Biochemical Pharmacology, Vol. 52, pp. 1831–1841, 1996.

ISSN 0006-2952/96/$17.00 + 0.00
PII S0006-2952(96)00505-9

Expression of Glutathione S-transferases Ya, Yb1, Yb2, Yc1 and Yc2 and Microsomal Epoxide Hydrolase Genes by Thiazole, Benzothiazole and Benzothiadiazole

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ABSTRACT. The effects of thiazole (TH), benzothiazole (BT) and benzothiadiazole (BZ) on the expression of hepatic glutathione S-transferases (GSTs) Ya, Yb1, Yb2, Yc1 and Yc2 and microsomal epoxide hydrolase (mEH) genes were compared in rats. TH treatment resulted in 4- to 24-fold increases in GST Ya mRNA levels at 24 hr posttreatment; the ED_{50} value was 70 mg/kg. GST Ya mRNA levels were elevated 13-, 20-, 20- and 9-fold at 12, 24, 48 and 72 hr following 100 mg/kg of TH treatment, respectively, as compared with the control. BT was a moderate inducer of GST Ya with a maximal 18-fold increase observed, whereas BZ treatment caused a transient increase in GST Ya mRNA at 12 hr posttreatment, followed by a return to a 4-fold relative increase at 24 hr or afterward. Treatment of rats with TH at the dose of 100 mg/kg resulted in an ~10-fold increase in either Yb1 or Yb2 mRNA levels at 24 hr posttreatment. BT-treated rats showed 7- and 3-fold increases in the GST subunit Yb1 and Yb2 mRNA levels at 24 hr posttreatment. BZ was the least effective in modulating either GST Yb1 or Yb2 mRNA, resulting in <2-fold changes. GST Yc1 and Yc2 mRNA levels were increased ~8-fold at the dose of 200 mg/kg of TH. BT minimally affected GST subunit Yc1 and Yc2 mRNA levels, with a maximal 4-fold relative increase observed. BZ was the least effective in enhancing Yc1 and Yc2 mRNA levels. Protein levels for GST subunit Ya, Yb1, Yb2 and Yc were also elevated in response to TH by 3-, 2-, 2- and 2-fold, respectively. Thus, TH was effective in modulating both constitutive and inducible GST gene expression. BT or BZ was much less effective in increasing the expression of GST subunits. These RNA and Western blot analyses revealed that the levels of major GST were differentially increased after treatment with these thiazoles, exhibiting a rank order of GST expression of TH > BT > BZ. mEH expression by these compounds appeared to be consistent with that of GST Ya. The mRNA levels for GST Ya, Yb1, Yb2, Yc1 and Yc2 and mEH were also determined after treatment with triazole (TR), imidazole (IM), benzoxazole (BX), benzotriazole (BTR) or benzimidazole (BIM). TR, IM, BX or BTR caused increases in Ya, Yb1, Yc1 and Yc2 mRNA levels by 2- to 3-fold, whereas the agents failed to modulate the expression of GST Yb2. The fact that benzene, cyclohexane or n-hexane minimally affected the major GST or mEH mRNA levels provided evidence that certain heterocyclic compounds are more capable of modulating GST or mEH gene expression than hydrocarbons. These results corroborate evidence that the thiazoles differentially stimulate GST or mEH genes, with TH being the most efficacious; that thiazoles with carbocyclic ring are much less effective in increasing GST or mEH levels than is TH; and that the changes in these GST and mEH levels are primarily associated with increases in mRNA levels. *Copyright ©1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;12:1831–1841, 1996.

KEY WORDS. glutathione S-transferase; epoxide hydrolase; thiazole; benzothiazole; benzothiadiazole; gene expression

Derivatives of thiazoles, including TH,† BT and BZ, are employed as intermediates in the production of a number of therapeutic agents. BT derivatives exhibit analgesic and anti-inflammatory activities, possibly through the mechanism of 5-lipoxygenase and thromboxane synthetase inhibition [1, 2]. Other BT compounds have gastric antisecretory activity [3] or an anthelmintic effect. Anthelmintic drug efficacy of BTs and BZs depends on the magnitude and molecular dipole moment and the percentage of polar surface area [4]. Increasing lipophilicity, decreasing size and increasing electron withdrawal of the ring substituent seem to be important parameters for their pharmacological activities, as supported by QSAR analysis of certain BTs active as anticonvulsants [5, 6]. These sulfur-containing compounds have also been identified as constituents in naturally occurring substances [7, 8].

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†*Abbreviations:* GST, glutathione S-transferase; mEH, microsomal epoxide hydrolase; EDTA, ethylenediamine tetraacetic acid; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TH, thiazole; BT, benzothiazole; BZ, benzothiadiazole; TR, triazole; IM, imidazole; BX, benzoxazole; BTR, benzotriazole; BIM, benzimidazole; AFB1, aflatoxin; EpRE, electrophile responsive element.

Received 12 March 1996; accepted 20 June 1996.

TH causes nephrotoxicity in mice, as supported by marked increases in serum urea nitrogen concentration [9]. Mizutani *et al.* suggested that nephrotoxicity of TH might be due to microsomal epoxidation of the 4,5-double bond, and the resulting epoxide could be decomposed following hydrolysis to form thioformamide, a potential toxic metabolite [9]. Depletion of glutathione by buthionine sulfoximine further increases TH-inducible tubular necrosis [9].

BTs are substrates and inhibitors of hepatic aldehyde oxidase, whereas heterocycles consisting of 5- or 6-membered ring thiazole or oxazole are neither substrates nor inhibitors [10]. A common feature of the compounds active in inhibition of peroxidase and cyclooxygenase is the presence of an -NCS or free -SH groups that are present in TH [11]. Derivatives based on TH, oxazole and IM, including 4-arylthiazole, exhibit an anti-anoxic effect and antilipid peroxidation [12]. Thus, THs have a wide variety of biological activities.

The induction of cytochrome P450 1A1 by BT derivatives has been reported [13]. The fact that certain BTs can be metabolized primarily by glucuronide and sulfate conjugation implicates the role of phase II conjugating detoxifying enzymes in the metabolism of these compounds [14]. In fact, GST appears to be involved in the metabolism of 2-methylthiobenzothiazole in the rat liver, leading to S-(2-benzothiazolyl)glutathione formation after oxidation into its methylsulphoxide and methylsulphone [15].

The metabolic profile of BT has been studied in guinea pigs. Five urinary cleavage metabolites of BT include 2-methylmeraptoaniline, 2-methylsulphinylaniline, 3-methylsulphinylaniline, 2-methylsulphinylphenylhydroxylamine and 3-methylsulphinylphenylhydroxylamine. The first three are excreted in either conjugated or unconjugated forms [16]. BT is easily degraded in activated sludge systems, suggesting that this compound is relatively unstable in a microbiological metabolizing system [17].

