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INHIBITION OF CYTOCHROME P4502E1 EXPRESSION BY ORGANOSULFUR COMPOUNDS ALLYLSULFIDE, ALLYLMERCAPTAN AND ALLYLMETHYLSULFIDE IN RATS

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Abstract—Cytochrome P4502E1 (CYP2E1) is active in both detoxication and activation of small organic molecules. The effects of organosulfur compounds including allylsulfide (AS), allylmercaptan (AM) and allylmethylsulfide (AMS) on the expression of CYP2E1 were examined in rats. 4-Nitrophenol, aniline hydroxylase and *N*-nitrosodimethylamine demethylase activities, the rates of which represent the level of CYP2E1, decreased in hepatic microsomes isolated from rats treated with AS in a time-dependent manner by 45% to 90%, as compared to control. Pyrazine-induced hepatic microsomes exhibited ~5-fold increases in CYP2E1-catalysed metabolic activities, whereas the hepatic microsomes obtained after treatment of animals with both AS and pyrazine showed rates comparable to or less than those in control microsomes. AM or AMS suppressed constitutive and pyrazine-inducible levels of CYP2E1 similarly to AS. Immunoblot analyses of hepatic microsomes, using an anti-CYP2E1 antibody, showed that AS, AM and AMS significantly suppressed constitutive levels of CYP2E1 apoprotein after 24, 48 and 72 hr. Time-dependent induction of CYP2E1 by pyrazine was also completely blocked by treatment of animals with AS throughout the experimental period, as evidenced by immunoblot analysis. The levels of CYP2E1 apoprotein in the hepatic microsomes isolated from animals treated with both AM and pyrazine, or with both AMS and pyrazine were comparable to those in control hepatic microsomes at days 1–3 post-treatment. Treatment of rats with each of these organosulfur compounds caused no significant changes in the levels of CYP2E1 mRNA, as assessed by slot and northern blot analyses, suggesting that post-transcriptional regulation may be associated with the suppression of CYP2E1 apoprotein levels. The results of metabolic activities, immunoblot analyses and RNA blot analyses demonstrated that these organosulfur compounds are effective in suppressing constitutive and inducible expression of CYP2E1.

CYP2E1§ is an ethanol-inducible form of the cytochrome P450 superfamily. CYP2E1 is active in the metabolism of a variety of small organic molecules including acetaminophen, aliphatic alcohols, acetone, carbon tetrachloride, *N*-nitrosodimethylamine (NDMA), benzene, phenol, pyridine, 4-nitrophenol (4-NP), anilines, diethylether, pyrazole, halothane and tetrafluoroethane [1–12]. The reactive intermediates formed during the metabolism of therapeutic agents, toxicants and carcinogens by this enzyme are frequently capable of covalently binding to tissue macromolecules, and causing tissue damage [2, 11–19]. Clinical studies in humans have shown the development of severe hepatotoxicity from moderate doses of acetaminophen in chronic alcoholics [2], and it has been suggested that the hepatotoxicity may result from CYP2E1-catalysed metabolism of acetaminophen [13]. The metabolism of carbon tetrachloride to a hepatotoxic intermediate appeared to be associated with increased levels of CYP2E1 [14, 15]. In addition, CYP2E1 plays a critical role in the metabolism of nitrosamines, which are potent carcinogens and require metabolic activation to exert their carcinogenic effect [5–7]. This enzyme was also identified as the principal enzyme metabolizing benzene and phenol [9], known hematotoxic compounds, which are associated with development of acute myelogenous leukemia [16, 17]. Hydroxylated benzene derivatives such as phenol, catechol, hydroquinone, benzoquinone and benzenetriol have been shown to induce DNA damage through the production of oxygen radicals [18]. CYP2E1 may also contribute to basal microsomal NADPH-dependent lipid peroxidation [19]. Thus, CYP2E1 may potentiate the toxicity of many xenobiotics via metabolic activation and/or accumulation of reactive metabolites.

