjugation is (are) not known. Using a cDNA containing the entire coding region of the GST Ya gene, we have investigated the steady state GST mRNA levels, nuclear transcription, and DNA methylation in rat liver following dietary exposure to oltipraz. Our results indicate that the initial changes in the rate of hepatic conjugation of AFB₁ with glutathione correlate with changes in the steady state levels of GST Ya mRNA and transcription of the GST Ya gene. Thus, expression of the rat liver GST Ya gene appears to be principally regulated by oltipraz at the transcriptional level.

MATERIALS AND METHODS

Animals and Diets. Male F344 rats, 75-100 g (Charles River Breeding Laboratories, Wilmington, MA), were housed under controlled conditions of temperature, humidity, and lighting. Food and distilled water were available ad libitum. Purified diet of the AIN-76A formu...
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lacking the recommended addition of 0.02% ethoxyquin (16) was used, and fresh diet was provided to animals daily. Oltipraz was incorporated into the diet at a final concentration of 0.075% unless otherwise indicated and was stored at 4°C. Oltipraz (RP 35,972) was kindly provided by Rhône-Poulenc (Paris) and was greater than 99% pure as adjudged by high performance liquid chromatography (17).

Assay of Glutathione S-Transferase Activity. Rats maintained on the experimental diets as indicated were stunned and killed by cervical dislocation, and livers were removed and homogenized with a Polytron homogenizer in 4 volumes (w/v) of 50 mm Tris-HCl buffer, pH 7.0, containing 0.25 mm sucrose. Homogenates were centrifuged at 150,000 X g for 60 min and the resulting supernatant fluid was stored at -80°C for subsequent use in assays for GST activity, with CDNB or DCNB as substrates (18). Protein was determined by the method of Bradford (19), using bovine serum albumin as standard.

Determination of GST Ya Protein Level by ELISA. GST Ya levels in 105,000 X g supernatants were measured using an enzyme-linked immunosorbent assay. Purified rat liver GST Ya and anti-rat GST Ya antibody were purchased from Medlabs (Dublin, Ireland). The anti-GST Ya antiserum does not cross-react with Yb, and Yb, subunits and shows very weak cross-reactivity with Yc (<2%) (20). Flat-bottomed, 96-well microtiter plates were coated with 0.2 ml of 0.1 mm sodium carbonate buffer, pH 9.6, containing either serial dilutions of the hepatic supernatant or purified rat liver GST Ya, and were incubated overnight at 4°C. Plates were then washed three times with TPBS, incubated for 2 h with TPBS containing 5% bovine serum albumin, and washed three times again with TPBS. Anti-rat GST Ya antibody diluted 1:5,000 was added to each well, in 0.2 ml TPBS, and incubated for 2 h at room temperature. The plates were washed as before, and 0.2 ml of a 1:10,000 dilution of peroxidase-labeled goat anti-rabbit IgG (Kirkegaard and Perry, Gaithersburg, MD) was added to each well for 1 h. The wells were washed repetitively with TPBS, as previously described, and then allowed to react with 0.1 ml/well of a 0.4 mg/ml solution of ortho-phenylenediamine, a 0.05 mm sodium citrate buffer, pH 5.0, supplemented with 0.0005% H2O2 just prior to use. After a 10-min incubation, the reaction was stopped by the addition of 50 µl H2SO4 and the subsequent color development was measured at 490 nm using a Biotek microplate autoreader.

Isolation of RNA and RNA Blot Analysis. Livers were frozen in liquid nitrogen and subsequently homogenized in 16 parts (w/v) of guanidine isothiocyanate stock solution, and RNA was purified by the method of Chirgwin et al. (21). For RNA slot blots, 5 µg of total RNA were denatured in formaldehyde and serially diluted onto nitrocellulose paper (22). The GST Ya complementary DNA clone pGTB38 (23) was the generous gift of Dr. Cecil B. Pickett, Merck Frost Centre for Therapeutic Research (Pointe-Claire-Dorval, Québec, Canada), and was radiolabeled with [32P]-dCTP, by standard nick translation, to a specific activity of 1 X 106 cpm/µg (24). Filters were hybridized for 16 h at 37°C in 50% formamide, 3X Denhardt’s (0.06% Ficoll, 0.06% bovine serum albumin, and 0.06% polyvinylpyrrolidone), 0.45 mm sodium chloride, 0.045 mm sodium citrate (pH 7.4), 0.5% SDS, 10% dextran sulfate. After hybridization, filters were washed in 0.15 mm chloride, 0.015 mm sodium citrate, 0.5% SDS, at 60°C. Washed filters were exposed to Kodak XAR-5 film, and the level of hybridized RNA was determined by densitometric scanning of autoradiographs. To ensure that equal amounts of RNA were loaded, RNA blots were stripped of probe and rehybridized to a human ß-actin probe, a gift of Dr. Lawrence Kedes, Stanford University (Palo Alto, CA) (25).