Given these wide uses and applications of TH derivatives and their toxicological significance, we were interested in the gene expression of GSTs and mEH in response to TH and BTs and the molecular structural basis for GST gene expression. Previous studies have shown that TH and pyrazine, nitrogen- and sulfur-containing prototypic heterocycles efficaciously induce mEH with substantial increases in mEH mRNA levels [18, 19]. As part of a complete evaluation of the effects of N- and S-containing heterocycles on drug-metabolizing enzyme expression, expression of five major GST subunits, including GST Ya, Yb1, Yb2, Yc1, Yc2 and mEH, was compared. The present study establishes that TH, BT and BZ differentially induce GST and mEH proteins and that the resulting changes in these detoxifying enzymes are primarily associated with increases in their mRNA levels.

Toward the end of this study, other representative heterocyclic compounds and cyclic or linear aliphatic hydrocarbons devoid of heteroatom(s) were used to compare their efficacy further in inducing the detoxifying enzymes.

MATERIALS AND METHODS

Materials

[α-^{32}P]dATP (185 TBq/mmol) and [γ-^{32}P]ATP (185 TBq/mmol) were purchased from NEN (Arlington Heights, IL, USA). TH, BT, BZ, TR, IM, BX, BTR, BIM, benzene, cyclohexane and n-hexane were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Chemical structures of the compounds are shown in Fig. 1. Biotinylated goat anti-rabbit IgG, streptavidin-conjugated horseradish peroxidase, and random prime-labeling and 5′-end-labeling kits were purchased from Promega (Madison, WI, USA). Anti-GST Ya, Yb1, Yb2 and Yc were purchased from Biotrin International (Dublin, Ireland). Most of the reagents in the molecular studies were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Animal Treatment

Male Sprague-Dawley rats (180–220 g) were treated with TH, BT or BZ in aqueous or corn-oil solution (10–200 mg/kg body weight/day, i.p., 1–3 days). Rats were intraperitoneally treated with either TR, IM, BX, BTR or BIM in aqueous or corn-oil solution at the daily dose of 200 mg/kg for 3 days and were killed at 24 hr after the last treatment. In a time-dependent study, animals were killed at different time points after a daily injection of each compound. Animals were fasted 16 hr prior to killing. Each data point

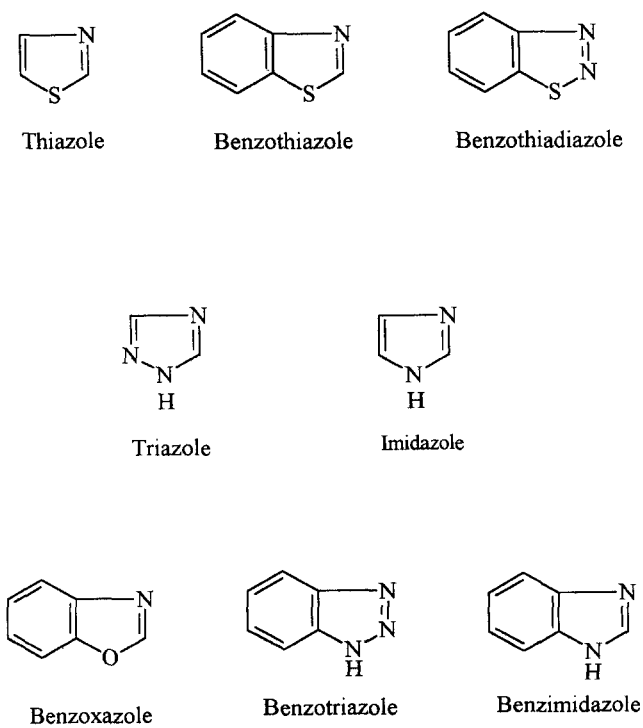

FIG. 1. Chemical structures of the thiazoles, azoles and benzoazoles.

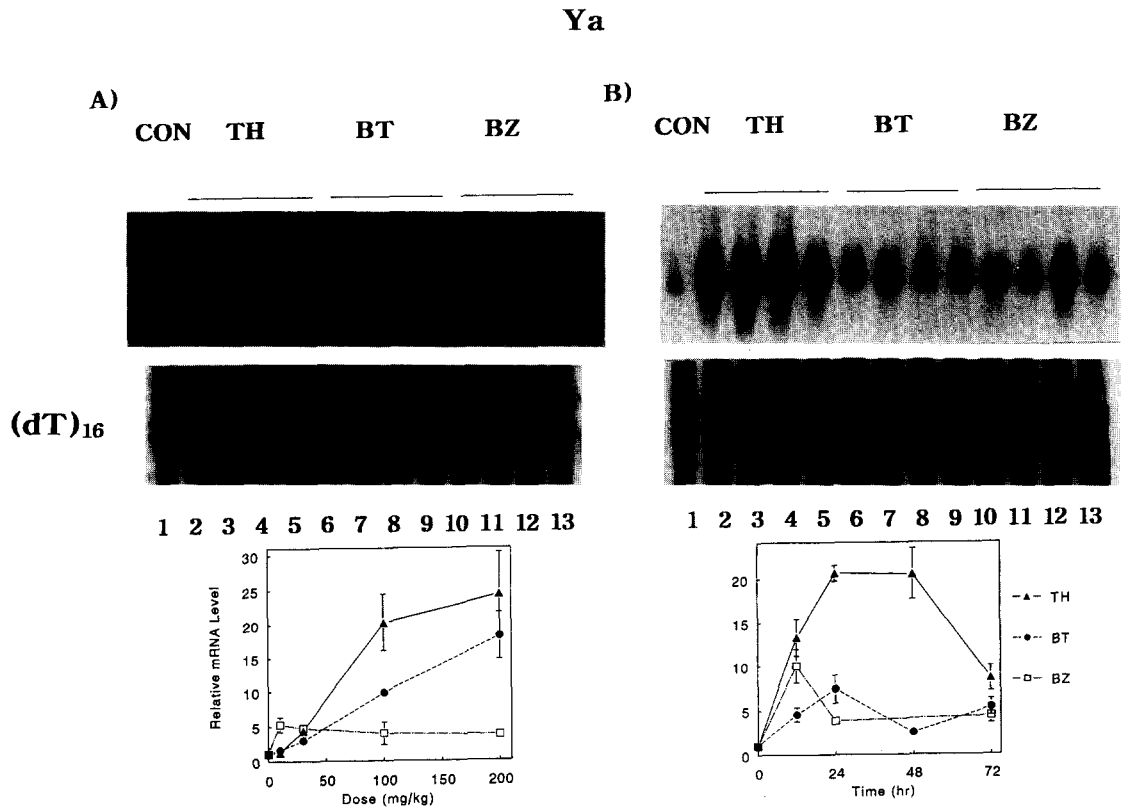

FIG. 2. RNA blot analyses of mRNA levels in hepatic GST Ya after TH, BT or BZ treatment. (A) Northern blot analysis was performed to examine GST Ya levels in total RNA fractions (20 μg each) isolated from rats at 24 hr after treatment with vehicle (lane 1), with TH at the dose of 10, 30, 100, or 200 mg/kg b.w. (lanes 2–5, respectively), with BT (lanes 6–9, respectively) or with BZ (lanes 10–13, respectively) at the same doses. Relative increases in GST Ya mRNA levels were depicted as a function of dose. GST Ya levels were assessed by scanning densitometry of the Northern blots. Each point represents the mean ± SD (N = 4 or 5). (B) Northern blot analysis for GST Ya mRNA levels in total RNA isolated from rats treated at the daily dose of 100 mg/kg b.w. Shown are the hepatic RNA levels in vehicle-treated animals (lane 1) or in rats at 12, 24, 48 and 72 hr after treatment with TH (lanes 2–5, respectively), BT (lanes 6–9, respectively) or BZ (lanes 10–13, respectively). The amount of RNA loaded in each lane was assessed by rehybridization of the stripped membrane with ^{32}P-labeled poly(dT)$_{16}$. The representative stripped membrane rehybridized is shown. Relative changes in GST Ya mRNA levels were plotted by following scanning densitometry.

