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ENHANCED EXPRESSION OF RAT MICROSOMAL EPOXIDE HYDROLASE GENE BY ORGANOSULFUR COMPOUNDS

NAK DOO KIM,* SANG GEON KIM† and MI KYONG KWAK

College of Pharmacy, Seoul National University and †College of Pharmacy, Duksung Women's University, Seoul, Korea

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Abstract—The effects of organosulfur compounds including allylsulfide (AS), allylmercaptan (AM) and allylmethylsulfide (AMS) on the expression of microsomal epoxide hydrolase (mEH) protein and its mRNA were examined in rats. The levels of mEH induction were examined with or without concomitant treatment of animals with pyrazine, a strong inducer of mEH, in order to establish whether a common molecular basis exists for mEH induction between these structurally different xenobiotics. Immunoblot analyses using anti-rat mEH antibody showed that treatment with AS caused an ~4-fold increase in hepatic mEH protein levels relative to controls whereas treatment with both AS and pyrazine resulted in only minimal additive increases in the elevation of mEH. Administration of AM to rats resulted in a comparable increase in mEH levels to that caused by AS, whereas an ~2-fold increase was noted after AMS treatment, as compared to control. mEH levels in the hepatic microsomes isolated from animals treated with both AMS and pyrazine were, however, ~50% less than those from pyrazine-treated rats. Thus, AS and AM appeared to be more effective than AMS in elevating mEH, as evidenced by immunoblot analyses. The levels of mEH mRNA were increased 10–16-fold following treatment with either AS or AM, while AMS caused a 3–7-fold increase relative to control, as assessed by slot blot analysis probed with a 1.3 kb mEH cDNA. Time-dependent increases in mRNA levels by each of these organosulfur compounds were consistent with those in mEH protein levels at 3 days. A marginal additive increase in mEH mRNA levels was noted following co-administration of either AS or AM with pyrazine, whereas treatment with both AMS and pyrazine decreased mEH mRNA levels by 55%. Significant mEH mRNA increases in poly(A)$^+$ RNA fractions were confirmed by northern blot analysis. The results demonstrate that these organosulfur compounds are inducers of mEH and that the induction involves increases in its mRNA.

Microsomal epoxide hydrolase (mEH‡) catalyses the hydration of epoxide intermediates formed by cytochromes P450. The expression of mEH is primarily associated with detoxication, protecting the cellular macromolecules from reactive epoxide intermediates [1–4], although mEH also provides the metabolic precursors of the vicinal diol-epoxides, which play a critical role in the carcinogenicity of several polycyclic aromatic hydrocarbons [5].

Diallylsulfide, a component of garlic oil, has recently been shown to both suppress the activity of cytochrome P4502E1 (CYP2E1) and exert a potent inhibitory effect on the induction of colon and liver cancer by chemical carcinogens [6, 9]. Our laboratories have also shown that certain organosulfur compounds cause a marked suppression of hepatic CYP2E1 expression in rats [10]. The selective suppression of CYP2E1 seemed to be associated with its anticarcinogenic properties [6]. Although the effects of diallylsulfide on the expression of CYP2E1 have been examined, no information is yet available regarding the role of the organosulfur compounds in the expression of mEH. Thus, the present study was initiated to establish whether allylsulfide (AS), allylmercaptan (AM) and allylmethylsulfide (AMS) elevate mEH protein and whether mEH induction by these compounds involves an increase in its mRNA levels. Furthermore, given the observations that pyrazine substantially modulates the levels of mEH protein, along with simultaneous increases in CYP2E1 and glutathione *S*-transferase [11, 12] levels, we examined whether a common molecular basis exists between these organosulfur compounds and pyrazine for mEH induction. The organosulfur compounds employed in this study appeared to be effective inducers of mEH, as evidenced by both immunoblot and RNA blot analyses.

* Corresponding author: Dr Nak Doo Kim, Laboratory of Pharmacology, College of Pharmacy, Seoul National University, Seoul 151-742, Korea.