The level of CYP2E1 expression may be correlated with the rate of toxic product generation from a wide variety of small organic compounds. CYP2E1 is inducible by many structurally diverse compounds such as ethanol, acetone, trichloroethylene, isoniazid, pyrazole, benzene, pyridine, thiazole,

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§ Abbreviations: CYP2E1, cytochrome P4502E1, SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NDMA, *N*-nitrosodimethylamine; 4-NP, 4-nitrophenol; AS, allylsulfide; AM, allylmercaptan; AMS, allylmethylsulfide; GST, glutathione *S*-transferase; mEH, microsomal epoxide hydrolase.

pyrazine and pyridazine as well as pathophysiological conditions and diverse states such as fasting, streptozotocin- and alloxan-induced diabetes, and spontaneous diabetes [4, 8–10]. Thus, it is highly likely that exposure to inducers of CYP2E1 in conjuction with exposure to toxic substrates for CYP2E1 results in deleterious effects.

Diallyl sulfide, a component of garlic oil, has recently been shown to suppress CYP2E1 activity and exert a potent inhibitory effect on the induction of colon and liver cancer induced by chemical carcinogens [20, 21]. In the present study, the effects of allylsulfide (AS), allylmercaptan (AM) and allylmethylsulfide (AMS) on the expression of hepatic CYP2E1 in rats were examined using metabolic activity, immunoblot and RNA blot analyses. Additional experiments examined whether these organosulfur compounds were capable of blocking the chemically enhanced expression of CYP2E1. This study was initiated with the aim of obtaining preclinical pharmacological data on organosulfur compounds, which could be used as chemoprotective (i.e. hepatoprotective) agents functioning through selective modulation of drug-metabolizing enzymes.

MATERIALS AND METHODS

Materials. AS, AM, AMS and pyrazine were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [γ-^{32}P]dATP (3000 mCi/mmol) was 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.). 1-Chloro-2,4-dinitrobenzene was purchased from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Biotinylated donkey anti-goat IgG and biotinylated goat anti-rabbit IgG, and streptavidin-conjugated horseradish peroxidase were purchased from BRL (Bethesda, MD, U.S.A.). 4-NP, aniline, 4-nitrocatechol and other reagents in the molecular studies were obtained from Sigma.

Treatment of animals. Male Sprague–Dawley rats (180–230 g, ~6 weeks of age) were given each of the organosulfur compounds and/or pyrazine (200 mg/kg body wt/day, i.p., 1–3 days) and fasted 16 hr before killing. Four different microsomal preparations were isolated from groups of animals (N = 8) and utilized for each treatment.

Subcellular fractionation. Microsomes were prepared by differential centrifugation as described previously [10], washed in pyrophosphate buffer and stored at −70° until used. Microsomal fractions stored in the absence of glycerol were used within a month and for long-term storage 20% glycerol was added. Protein was assayed by the method of Lowry *et al.* [22].

4-NP hydroxylase assay. This was perfomed as described previously [10]. Reaction mixtures contained 100 mM potassium phosphate buffer (pH 6.8), 1.0 mM ascorbic acid, 1 mM NADPH, 1 mg of hepatic microsomes and 100 μM 4-NP in a total vol. of 1.0 mL. The 4-nitrocatechol formed was determined spectrophotometrically.

Aniline hydroxylase. Aniline hydroxylase was measured by *p*-aminophenol formation as described by Brodie and Axelrod [23].

NDMA demethylase activity. The metabolism of NDMA was measured using reaction mixtures containing 1.0 mg protein, 1.0 mM NADPH, 1.0 mM NDMA and 0.1 M potassium phosphate buffer (pH 7.4) in a total vol of 1.0 mL. Formaldehyde production from NDMA was assayed according to the procedure of Nash [24].

Gel electrophoresis. SDS–PAGE analysis was performed according to Laemmli [25] using a BioRad Mini-protean II apparatus. Microsomal proteins were analysed using 7.5% separating gels.

Immunoblot analysis. Immunoblot analysis was as described previously [26]. Microsomal proteins were separated by 7.5% SDS–PAGE and electrophoretically transferred to nitrocellulose paper which was immunoblotted with anti-CYP2E1 antibody. Biotinylated donkey anti-goat IgG was used as the secondary antibody and color was developed using streptavidin–horseradish peroxidase and 4-chloro-1-naphthol.