consisted of samples pooled with groups of animals. Three rats were employed in each treatment group. Results were confirmed with different groups of animals.

Isolation of Microsomal and Cytosolic Proteins

Hepatic microsomal and cytosolic fractions prepared by differential centrifugation were washed in pyrophosphate buffer and stored in 50 mM Tris acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol. Microsomal and cytosolic preparations were stored at −80°C until use. Protein content was determined by the method of Lowry *et al.* [20].

Gel Electrophoresis

SDS-PAGE analysis was performed according to Laemmli [21] by using a Hoefer gel apparatus.

Immunoblot Analysis

Immunoblot analysis was performed according to previously published procedures [22, 23]. Microsomal or GST proteins were separated by 8% and 12% SDS-PAGE, respectively, and electrophoretically transferred to nitrocellulose paper, which was incubated with rabbit anti-rat GST Ya, Yb1, Yb2, Yc and mEH antibodies. Biotinylated goat anti-rabbit IgG was used as the secondary antibody. Immunoreactive protein was visualized by incubation with streptavidin-horseradish peroxidase, followed by addition of 4-chloro-1-naphthol and hydrogen peroxide.

Isolation of Total RNA

Total RNA was isolated by using the improved single-step method of thiocyanate-phenol-chloroform RNA extraction according to the method of Puissant and Houdebine [24].

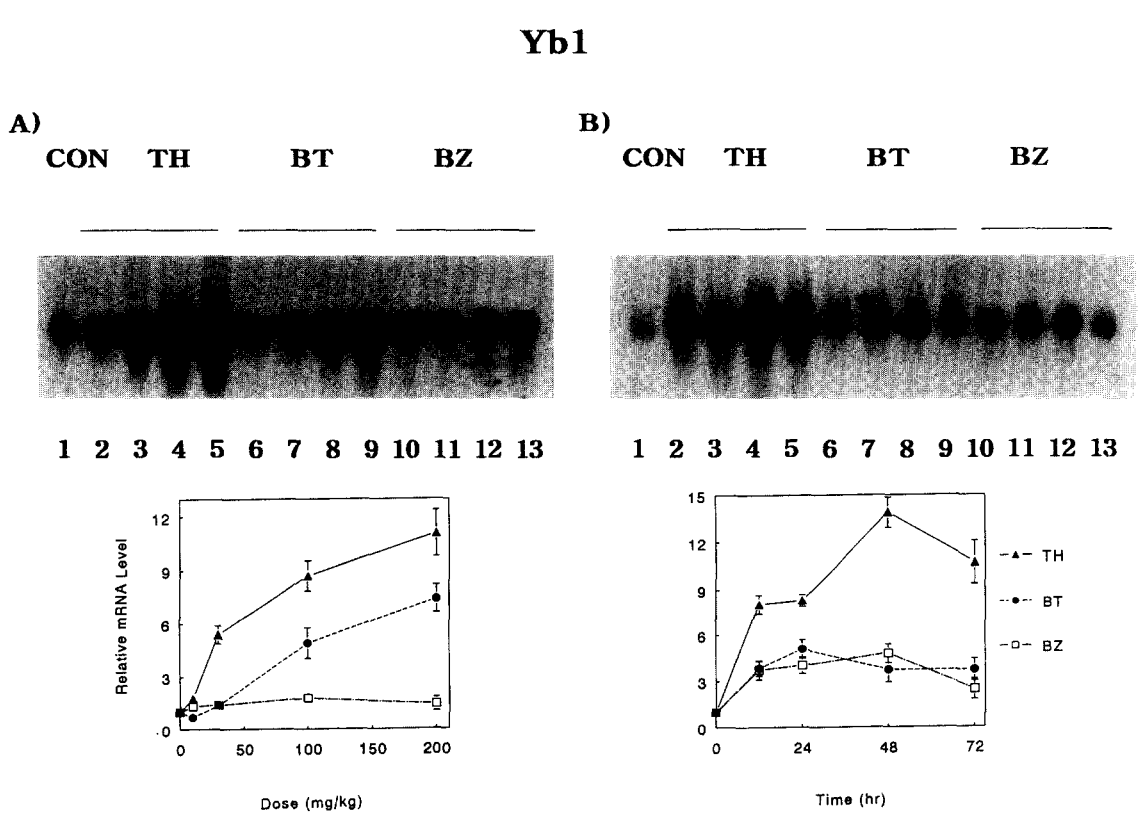

FIG. 3. RNA blot analyses of hepatic GST Yb1 mRNA levels after TH, BT or BZ treatment. (A) Northern blot analysis was performed with the samples isolated after various doses of thiazoles. (B) Time course of GST Yb1 mRNA levels at the daily dose of 100 mg/kg b.w. The lanes represent the same conditions as those shown in Fig. 2.