‡ Abbreviations: mEH, microsomal epoxide hydrolase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; AS, allylsulfide; AM, allylmercaptan; AMS, allylmethylsulfide; GST, glutathione *S*-transferase; CYP2E1, cytochrome P4502E1.

MATERIALS AND METHODS

Materials. AS, AM, AMS and pyrazine were purchased from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). [α-^{32}P]dATP (3000 Ci/mmol) and [γ-^{32}P]dATP (3000 mCi/mmol) were purchased from Amersham (Arlington Heights, IL, U.S.A.). 5′-End and random prime labeling kits were purchased from BRL (Gaithersburg, MD, U.S.A.). Biotinylated goat anti-rabbit IgG and streptavidin-conjugated horseradish peroxidase were purchased from BRL (Bethesda, MD, U.S.A.). Other reagents in the molecular studies were obtained from the

Sigma Chemical Co. (St Louis, MO, U.S.A.). Male Sprague–Dawley rats were provided by the animal care facility at Seoul National University (Seoul, Korea).

Subcellular fractionation. Rats were treated with each organosulfur compound (four to eight rats for each treatment) and/or pyrazine (200 mg/kg body wt/day, i.p., 1–3 days) and fasted 16 hr before killing. Hepatic microsomes were prepared from homogenates in 0.1 M Tris acetate buffer (pH 7.4) containing 0.1 M potassium chloride and 1 mM EDTA by centrifugation at 10,000 *g* for 30 min and subsequently at 100,000 *g* for 90 min, washed in pyrophosphate buffer and stored at −70° until used, as described previously [13]. Protein was assayed by the method of Lowry *et al.* [14] using bovine serum albumin as a protein standard.

Gel electrophoresis. SDS–PAGE analysis was performed according to Laemmli [15] using BioRad Mini-protean II apparatus. Microsomal proteins were analysed using a 7.5% gel.

mEH protein purification and the production of antisera. Hepatic mEH protein was purified from thiazole-induced rat hepatic microsomes. Briefly, liver microsomes isolated from rats treated with thiazole were suspended in 0.2 M potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA. Tergitol NP-10 (10%) was added to yield a final concentration of 1% while stirring, and stirred for an additional 30 min. Unsolubilized microsomes were sedimented by centrifuging for 1 hr at 100,000 *g* and the supernatant was dialysed against 5 mM potassium phosphate buffer (pH 7.25) containing 0.05% NP-10 (buffer A) overnight with two changes. The dialysate was applied to a DEAE-cellulose column (Whatman DE-52) equilibrated with buffer A and the column was washed with buffer A. The eluate was pooled and concentrated using an Amicon ultrafiltration apparatus (Amicon, Beverly, MA, U.S.A.). The homogeneity was determined electrophoretically by SDS–PAGE analysis and Coomassie brilliant blue staining. The purified mEH protein exhibited a single band in SDS–PAGE analyses. N-terminal amino acid sequencing on the mEH protein transferred onto Immobilon PVDF membrane following gel electrophoresis was performed using a gas-phase sequencer. The analyses revealed that a 16 N-terminal amino acid sequence of the purified mEH protein was identical to that of the published mEH sequence. Only single peaks were observed at each cycle, indicating that no other protein co-purified with mEH. Antibody was produced in a rabbit using purified mEH protein, as described previously [16]. Immunochemical specificity of the anti-mEH antibody was confirmed with an authentic anti-mEH antibody which was kindly provided by Dr C. B. Kasper at the University of Wisconsin (U.S.A.).

Immunoblot analysis. Immunoblot analysis was performed according to the previously published procedure [16, 17]. Microsomal proteins were separated by 7.5% SDS–PAGE and electrophoretically transferred to nitrocellulose paper which was immunoblotted with anti-mEH antibody. Biotinylated goat anti-rabbit IgG was used as the secondary antibody and color was developed using streptavidin–horseradish peroxidase and 4-chloro-1-naphthol. Serially diluted samples were analysed repeatedly for comparison of band intensities in order to quantify relative increases in mEH. Further analysis of band intensity using a laser scanning densitometer was performed ($r = 0.97$–0.99).