Isolation of total RNA and poly(A)$^+$ RNA. Total RNA was isolated using an improved single-step thiocyanate–phenol–chloroform RNA extraction according to the methods of Cathala *et al.* [27] and Chomczynski and Sacchi [28], as modified by Puissant and Houdebine [29]. Poly(A)$^+$ RNA was isolated from the total RNA using an oligo(dT)-cellulose column according to the method of Jacobson [30].

Oligonucleotide synthesis. A 19-mer CYP2E1-specific oligonucleotide (5′-d(CAAAGCCAACT-GTGACAGG)-3′) was synthesized using a DNA synthesizer (Research Genetics, Huntsville, U.S.A.), as described previously [31].

RNA slot blot hybridization. Slot blots were obtained using a Schleicher & Schuell slot blot system (Minifold II), as described previously [26, 32]. 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.

Blots were incubated in the hybridization buffer containing 6× SSPE (1× SSPE: 0.15 M NaCl, 10 mM NaH_2PO_4 and 1 mM Na_2EDTA, pH 7.4), 200 μg/mL of sonicated salmon sperm DNA, 0.1% SDS and 5× Denhardt's solution [0.1% Ficoll, 0.1% polyvinylpyrrolidine and 0.1% bovine serum albumin (Pentex Fraction V)] at 53° for 1 hr without probe. Hybridization was performed in the same solution at 53° for 18 hr with a 5′-end-labeled oligonucleotide probe. Blots were washed at the hybridization temperature for 1 hr with three changes of a solution of 6× SSPE and 0.1% SDS. Membranes were exposed to Kodak X-Omat AR film in a cassette containing intensifying screens at −80°.

Scanning densitometry. Scanning densitometry was performed with a Soft Laser Scanning Densitometer (Model, SLR-1D/2D, Bio-Med Instrument Incorporation, 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. The quantitation of the mRNA loaded on the slot blot was accomplished by hybridization of stripped membranes with ^{32}P-end-labeled poly(dT)$_{16}$,

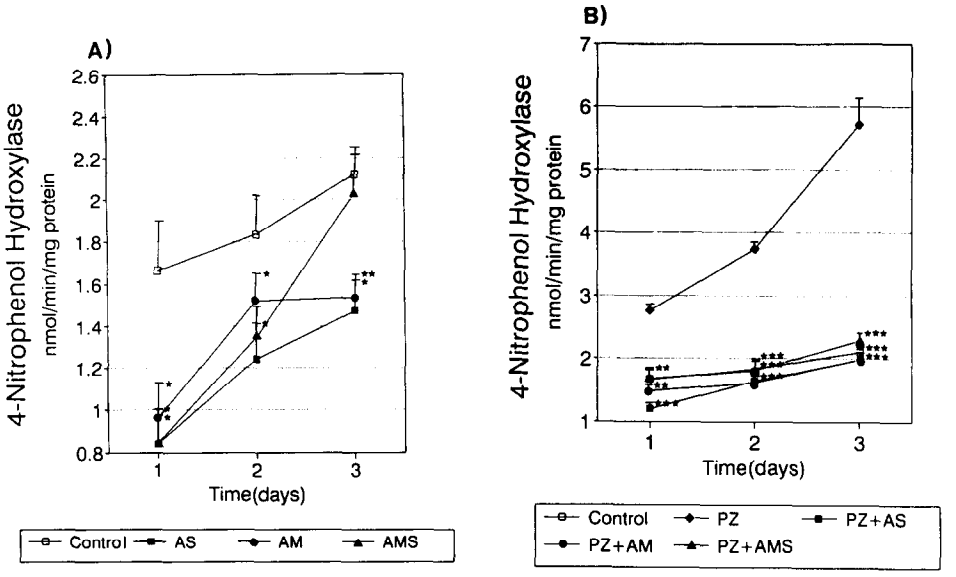

Fig. 1. 4-NP hydroxylase activity in hepatic microsomes. (A) Time-dependent changes in 4-NP hydroxylase activity in microsomes isolated from rats treated with either AS, AM or AMS, as compared with those from corn oil-treated rats. (B) Time course of microsomal 4-NP hydroxylase activity following co-treatment of rats with each of the organosulfur compounds and pyrazine (PZ) was compared to that obtained from rats treated with PZ alone. Limit of sensitivity for the assay was 0.33–0.50 nmol 4-nitrocatechol/min/mg protein. Values are means ± SE from different microsomal preparations isolated from eight animals. Symbols indicate significant difference from either vehicle (A)- or PZ (B)-treated animals at the respective time, as analysed by Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