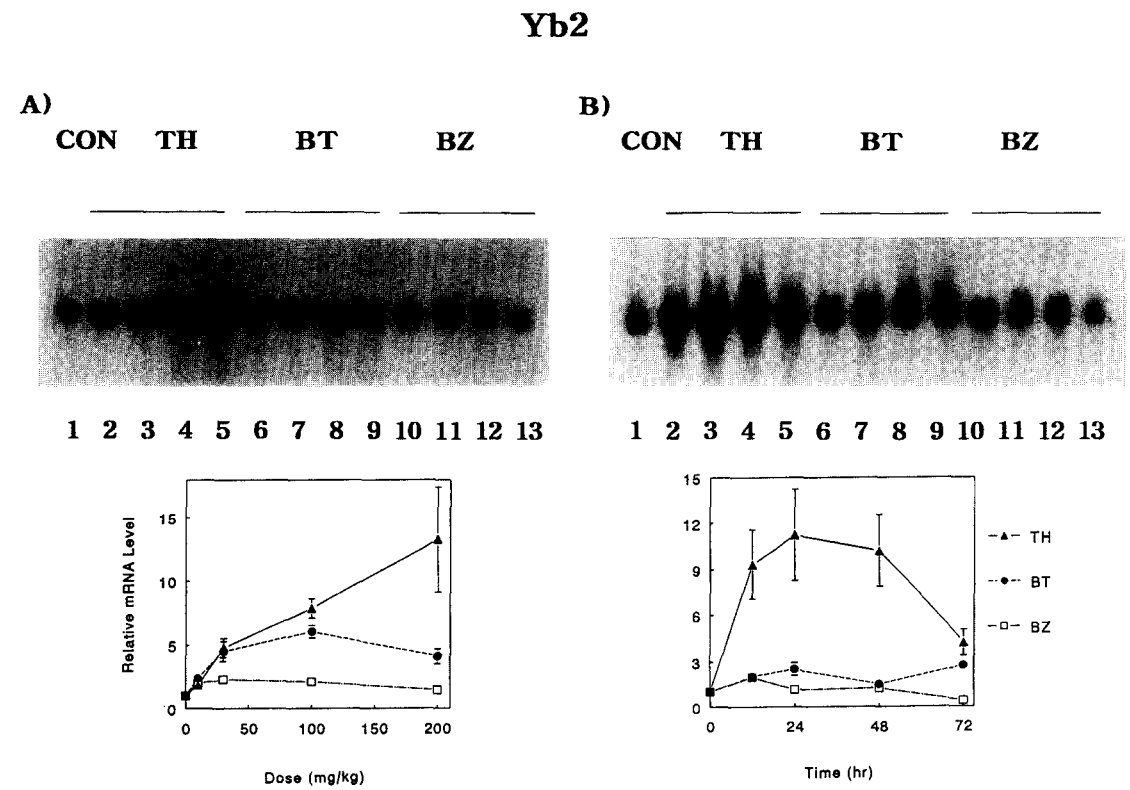

FIG. 4. RNA blot analyses of hepatic GST Yb2 mRNA levels after TH, BT or BZ treatment. (A) Northern blot analysis was performed with the samples isolated after different doses of thiazoles. (B) Time course of GST Yb2 mRNA levels at the daily dose of 100 mg/kg b.w. The lanes represent the same conditions as those shown in Fig. 2.

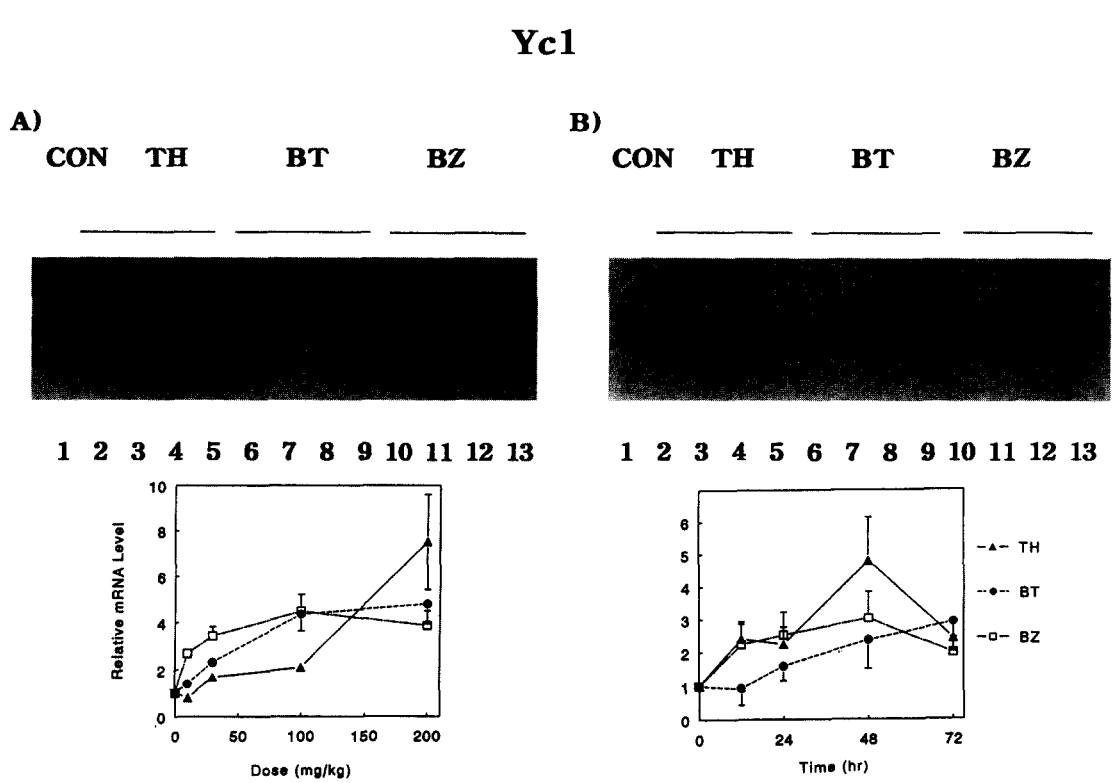

FIG. 5. RNA blot analyses of hepatic GST Yc1 mRNA levels after TH, BT or BZ treatment. (A) Northern blot analysis was performed with the samples isolated after different doses of thiazoles. (B) Time course of GST Yc1 mRNA levels at the daily dose of 100 mg/kg b.w. The lanes represent the same conditions as those shown in Fig. 2.

Preparation of cDNA Probes for GST Subunits and mEH

Specific cDNA probes for GST genes Ya (287–684), Yb1 (643–963), Yb2 (415–942), Yc1 (122–488) and Yc2 (122–530) were amplified by reverse transcriptase-polymerase chain reaction by using the selective primers for each gene [25–28]. cDNA derived from hepatic poly(A)$^+$ RNA obtained from rats treated with pyrazine was cloned in a pGEM + T vector (Promega). A cDNA for mEH was prepared as described previously [29].

Northern Blot Hybridization

Northern blot was done according to the procedures described previously [29, 30]. Briefly, total RNA isolated from rat livers was resolved by electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde and transferred to supported nitrocellulose paper. The nitrocellulose paper was baked in a vacuum oven at 80°C for 2 hr. The blot was incubated with hybridization buffer containing 50% deionized formamide, 5× Denhardt's solution [0.1% Ficoll, 0.1% polyvinylpyrrolidine and 0.1% bovine serum albumin (Pentex Fraction V)], 0.1% SDS, 200 μg/mL of sonicated salmon sperm DNA and 5× SSPE (1× SSPE: 0.15 M NaCl, 10 mM NaH_2PO_4 and 1 mM Na_2EDTA, pH 7.4) at 42°C for 1 hr without probe. Hybridization was performed at 42°C for 18 hr with a heat-denatured cDNA probe, which was random prime labeled with [α-^{32}P]dATP. Filters were washed twice in 2× SSC and 0.1% SDS for 10 min at room temperature and twice in 0.1X SSC and 0.1% SDS for 10 min at room temperature. The filters were finally washed in the solution containing 0.1× SSC and 0.1% SDS for 60 min at 60°C. After quantitation of mRNA levels, the membranes were stripped and rehybridized with ^{32}P-end-labeled poly(dT)$_{16}$ to quantify the amount of RNA loaded onto the slots. Duplicate Northern blot analyses were performed on different mRNA samples.