Isolation of total and poly(A)$^+$RNA. Total RNA was isolated using the improved single-step method of thiocyanate–phenol–chloroform RNA extraction according to the methods of Cathala *et al.* [18] and Chomczynski and Sacchi [19], as modified by Puissant and Houdebine [20]. Poly(A)$^+$ RNA was isolated from the total RNA using an oligo(dT)-cellulose column according to the method of Jacobson [21].

RNA slot blot hybridization. A 1.3 kb mEH cDNA was used to probe the levels of mEH mRNA [22, 23]. Slot blot was performed using a Schleicher & Schuell slot blot system (Minifold II), as described previously [17, 22, 23]. RNA was serially diluted in 15× SSC (1×SSC: 150 mM NaCl, 15 mM sodium citrate) applied to slots according to the manufacturer's protocols. The nitrocellulose paper was baked in a vacuum oven at 80° for 2 hr. Hybridization was performed with the hybridization buffer containing 50% deionized formamide at 42° for 18 hr with mEH cDNA (1.3 kb), which was random prime-labeled with [α-^{32}P]dATP. Blots were washed in 2× SSC and 0.1% SDS for 10 min at room temperature twice and in 0.1 × SSC and 0.1% SDS for 10 min at room temperature twice. Membranes were finally washed in the solution containing 0.1×SSC and 0.1% SDS for 60 min at 60°. Membranes were exposed to Kodak X-Omat AR film in a cassette containing intensifying screens at −70°. Slot-blotted membranes were stripped and re-hybridized with ^{32}P-end-labeled poly(dT)$_{16}$ to quantitate the amount of mRNA loaded onto the slots. Loading of equal amounts of RNA was ensured.

Northern blot hybridization. Northern blot analysis was performed as described by Sambrook *et al.* [24]. Poly(A)$^+$ RNA isolated from rat livers was resolved by electrophoresis in 1% agarose gels containing 2.2 M formaldehyde and then transferred to nitrocellulose paper by capillary transfer. Blots were hybridized as described above.

Scanning densitometry. Scanning densitometry was performed with a Soft Laser Scanning Densitometer (Model, SLR-1D/2D, Bio-Med Instrument Inc., Fullerton, U.S.A.). The area of each lane or slot was integrated using the Laser and Camera Hard Disk Program, followed by background subtraction. Quantitation of the mRNA loaded on the slot blot was accomplished by hybridization of stripped membranes with ^{32}P-end-labeled poly(dT)$_{16}$, and the relative change in mRNA was determined by normalization of the hybridization signal to the mRNA loaded onto the slots.

RESULTS

mEH immunoblot analyses of hepatic microsomes

Immunoblot analyses employing anti-rat mEH antibody revealed a significant increase in mEH protein at 48 and 72 hr following treatment of rats with either AS, AM or AMS (200 mg/kg body wt/day, 1–3 days), as compared to those from corn oil-treated animals (control). mEH levels in the hepatic