DNA Isolation and Southern Blot Analysis. Isolation of high molecular weight DNA was carried out as described by Goelz et al. (26). For analysis of the DNA methylation pattern, genomic DNA (10 µg) was digested with methylation-sensitive restriction endonucleases Mspl (5 units/µg DNA) or HpaII (10 units/µg DNA), according to manufacturer’s specifications. In some cases double digestes using the enzyme PstI, which is known to cut within the coding region of the GST Ya gene, were done to facilitate analysis. Digested DNA was separated by electrophoresis on a 0.9% agarose gel and transferred to nitrocellulose paper (27). Filters were then hybridized and washed as above.

Nuclear Run-off Transcription Assay. Nuclei were isolated from rat liver (28) and the transcription reaction was carried out by incubating freshly prepared nuclei for 30 min at 37°C, in 300 µl of a reaction mixture containing 10 mm Tris-HCl, pH 8.0, 5 mm MgCl2, 150 mm KCl, 20% glycerol, 0.4 mm each ATP, GTP, and CTP, and 500 µCi [a-32P]-UTP (700 Ci/mmol; New England Nuclear, Boston, MA), as previously described (29). The 32P-labeled RNA was isolated by centrifugation through guanidinium isothiocyanate-cesium chloride and resuspended in 10 mm Tris-HCl, pH 7.5, and 1 mm EDTA. Equal numbers of counts were hybridized to an excess of denatured plasmid DNA immobilized on nitrocellulose filters. After hybridization for 3 days at 42°C, filters were washed under stringent conditions with 0.015 mm sodium chloride, 0.0015 mm sodium citrate, 0.05% SDS, at 60°C. Autoradiographs were analyzed by densitometry and the results were normalized by comparison to the transcriptional level of actin, which remained stable. Hybridization to the carrier plasmid, pBR322, was also monitored and was absent in all cases.

RESULTS

Effects of Oltipraz on GST Enzyme Activity and GST Ya Protein and mRNA Levels. The dose-response characteristics for the induction of hepatic GST activity by oltipraz are shown in Fig. 2A. The highest concentration of oltipraz tested, 0.1% in the diet, induced GST activities 3.2- (DCNB) to 4.5-fold (CDNB) after 1 week of feeding. Concentrations as low as 0.02% in the diet yielded modest but statistically significant increases in enzyme activity (50%), whereas 0.01% oltipraz was without effect on hepatic GST activities. Because we have recently observed that feeding 0.075% oltipraz to rats during the period of AFB1 exposure completely inhibits the development of hepatocellular carcinomas,4 we used this dietary concentration for all subsequent experiments. The time course for the induction of GST specific activities in rat liver cytosols following addition of 0.075% oltipraz to the diet is depicted in Fig. 2B. Within 1 day on the oltipraz diet, GST activities were significantly enhanced above 1.5-fold. Maximal induction, as measured with either DCNB (2.7-fold) or CDNB (3.5-fold) as substrate, was observed after 5 days of feeding the diet. Maximal enzyme induction was sustained as long as oltipraz was included in the diet. However, as previously observed with the feeding of ethoxyquin (14), removal of oltipraz from the diet resulted

Fig. 1. Structure of 5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione (oltipraz).

Fig. 2. Dose response and time course for induction of hepatic GST activity by dietary oltipraz. Left, dose response for the induction of glutathione S-transferase activity following feeding oltipraz for 7 days. Right, time course. Rats were placed on a diet containing 0.075% oltipraz and were sacrificed at the indicated times. Points, means of duplicate determinations done on each of four animals.
in diminution of GST activities such that baseline activities were reached in 7–10 days (data not shown). As shown in Fig. 3, the time course for the expression of GST Ya protein rat liver after feeding oltipraz is very similar to that observed for GST activity. A 3-fold elevation of GST Ya protein was seen within 3 days on the oltipraz diet.