Scanning Densitometry

Scanning densitometry was performed with a Microcomputer Imaging Device (Model M1, Imaging Research, St. Catharines, Ontario, Canada). The area of each lane was integrated by using MCID software (version 4.20, rev 1.0), followed by background subtraction.

RESULTS

Effects of TH, BT and BZ

EXPRESSION OF GST Ya BY THIAZOLES. RNA blot analyses were done to determine the mRNA expression of major GST genes in response to TH, BT or BZ. Northern blot analyses were performed to examine changes in GST Ya. Treatment of animals with TH resulted in 4- to 24-fold increases in the mRNA levels of GST Ya subunit at 24 hr

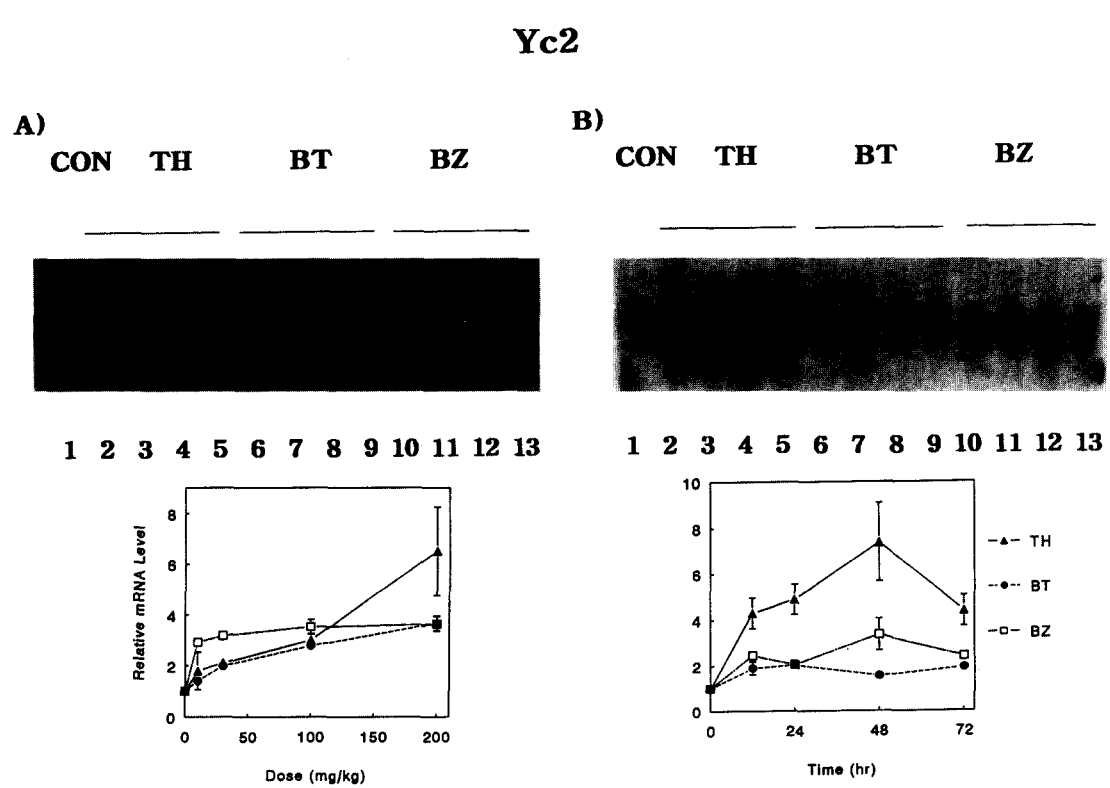

FIG. 6. RNA blot analyses of hepatic GST Yc2 mRNA levels after TH, BT or BZ treatment. (A) Northern blot analysis was performed with the samples isolated after different doses of thiazoles. (B) Time course of GST Yc2 mRNA levels at the daily dose of 100 mg/kg b.w. The lanes represent the same conditions as those shown in Figure 2.

posttreatment at the doses of 10–200 mg/kg; the ED_{50} value was 70 mg/kg (Fig. 2A). An experiment was designed to examine GST mRNA expression by these thiazoles as a function of time. Time courses in relative mRNA levels in the expression of GST major subunits in response to TH, BT or BZ are depicted in Fig. 2B. Northern RNA blot analyses performed with hepatic total RNA revealed substantial time-dependent increases in GST Ya mRNA levels from 12 hr to 72 hr time points. Hepatic GST Ya mRNA levels were elevated 13-, 20-, 20- and 9-fold at 12, 24, 48 and 72 hr following TH treatment, respectively, relative to control. Thus, the elevation in GST Ya mRNA levels peaked at 24 hr and plateaued at 48 hr posttreatment.

BT appeared to be a moderate inducer of GST Ya, with a maximal 18-fold increase being observed at the dose of 200 mg/kg, whereas dose of less than 100 mg/kg resulted in an ~3-fold induction (Fig. 2A). Changes in Ya mRNA after BT fluctuated somewhat, resulting in 5- to 7-fold increases over the time points examined (Fig. 2B).

BZ was even less effective in modulating GST Ya expression, resulting in 4- to 5-fold relative increases (Fig. 2A). In contrast with TH or BT, BZ treatment exhibited a transient increase in GST Ya mRNA at 12 hr posttreatment, decreasing to an ~4-fold increase relative to control at 24 hr or afterward (Fig. 2B).

EXPRESSION OF GST Yb1 AND Yb2 BY THIAZOLES. Treatment of rats with TH at the dose of 100 mg/kg resulted in ~10-fold increases in both Yb1 and Yb2 mRNA levels at 24 hr posttreatment (Figs. 3A, 4A). Maximal increases in GST Yb1 and Yb2 by TH were also observed at these time points (Figs. 3B, 4B).

BT-treated rats showed 7- and 3-fold increases in GST subunit Yb1 and Yb2 mRNA levels at 24 hr posttreatment at the dose of 200 mg/kg (Figs. 3A, 4A). The increase in GST Yb mRNA levels in response to 100 mg/kg of BT was 50% less than that in GST Ya mRNA.

BZ was the least effective in modulating either GST Yb1 or Yb2 even at the dose of 200 mg/kg, resulting in ~2-fold changes (Figs. 3A, 4A). BT- or BZ-induced increases in Yb1 or Yb2 mRNA levels observed as a function of time were substantially less than those caused by TH, resulting in <4-fold increase (Figs. 3B, 4B). Thus, the rank order of Yb1 and Yb2 mRNA increases by the thiazoles was the same as that in Ya (or mEH) gene expression.