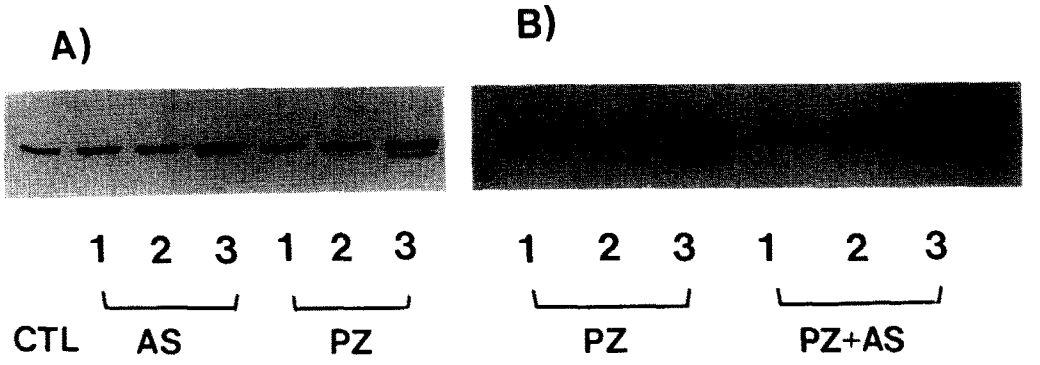

Fig. 1. Immunoblot analyses of rat hepatic microsomes with anti-mEH antibody. (A) mEH levels in rats treated with either AS or pyrazine (PZ). (B) mEH levels in rats treated with PZ or with both AS and PZ. Each lane was loaded with 1 μg of rat liver microsomes isolated from rats treated with corn oil (CTL), AS or AS with PZ for 1, 2 or 3 consecutive days. Blots exhibit time-dependent increases in mEH levels following AS and/or PZ treatment. Whereas PZ caused a 43 kDa mEH-related protein to appear, AS co-administration blocked the PZ effect.

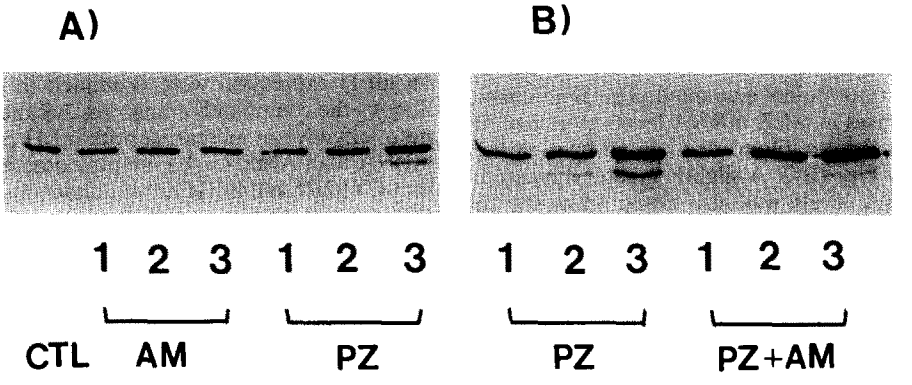

Fig. 2. Immunoblot analyses of rat hepatic microsomes with anti-mEH antibody. (A) mEH levels in rats treated with either AM or pyrazine (PZ). (B) mEH levels in rats treated with PZ or with both AM and PZ. Each lane (1 μg) contained rat liver microsomes isolated from rats treated with corn oil (CTL), AM or PZ for 1, 2 or 3 consecutive days. These blots exhibit time-dependent increases in mEH levels following AM and/or PZ treatment.

microsomes from AS-treated animals were increased in a time-dependent manner compared to that in vehicle-treated rats with a maximal increase of ~4-fold being noted 3 days post-treatment, which was comparable to that caused by pyrazine treatment (Fig. 1A). Concomitant treatment of rats with AS and pyrazine resulted in less than additive increases in the mEH protein levels as shown in Fig. 1B. AM-induced hepatic microsomes exhibited a lesser increase in mEH protein levels than AS-induced microsomes (Fig. 2A) (i.e. ~2–3-fold increases relative to control microsomes). A 5-fold increase was observed following treatment of animals with both AM and pyrazine, which was comparable to treatment with pyrazine alone (Fig. 2B).

Treatment of animals with a methyl derivative of AS, AMS, gave only a minimal increase in mEH levels (i.e. ~2-fold), as compared to vehicle-treated animals (Fig. 3A). mEH levels in hepatic microsomes, when treated with both AMS and pyrazine, were comparable to the microsomes obtained from the animals treated with AMS alone, which were ~50% less than those in pyrazine-induced microsomes (Fig. 3). These immunoblot analyses indicate that AMS partially blocks the pyrazine-inducible increase in mEH levels 48 and 72 hr post-treatment. Thus, AS appeared to be most effective in elevating mEH levels among the three compounds examined.