In order to investigate the mechanisms by which oltipraz increases hepatic GST levels and activities, the time course for increases in hepatic GST Ya mRNA levels was examined following introduction of oltipraz into the AIN-76A diet. As shown in Fig. 4, significant elevations in the levels of mRNA coding for subunit Ya were evident within 24 h; however, a maximal increase of 3-fold was not observed until day 3. Interestingly, mRNA levels for the GST Ya subunit decreased after this time, despite sustained feeding of oltipraz. This observation contrasts with the observation that the specific activities of GST for DCNB or CDNB, as well as GST Ya protein levels, did not decline over this latter period.

Total cellular RNA, isolated from the livers of animals fed oltipraz for 1, 3, 5, and 7 days or control animals, was subjected to Northern blot analysis as well. Oltipraz treatment did not alter the expected GST RNA size. Furthermore, the stimulatory effects of oltipraz on GST mRNA level demonstrated by slot blot analysis were confirmed by the Northern blots (Fig. 4).

Analysis of GST Transcription. The transcripational activity of the GST Ya gene was determined by incubating purified rat liver nuclei in an in vitro transcription system. In this assay, nuclei were transcribed in vitro to allow the in vivo-initiated nascent RNA transcripts to elongate in the presence of [\(^{32}P\)]-UTP. Labeled nuclear GST Ya mRNA was then quantitated by filter hybridization, thus measuring a relative rate of transcription at the time of cell lysis. Fig. 5 shows the time course for the increase in relative rates of GST Ya gene transcription following dietary administration of oltipraz. A 2-fold increase in the transcripational activity of the GST Ya gene was observed 24 h after oltipraz feeding and reached a maximum of 2.4-fold at 3 days, the time of maximal increase in GST Ya mRNA levels (Fig. 4). Thus, it would appear that the elevation in transcripational activity is sufficient to account for the increase in GST Ya mRNA levels determined by the RNA blot hybridization experiments. Furthermore, in parallel with mRNA levels, transcriptional activity decreased despite sustained feeding of oltipraz and returned to near control levels at 7 days.

Since little initiation of transcription takes place in isolated nuclei and the nascent RNA transcripts are stable, the transcription rates presumably reflect genes which have been initiated in vivo by RNA polymerase II. To determine the dependence of the RNA transcripts completed in vitro on RNA polymerase II activity, transcripational activity of the GST Ya gene was determined at the time of maximal activity (3 days) in the presence of 2 \(\mu\)g/ml \(\alpha\)-amanitin, a specific inhibitor of RNA polymerase II. As previously observed for the induction of hepatic GST Ya gene transcription by phenobarbital and 3-methylcholanthrene in the rat (30), the transcription of GST Ya mRNA following oltipraz treatment was nearly completely blocked by the addition of \(\alpha\)-amanitin (data not shown).

Changes in DNA Methylation of the GST Ya Gene. The correlation which has been observed between the undermethylation of DNA within and around a specific gene and its expression (31) prompted an examination of the methylation
of the GST Ya gene during stimulation with oltipraz. To detect the degree of methylation in the GST Ya gene, the methylation-sensitive isoschizomeric restriction enzymes Mspl and HpaII were used to digest genomic DNA. Both of the enzymes recognize the sequence CCGG. However, HpaII does not cleave DNA if the internal deoxyctosine nucleotide is methylated, while Mspl cuts regardless of whether the internal cytosine in its recognition sequence is methylated. Mspl digestion yielded the restriction pattern in Fig. 6, which did not change over 7 days of oltipraz treatment (data not shown). Several CCGG sequences within the GST Ya gene of the uninduced rat were constitutively methylated, as evidenced by the relative insensitivity to cleavage by HpaII. However, treatment with oltipraz for up to 7 days was not associated with any change in the HpaII restriction pattern. Double digestions using HpaII or Mspl and PstI (which is known to cleave at several sites within the coding sequences of the gene) also failed to demonstrate any change in restriction pattern after 3 days of oltipraz feeding. These data suggested that the GST Ya gene is methylated at internal sites; however, oltipraz-mediated modulation of transcriptional activity is not associated with changes in methylation within the gene. The methylation status of putative 5' regulatory sequences for the GST gene was not examined in these studies.