EXPRESSION OF GST Yc1 AND Yc2 BY THIAZOLES. GST Yc1 and Yc2 mRNA levels were increased 8- and 7-fold, respectively, in response to 200 mg/kg of TH (Figs. 5A, 6A). However, TH at the doses of 100 mg/kg or less was minimally effective in modulating the levels of Yc subunits (~2-fold). Nonetheless, TH was effective in modulating both constitutive and inducible GST gene expression. TH-induced peak increases in the subunits Yc1 and Yc2 mRNA were observed at 48 hr after treatment, with 5- and

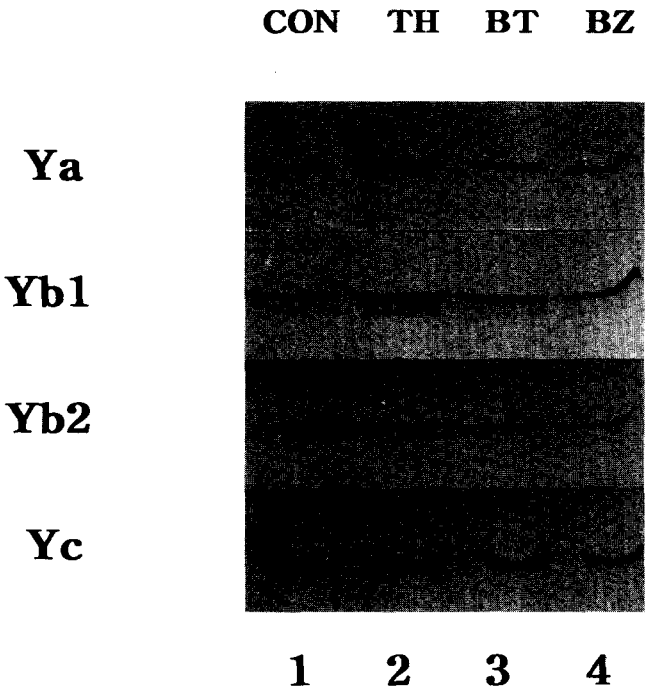

FIG. 7. Immunoblot analyses of rat hepatic cytosolic fraction with rabbit anti-rat GST antibodies. Each lane was loaded with 1.5 μg of cytosolic proteins for Ya or Yc or with 3 μg of the proteins for Yb1 or Yb2 immunoblots. These blots exhibit protein levels in GST subunits Ya, Yb1, Yb2 or Yc in liver cytosolic fractions isolated from vehicle- (lane 1), TH- (lane 2), BT- (lane 3) or BZ- (lane 4) treated rats (100 mg/kg b.w./day, i.p., 3 days). Animals were killed 24 hr after the last treatment to obtain cytosolic fraction. Duplicate immunoblot analyses were performed on different cytosolic samples.

7-fold increases, respectively, being noted (Figs. 5B, 6B). The mRNA levels for Yc1 and Yc2 were decreased from the maximal increases by 30% after 3 consecutive daily treatments.

GST subunit Yc1 and Yc2 mRNA levels were minimally affected by BT as a function of dose, resulting in a maximal 4-fold increase at the dose of 200 mg/kg (Figs. 5A, 6A). Although BT was more lipophilic than TH, rats treated with BT exhibited ~2-fold changes in Yc2 mRNA levels. Among the thiazoles, BZ appeared to be the least effective in modulating GST Yc mRNA levels (Figs. 5A, 6A). Approximately 2- to 4-fold changes were observed at the dose of 200 mg/kg. Both BT and BZ were minimally effective in modulating GST Yc1 or Yc2 as a function of time (Figs. 5B, 6B). The Yc2 subunit mRNA level was more rapidly increased than were other subunits.

These RNA blot analyses revealed that levels of major GST mRNA were differentially increased after treatment with the thiazoles, with a rank order of GST Ya mRNA expression of TH > BT > BZ. The Northern blot analyses also demonstrated the integrity of employed RNA and the specificity of the probe as detected by the single bands. These results demonstrate that the thiazoles differentially induced GST genes, with TH being the most efficacious, and that the changes in these GST levels were primarily associated with increases in their mRNA levels.

WESTERN BLOT ANALYSES OF GST PROTEINS. Expression of GST subunits was assessed by immunochemically quantifying protein levels. Hepatic cytosol prepared from rats treated with TH for 3 consecutive days exhibited significant increases in GST protein levels, including subunits Ya, Yb1, Yb2 and Yc by 3-, 2-, 2- and 2-fold, respectively (Fig. 7). TH-induced GST Ya most prominently among the forms of GSTs examined, whereas GST Yb1 and Yb2 were moderately elevated by the agent. BT and BZ were much less effective in increasing the expression of GST subunits. In particular, GST Yc induction by BT was minimal. Hepatic cytosol produced from rats treated with BZ failed to increase the levels of GST proteins.

RNA BLOT ANALYSES OF HEPATIC mEH mRNA LEVELS. Northern blot analyses were performed to examine mEH mRNA levels in total RNA fractions isolated from rats at 24 hr after treatment with TH, BT or BZ (Fig. 8A, B). Treatment of animals with TH at the dose of 10, 30, 100 or 200 mg/kg b.w. resulted in 1-, 9-, 20- and 25-fold increases in mEH mRNA levels (Fig. 8A). TH maintained the maximal 20-fold increases in mEH mRNA levels after multiple daily treatments at the dose of 100 mg/kg. BT failed to increase mEH mRNA levels except at the dose of 200 mg/kg. BZ caused no increase in mEH mRNA levels at the doses employed. Thus, thiazoles with carbocyclic rings were much less effective in increasing mEH mRNA levels than was TH. BT treatment resulted in a minimal 4-fold increase at 3 days after treatment. Again, BZ failed to affect mEH gene expression after 3 consecutive daily treatments.

IMMUNOBLOT ANALYSIS FOR mEH LEVELS. mEH induction was monitored by measuring mEH protein levels immunochemically (Fig. 9). Whereas treatment of animals with TH resulted in marked induction of mEH, hepatic microsomes isolated from rats treated with BT caused a minimal 2-fold increase. BZ failed to induce mEH. The rank order of mEH induction by these azole heterocycles was TH > BT > BZ.

Effects of Other Heterocyclic or Nonheterocyclic Compounds

The mRNA levels for GST Ya, Yb1, Yb2, Yc1 and Yc2 were determined in rats treated with TR, IM, BX, BTR or BIM at the dose of 200 mg/kg for 3 days to compare the efficacy of GST induction by other representative heterocycles (Fig. 10). TR, IM, BX and BTR caused increases in Ya, Yb1, Yc1 and Yc2 mRNA levels by 2- to 3-fold, as compared with vehicle-treated animals. TR treatment caused an ~8-fold increase in the mEH mRNA level, relative to control. Most of the compounds examined minimally modulated the expression of Yb2 mRNA levels.