and the relative change in mRNA was determined by normalization of the hybridization signal to the mRNA loaded onto the slots.

RESULTS

Metabolic activities of CYP2E1

4-NP, aniline hydroxylase and NDMA demethylase activities, the rates of which reflect the level of CYP2E1, were decreased in hepatic microsomes isolated from rats treated with AS, AM or AMS, as compared to the hepatic microsomes obtained from corn oil-treated rats (Figs 1A, 2A and 3A). Treatment of animals with corn oil, which was used as a vehicle, caused a 40–80% increase in the rate of hepatic microsomal 4-NP hydroxylase activity as compared to that present in hepatic microsomes from untreated animals [10]. The result was consistent with previously reported values (i.e. 1.7 and 1.2 nmol 4-nitrocatechol/min/mg protein for corn oil-treated and vehicle-treated microsomes, respectively) [10]. Hepatic microsomes isolated from the rats treated daily with either AS or AM significantly decreased the rate of 4-NP hydroxylase activity. Microsomes from AS- or AM-treated animals had decreased 4-NP hydroxylase activity by 42–49% 24 hr post-treatment whereas 4-NP hydroxylase activity had decreased by 20–30% at 48 and 72 hr. AMS appeared to be less effective than AS or AM in suppressing 4-NP hydroxylase activity, as shown in Fig. 1A.

The effects of concomitant treatment of animals with the organosulfur compounds and pyrazine, a potent inducer of CYP2E1, on CYP2E1-catalysed activity was examined. A time-dependent increase in microsomal 4-NP hydroxylase activity was observed after treatment of rats with pyrazine, with a ~5-fold increase observed 3 days post-treatment (i.e. 5.71 nmol/min/mg protein), relative to microsomes from untreated animals (Fig. 1B). The increase in microsomal hydroxylase activity caused by pyrazine was completely inhibited by concomitant treatment of animals with either AS, AM or AMS (Fig. 1B). Treatment of rats with both AM and pyrazine resulted in comparable inhibition of the 4-NP hydroxylase activity with a value of 1.99 nmol 4-nitrocatechol/min/mg protein at 3 days post-treatment (Fig. 1B). AMS caused less inhibition of the pyrazine-inducible increase in 4-NP hydroxylase activity than AS or AM, with a value of 2.29 nmol 4-nitrocatechol/min/mg protein, which was slightly greater than that in the microsomes from vehicle-treated rats (Fig. 1B).

Virtually complete suppression of aniline hydroxylase activity occurred in hepatic microsomes isolated 3 days after treatment of animals with either AS, AM or AMS relative to corn oil treatment (i.e. control microsomes), as shown in Fig. 2A. Pyrazine-induced hepatic microsomes isolated 3 days post-treatment exhibited an enhanced rate of aniline hydroxylase activity with a value of 1.64 nmol 4-aminophenol/min/mg protein (Fig. 2B). Co-administration of each organosulfur compound efficiently inhibited pyrazine-mediated increase in aniline hydroxylase activity to a level comparable to