**DISCUSSION**

Antioxidants such as BHA, butylated hydroxytoluene, and ethoxyquin have been known for many years to exert an anticarcinogenic effect when administered simultaneously with carcinogens. While numerous animal studies have documented that these protective effects could be achieved against a diverse array of chemical carcinogens having different target organ specificities, there have been few experiments designed to investigate the mechanisms of such protective actions. One of the earliest studies to implicate a role for the induction of GST in the protective actions of antioxidants was that of Talalay and co-workers (32), who showed that liver cytosols from BHA- or ethoxyquin-fed rats or mice exhibited much higher GST activities and that cytosols prepared from the livers of these rodents inhibited the mutagenic activity in urine from mice pretreated with benz[a]pyrene. Subsequent studies demonstrated that dietary administration of antioxidants increased GST activities in extrahepatic tissues such as lung, stomach, small intestine, and kidney (33). Initial studies on the molecular mechanisms of induction of GSTs by antioxidants were conducted by Pearson et al. (34), who observed a 20-fold increase in mRNA for the major GST in the livers of mice several days after feeding 0.75% BHA. Benson et al. (35) subsequently reported that significant increases in mRNA levels could be observed as early as 24 h after placing mice on the BHA diet. More recently, Pearson et al. (36) have studied the mechanisms of tissue-specific induction of murine GST mRNAs by BHA. In these studies, measurements of transcription rates in isolated nuclei indicated that increased GST mRNA levels were due to increased rates of transcription.

Similar findings were reported by Pickett and co-workers (30, 37), in rats given a single i.p. injection of either phenobarbital or 3-methylcholanganthe. The increase in GST enzyme content and/or activity was paralleled by an elevation in the translational activity of the Ya mRNA. Additionally, the relative transcriptional rates of the GST Ya gene were elevated between 5- and 8-fold after treatment with either of these agents. Interestingly, phenobarbital is also known to inhibit AFB1 carcinogenesis (38), but whether the protective mechanism involves induction of monooxygenases like cytochrome P-450 or Phase II enzymes such as GSTs or other factors remains to be evaluated.

Our results with oltipraz also indicate that the initial increase in hepatic GST activity correlates with changes in steady state levels of GST Ya mRNA. In addition, these inductions correlate with early changes in GST Ya transcription rates and GST Ya protein levels, as judged from nuclear run-off and ELISA assays, respectively. Thus, the initial response to dietary oltipraz in GST Ya regulation appears to be mediated at the transcriptional level. However, prolonged oltipraz treatment (5–7 days) is associated with persistent elevation of enzyme activities and Ya subunit protein levels, while GST Ya mRNA levels and transcription rates decline towards pretreatment levels. This disparity presumably reflects differences in the turnover rates of GST Ya protein and mRNA. One possible explanation for our observation that GST Ya mRNA levels decline after day 3 despite continued feeding of oltipraz may be derived from a consideration of the signalling mechanisms for Phase II enzyme induction by oltipraz. Talalay et al. (39) have suggested that common chemical signals regulate the induction of Phase II enzymes and that the presence of an olefin conjugated to an electron-withdrawing thioketone in the 1,2-dithiole-3-thione nucleus is critical to the activity of this class of inducers. It is possible that altered rates and routes of the metabolism of oltipraz induced during chronic feeding may diminish the bioavailability of the chemically active form of oltipraz. Thus, the diminishing rates of GST Ya transcription as a function of oltipraz feeding may reflect a parallel diminution in the steady state hepatic concentrations of oltipraz. The metabolism of oltipraz has been well characterized in several mammalian species including the rat. After p.o. administration to rats, oltipraz is rapidly (plasma t½ = 2.5 h) excreted in the urine as both free and conjugated metabolites (40, 41). Glucuronides represent the major form of urinary metabolites. We have previously reported that oltipraz is an effective inducer of glucuronyl transferases in rat liver (8). Thus, chronic feeding of oltipraz may serve to induce enzymes leading to its enhanced elimination. However, the effects of enzyme induction on the
kinetics of oltipraz disposition have not been evaluated. Studies by Seidegård et al. (42, 43) suggest that deficiencies in the levels of expression of GSTs in humans may be important determinants for susceptibility to lung cancer. Therefore, the modulation of GST expression in humans by potent inducers such as 1,2-dithiole-3-thiones may offer a realistic strategy for chemoprotection, as has been demonstrated in carcinogen-exposed animals. However, the efficacy in humans of agents identified in rodent assays as functional inducers of GSTs remains to be established. Nonetheless, the development and evaluation of 1,2-dithiole-3-thiones and other classes of enzyme inducers as chemoprotective agents in humans will be greatly facilitated by increased understanding of the roles of these enzymes in chemical carcinogenesis and the mechanisms that regulate their expression.

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