Previous research has shown that pyrazine induces both mEH and a mEH-related 43 kDa protein [25]. Concomitant treatment of animals with either AS, AM or AMS with pyrazine, however, caused the levels of pyrazine-inducible mEH-related 43 kDa protein to diminish notably; AMS was the most effective in suppressing the 43 kDa protein band intensity (Fig. 3).

Slot blot analysis of mEH mRNA levels

mEH mRNA levels were quantified by slot blot analyses using a 1.3 kb mEH cDNA as probe. mEH mRNA levels were increased in a time-dependent manner by each of these organosulfur compounds, as assessed by scanning densitometry of slot blot analyses (Fig. 4A, B). The time course and efficacy in mEH mRNA increases were consistent with those in mEH protein by the organosulfur compounds, although relative increases were more substantial in mRNA levels.

mEH mRNA levels were increased ~10-, 15- and 16-fold at 1, 2 and 3 days after AS treatment,

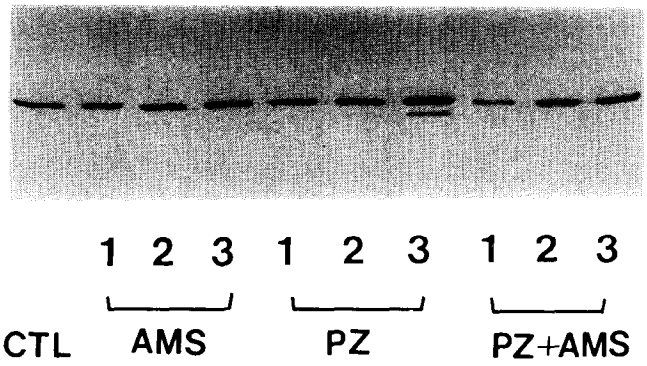

Fig. 3. Immunoblot analyses of mEH. This blot represents mEH levels in hepatic microsomes isolated from either AMS- or pyrazine (PZ)-treated rats or from both PZ and AMS-treated animals. Each lane contained 1 μg of rat liver microsomes isolated from corn oil (CTL)-, AMS-, PZ- or AMS plus PZ-treated rats.

respectively, whereas AM caused 8-, 12- and 16-fold increases at the same time points (200 mg/kg body wt/day), relative to vehicle-treated control animals (Fig. 4A, B-1 and B-2). Maximal increases were observed 3 days post-treatment for these xenobiotics among the time points examined. The time course of the increases in mEH mRNA levels by either AS or AM was comparable to that caused by pyrazine, as shown in Fig. 4B-2. A slightly greater increase in mEH mRNA levels was also noted following co-administration of either AS or AM with pyrazine (i.e. ~10 to 20%) than following treatment with either AS or AM alone, which suggests that organosulfur compounds may share a common molecular basis for mEH induction with pyrazine in spite of the distinct difference in their molecular structures. Whereas the relative maximal increases in mEH mRNA for AS or AM were 16-fold, AMS caused an ~7-fold increase in mEH mRNA levels. mEH mRNA increase by AMS plateaued 48 hr post-treatment, as shown in Fig. 4B-1. Rats treated with both AMS and pyrazine exhibited a decrease in hepatic mEH mRNA levels by ~50%, relative to pyrazine treatment, as shown in Fig. 4A and B-2. Thus, AMS appeared to block the pyrazine effect of elevating both mEH protein and mRNA, apparently serving as a partial agonistic mEH inducer. These changes in mEH mRNA were confirmed in poly(A)$^+$ RNA fractions (Fig. 5).

Northern blot hybridization analysis of mEH mRNA

Northern blot hybridization analysis was performed with the hepatic poly(A)$^+$ RNA isolated from rats treated with the organosulfurs and/or pyrazine for 3 days (Fig. 6). Northern blot analysis confirmed the rank order in increases in mEH mRNA levels among the compounds and integrity of the mRNA employed (Fig. 6); treatment of rats with AS gave the most significant changes in mEH mRNA, while concomitant treatment of animals with AS and pyrazine resulted in less than additive increase. Administration of both AM and pyrazine failed to result in an additive change in mEH mRNA levels. AMS appeared to partially block the effect of pyrazine in the elevation of mEH mRNA, consistent with the immunoblot and slot blot analyses.