The effects of aromatic or aliphatic hydrocarbons, including benzene, cyclohexane or n-hexane, on the expres-

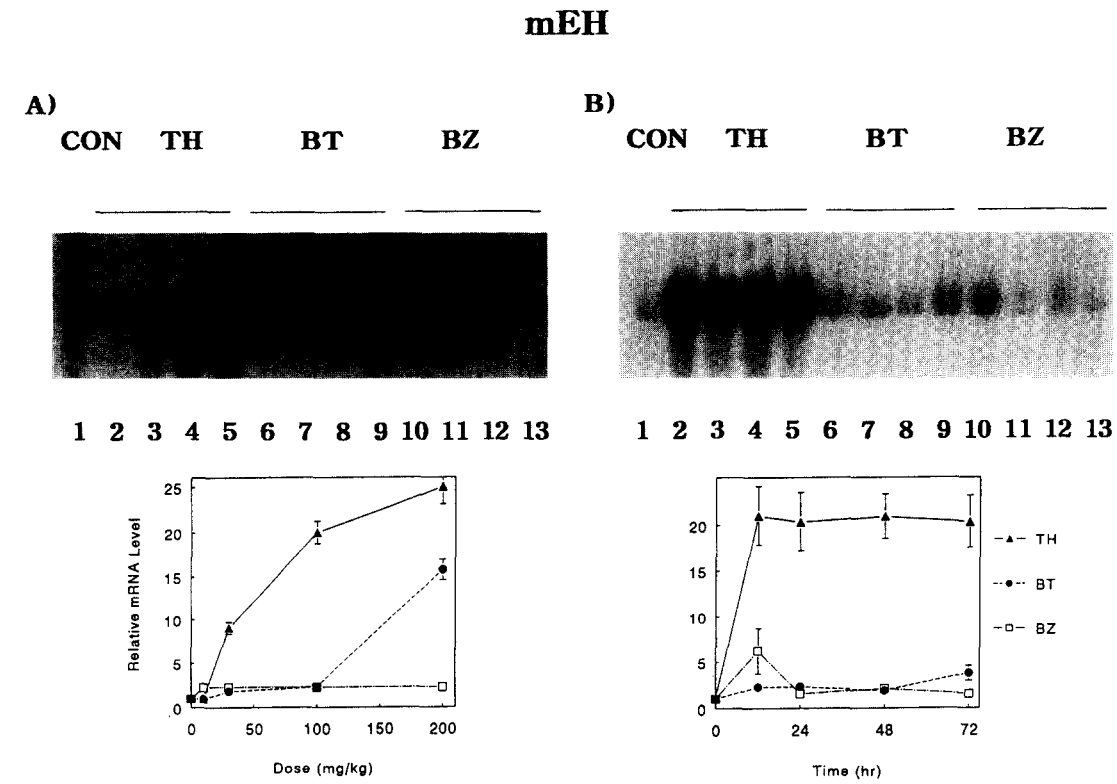

FIG. 8. RNA blot analyses of hepatic mEH mRNA levels after TH, BT or BZ treatment. (A) Northern blot analysis was performed with the samples isolated after different doses of thiazoles. (B) Time course of mEH mRNA levels at the daily dose of 100 mg/kg b.w. The lanes represent the same conditions as those shown in Fig. 2.

sion of major detoxifying enzymes were also examined. Animals treated with these compounds exhibited minimal changes in major GST or mEH mRNA levels (data not shown). These results provide evidence that certain heterocyclic compounds are selectively effective in modulating GST or mEH gene expression.

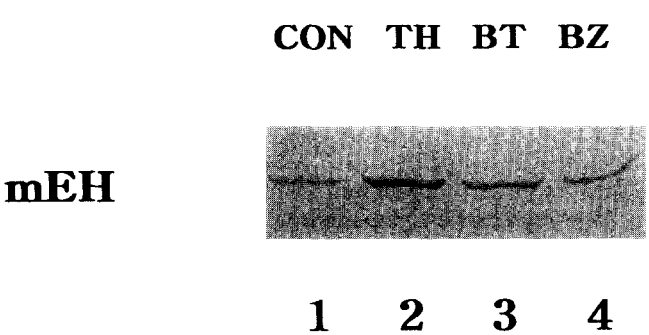

FIG. 9. Immunoblot analysis of rat hepatic microsomes with rabbit anti-rat mEH antibody. Each lane was loaded with 5 μg of rat liver microsomes. This representative blot exhibits mEH protein levels in the hepatic microsomes isolated after treatment with vehicle (lane 1), TH (lane 2), BT (lane 3) or BZ (lane 4) at the daily dose of 100 mg/kg b.w. for 3 days (i.p.). Microsomes were prepared at 24 hr after the last treatment. An apparent increase in mEH protein levels was noted after TH treatment, whereas BT caused a moderate increase. Duplicate immunoblot analyses were performed on different hepatic microsomes.

DISCUSSION

GSTs and mEH catalyze the detoxication of epoxide reactive intermediates. Previous studies have shown that levels of mEH mRNA are substantially increased after heterocycle treatment. Given that both mEH protein and mRNA levels are elevated after treatment with TH and that THs and BTs form part of the molecular structure of a number of natural products and therapeutic agents, this study was designed to examine the changes in the major GST gene expression in response to TH and representative thiazoles with carbocyclic rings and the molecular basis for the expression of the enzymes.

Previous studies have shown that mEH mRNA levels are elevated at early times following a single injection of TH [18, 19]. The time-dependent changes in GST Ya mRNA levels after TH treatment were comparable to those in mEH. Time courses in GST Ya gene expression by the thiazoles with carbocyclic rings were also quite comparable to those in mEH gene, with the maximal increase noted at 12–24 hr posttreatment, which suggests that these heterocyclic compounds may have a common molecular mechanism for GST Ya and mEH induction. Differential induction and limitation of maximal induction by these compounds may involve the antioxidant responsive element (ARE)/EpRE, which has been identified in the genes of phase II metabolizing enzymes. GST Ya gene is transcrip-