DISCUSSION

A marked suppression of rat hepatic CYP2E1 by AS, AM and AMS was described in the accompanying paper [10]. Effects of these organosulfur compounds on mEH expression were examined in this study. Among the compounds employed, AS and AM were more effective in increasing both mEH protein and mRNA than AMS. Given the previous observations that CYP2E1 and mEH are simultaneously induced in response to pyrazine treatment, each of these organosulfur compounds was administered to rats with pyrazine to examine whether there was additive or synergistic induction of mEH. Concomitant treatment of AS or AM with pyrazine was less than additive for both hepatic mEH protein and mRNA (i.e. ceiling effects), implying that these structurally different compounds share a common molecular mechanism for mEH induction. Interestingly, hepatic mEH protein and mRNA levels in the animals treated with both AMS and pyrazine were ~50% less than in pyrazine-treated rats, indicating that AMS partially blocks pyrazine induction of mEH, although AMS itself induced mEH. Previous research in our laboratories showed that treatment of rats with either thiazole or pyrazine increases hepatic mEH expression, whereas pyrazine treatment results in induction of another mEH-related 43 kDa protein, and that a distinct species difference exists between rats and rabbits in the induction of mEH with these xenobiotics [12, 25]. Immunoblot and N-terminal amino acid sequence analyses showed that the 43 kDa protein in pyrazine-induced rat hepatic microsomes is associated with mEH. Expression of another gene or post-translational modification may be involved in the regulatory mechanism for mEH and the induction of the 43 kDa protein. It is interesting that co-administration of organosulfur and pyrazine suppressed the expression of the pyrazine-inducible mEH-related 43 kDa protein, as assayed by immunoblot analysis (i.e. AS or AMS completely suppressed the induction of the 43 kDa protein). Blocking of 43 kDa protein expression indicates that other genes may also be modulated by these organosulfur compounds.

Studies in our laboratories have also shown that imidazole antimycotic agents including ketoconazole, clotrimazole, miconazole and econazole induce mEH

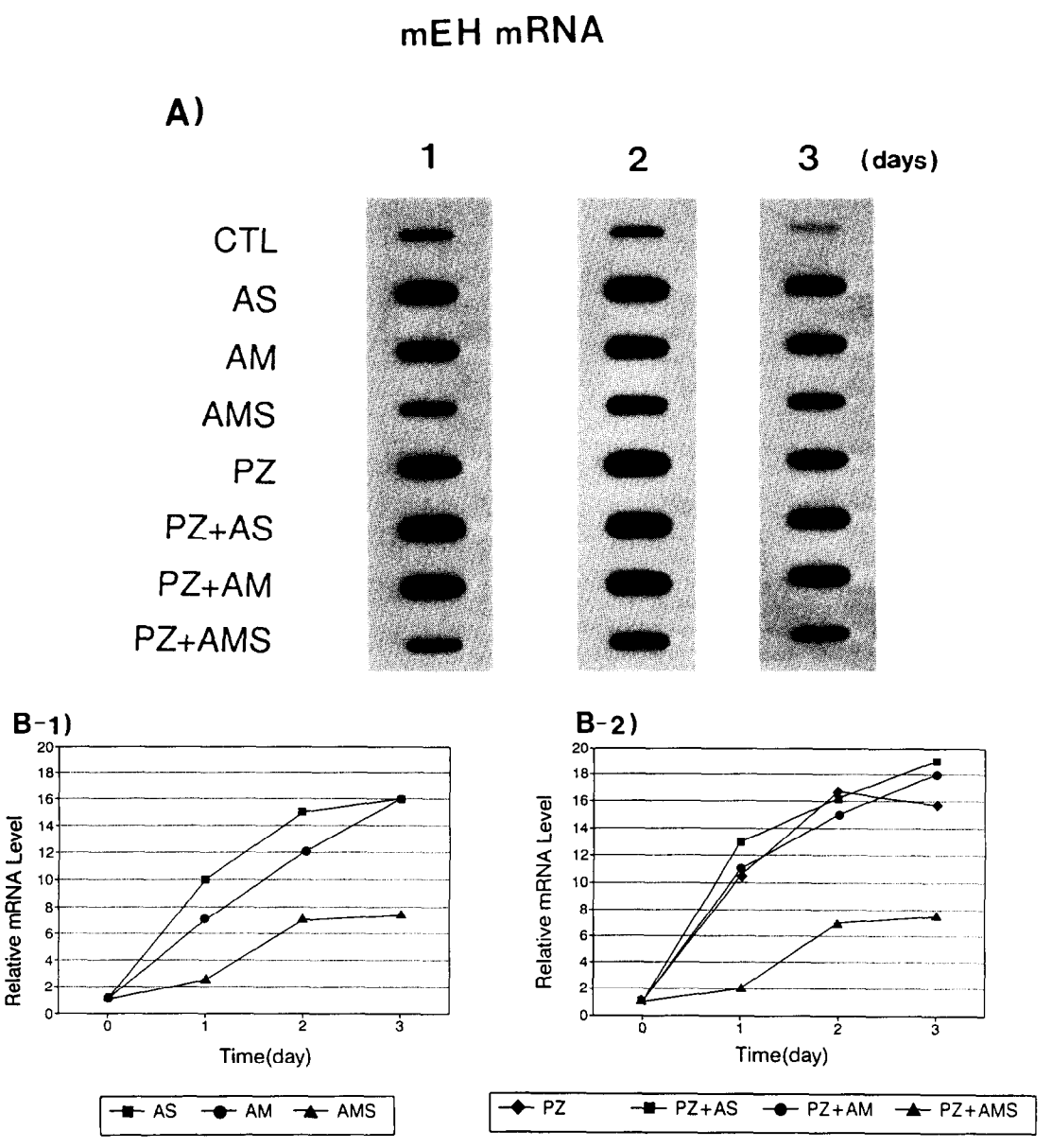

Fig. 4. Slot blot analyses of mEH mRNA in total RNA fractions. (A) The autoradiography shows the mEH mRNA levels at 1, 2 or 3 consecutive day(s) (columns 1 through 3) following corn oil (CTL), AS, AM, AMS, pyrazine (PZ), PZ plus AS, PZ plus AM and PZ plus AMS treatment (rows 1 through 8, respectively). Twenty micrograms of total RNA were blotted onto nitrocellulose membrane and hybridized with a random prime-labeled 1.3 kb mEH cDNA probe. (B) Relative changes in mEH mRNA following organosulfur treatment. The mRNA levels were quantitated by densitometric scanning of the slot blot and mEH signal intensities were normalized to the level in the control within the same membrane. Duplicate determinations were performed on mRNA samples isolated from two different groups of rats.

protein and mRNA and that the enhancement of mEH mRNA produced by these agents results from transcriptional activation, as evidenced by nuclear run-on analysis [22]. Studies in other laboratories also demonstrated that some xenobiotics including phenobarbital and nitrosamine induce mEH through transcriptional activation [26–28]. A number of laboratories including ours have shown that the levels of mEH mRNA highly correlated with those of mEH protein, and that immunochemically detectable levels of mEH are primarily associated with the catalytic activities of the enzyme [25]. Given the time-dependent increases in mEH mRNA following AS, AM or AMS treatment, it is tempting to suggest transcriptional activation as the molecular mechanism for induction although it is necessary to conduct further experiments to confirm this mechanism.