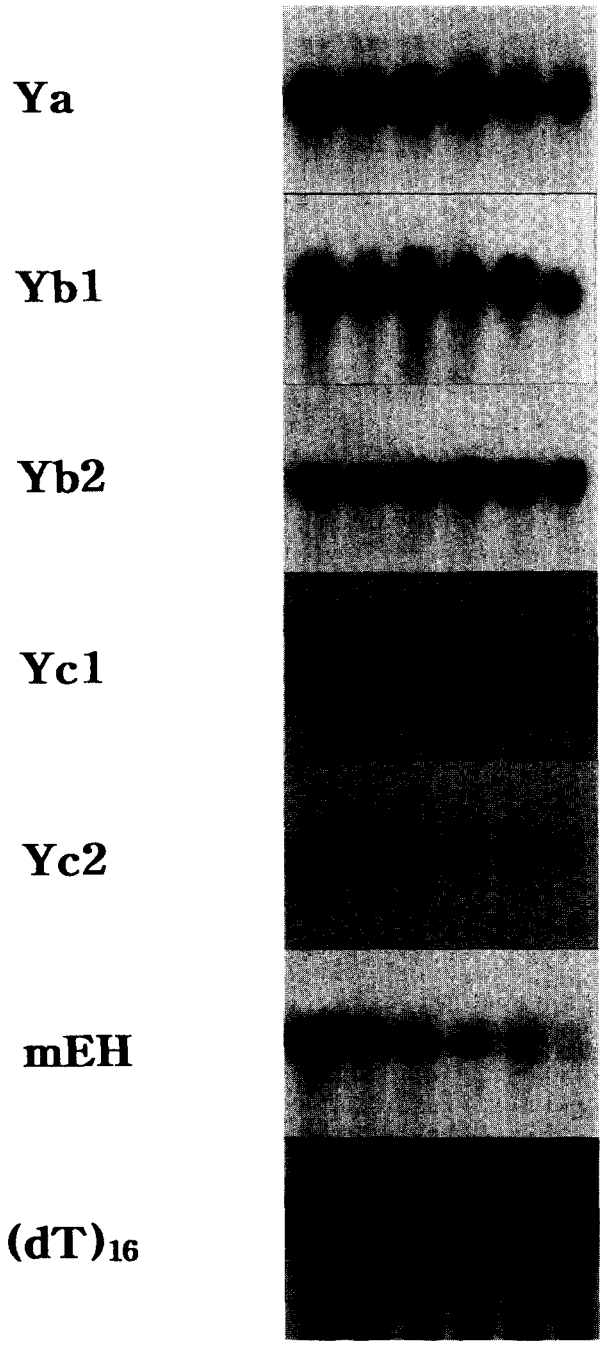

FIG. 10. RNA blot analyses of hepatic GST and mEH mRNA levels after TR, IM, BX, BTR or BIM treatment. Northern blot analyses were performed to examine changes in GST Ya, Yb1, Yb2, Yc1, Yc2 and mEH mRNA levels in total RNA fractions (20 μg each) isolated after treatment with TR (lane 1), IM (lane 2), BX (lane 3), BTR (lane 4) or BIM (lane 5) at the dose of 200 mg/kg for 3 days. Lane 6 represents the mRNA level in vehicle-treated animals. Animals were killed 24 hr after the last treatment to obtain total RNA fraction. The amount of RNA loaded in each lane was assessed by rehybridization of the stripped membrane with ^{32}P-labeled poly(dT)$_{16}$.

tionally regulated by xenobiotics, including phenolic antioxidants, through the expression of AP-1 transcriptional regulatory proteins. The complex of Fos/Jun (AP-1) binds to AP-1-like binding sites in the 5′-flanking region of GST Ya and Pi-class genes and is responsible for basal and inducible expression by xenobiotics [31, 32]. The ARE on the rat GST subunit Ya gene is also responsive to hydrogen peroxide, reactive oxygen species and phenolic antioxidants that undergo redox cycling [33]. Thus, the indication of certain GST gene expression is mediated by oxidative stress in response to a variety of chemical agents.

Because GST Yb1 and Yb2 are constitutively expressed to a greater extent than are other subunits, the relative changes in these subunits were less marked than those in Ya and Yc2. The induction of Yb1 or Yb2 was ~50% of GST Ya subunit expression, possibly because of the lack of ARE or AP-1 binding sites in the 5′-regulatory sequence, as in the mouse Yb1 gene [34].

Although the polypeptide sequencing of rat Yc1 and Yc2 is 90% homologous, the increase in Yc1 mRNA is usually less marked than that in Yc2 mRNA. The induction of the Yc1 subunit by these heterocycles was also much less than that of either Ya or Yc2, as was observed with ethoxyquin or oltipraz. Although 3-methylcholanthrene induces Ya and mu-class GSTs, this agent fails to induce Yc2 in rats. An interesting result of this study was the finding of the extent of Yc2 expression by TH, which is nephrotoxic. The exact mechanism by which TH operates and its role in GST expression in association with nephrotoxicity should be further clarified.

A number of synthetic compounds such as ethoxyquin, butylated hydroxyanisole and oltipraz protect the rat from the electrophilic attack of AFB1 epoxide. Ethoxyquin, which is used as an antioxidant in feed and food and as antidegradation agent for rubber, contains a carbocyclic ring and a nitrogen atom in its chemical structure. These agents serve as inducers of major forms of GST, including Yc2. Ethoxyquin induces the level of the Yc2 subunit by 10–15-fold with a 5-fold increase in AFB1-epoxide metabolism [35, 36]. High levels of Yc2 expression in fetal liver render the fetal rat resistant to AFB1. The difference in the expression of Yc in rats and mice accounts for the distinct susceptibility of these species to hepatotoxins [28, 36]. Because Yc2 is developmentally modulated in male and female rat liver, the expression of this form of GST may be hormonally regulated. Maturation-related sexual dimorphism in Yc2 has also been demonstrated. Because the expression of Yc2 is associated with protection against chemical carcinogenesis of certain hepatotoxicants including AFB1, chemical modulations in Yc2 may provide the basis for the design of chemopreventive agents [28].

This study clearly shows that thiazoles with carbocyclic rings make the molecules less effective in stimulating GST expression. Addition of nitrogen atom in the ring of TH makes it far less efficacious. Changes in lipophilicity may affect the absorption of the compounds, and the sulfur or nitrogen atoms in thiazoles probably act as either a ligand

or active moiety in the induction of the drug-metabolizing enzymes. The rank order of dipole moments of the compounds may be associated with the inducibility of the agents. Nonetheless, their dipole moments appeared not to be strictly related with the expression of GSTs and mEH. Rather, these agents can generate oxidative stress *in vivo*, which can lead to induction of the Yc2 and Ya subunits. In particular, TH and BT are prone to be under ring cleavage, probably through cytochrome P450 [9, 10]. Compounds containing a 5-membered TH ring may undergo attack at one of the heteroatoms. Substituted BTs also undergo conjugation after ring hydroxylation. Hence, ring cleavage products from TH or BT through oxidation (e.g. cleaved metabolites including sulphinyl or sulphonyl amines) may produce the reactive oxygen species, including hydroxyl free radical, which may be responsible for stimulating GST and mEH induction.

Among the 13 GST isozymes identified in rats, these five genes, which have distinct biochemical characteristics with different substrate specificities, are the major forms in the rat liver. This study establishes the rank order of heterocyclic compounds in the expression of the major GST isoforms and the extent of elevated expression for each of the major GST forms in response to thiazoles. It also demonstrates that TH is an effective inducer of GST Ya, Yb1, Yb2, Yc1 and Yc2. The lack of strong induction of GST and mEH by TR, IM, BX, BTR or BIM further supports the hypothesis that certain heterocyclic compounds are distinctly capable of modulating GST or mEH gene expression. The fact that benzene, cyclohexane or n-hexane failed to notably increase the major GST or mEH mRNA levels provides evidence that lipophilicity or pi-electron distribution alone might not be the factors responsible for modulating detoxification enzyme expression.

This work was supported by research grant 951-0710-001-02 from the Korean Science and Engineering Foundation (SGK) and in part by a grant form Ministry of Health and Welfare and a research grant from Duksung Women's University.

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