Preliminary studies in this laboratory have shown that these organosulfur compounds enhanced glutathione *S*-transferase (GST) activity and increased GST protein levels in rat hepatic tissue,

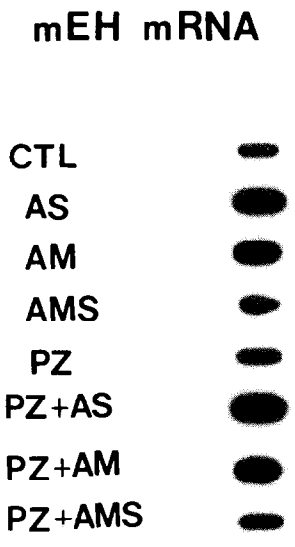

Fig. 5. Slot blot analyses of mEH mRNA in poly(A)$^+$ RNA fractions from rats treated with allylsulfides and/or pyrazine (PZ). One microgram of poly(A)$^+$ RNA isolated from rats was blotted onto a nitrocellulose membrane and hybridized with a ^{32}P-labeled mEH cDNA probe. The slots represent the levels of mEH mRNA 3 days after corn oil (CTL), AS, AM, AMS, PZ, PZ plus AS, PZ plus AM and PZ plus AMS treatment.

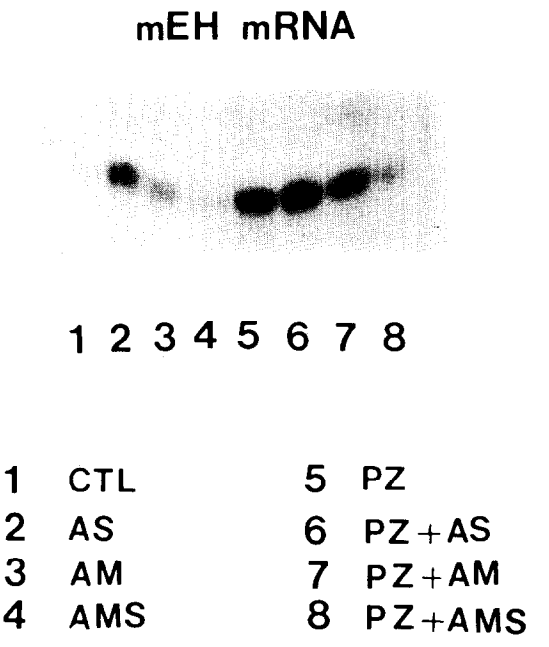

Fig. 6. Northern blot analyses of mEH mRNA in poly(A)$^+$ RNA fractions isolated from rats treated with organosulfurs and/or pyrazine (PZ). One microgram of poly(A)$^+$ RNA isolated from rats was fractionated in a 1% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose membrane and hybridized with ^{32}P-labeled mEH cDNA probe. Autoradiography shows the levels in mEH mRNA 3 consecutive days after corn oil (CTL), AS, AM, AMS, PZ, PZ plus AS, PZ plus AM and PZ plus AMS treatment (lanes 1 through 8, respectively).

which is in agreement with the study performed with AS, reported by Sparnins *et al.* [29]. The elevation of electrophilic detoxication systems (i.e. increase in glutathione levels, induction of GST, mEH and NADP(H):quinone reductase) has been recognised as characteristic of the action of certain xenobiotics. For example, the inducing effects of butylhydroxyanisole, butylhydroxytoluene and other food antioxidants on a number of phase II detoxication enzymes including mEH, GST, quinone reductase and glucuronyl transferases have been reported without a corresponding increase in the activities of Phase I cytochrome P450s [30, 31]. The anticarcinogenic effect of these antioxidants has been attributed to an increase in Phase II metabolizing enzymes. In addition, Oltipraz, which has a pyrazine moiety and sulfur atoms in its structure, has been shown to be active against the development of pulmonary and forestomach cancers induced by benzopyrene in rodents [32]. In view of the fact that epoxide intermediates formed by cytochrome P450 attack cellular macromolecules, mEH is primarily associated with the detoxication of xenobiotics. Acrylonitrile, for example, is converted to 2-cyanoethylene oxide by cytochrome P450, which is believed to be the ultimate mutagenic and carcinogenic species [25]. Epoxide hydrolase-mediated hydrolysis of the epoxide plays an important role in detoxication. Thus, induction of mEH by these organosulfurs may protect against chemical-induced tissue injury.

In summary, the present study demonstrates that AS, AM or AMS induce mEH through large increases in mRNA levels with AS and AM being the more effective.

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