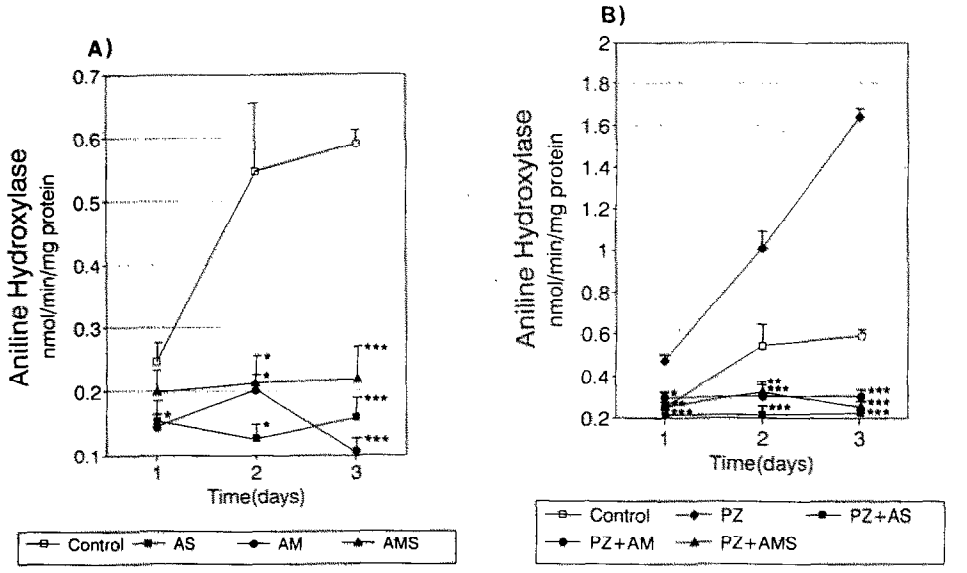

Fig. 2. Aniline hydroxylase activity in hepatic microsomes. (A) Aniline hydroxylase activity was measured in hepatic microsomes isolated from rats untreated or treated with either AS, AM or AMS. (B) Time course of microsomal aniline hydroxylase activity following treatment of rats with each of the organosulfur compounds and pyrazine (PZ) relative to the animals treated with PZ alone. Limit of sensitivity for the assay was 0.005–0.025 nmol 4-aminophenol/min/mg protein. Values are means ± SE from different microsomal preparations isolated from eight animals. Symbols indicate significant difference from either vehicle (A)- or PZ (B)-treated animals at the respective time, as analysed by Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

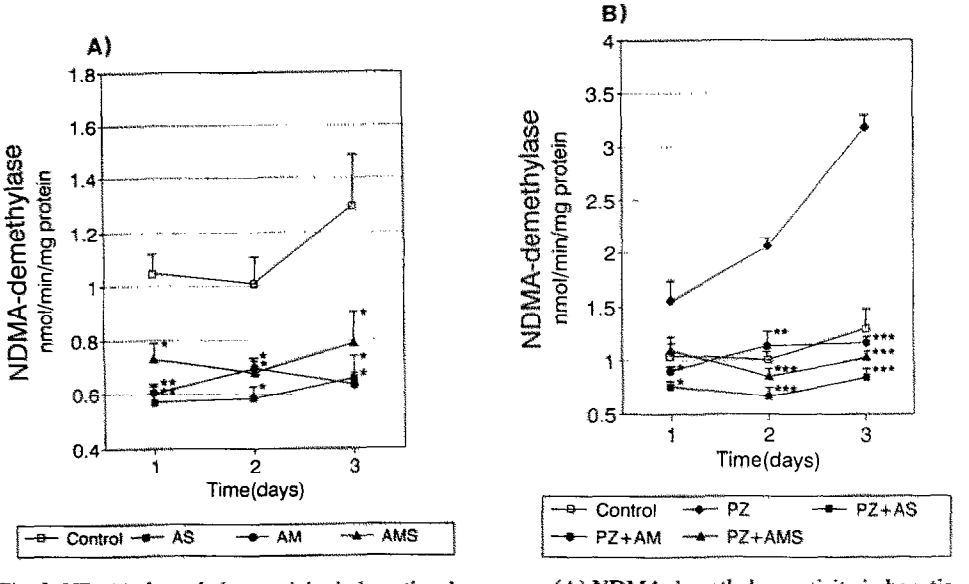

Fig. 3. NDMA demethylase activity in hepatic microsomes. (A) NDMA demethylase activity in hepatic microsomes isolated from rats treated with either AS, AM or AMS for 1, 2 or 3 consecutive days. (B) Microsomal NDMA demethylase activity was monitored following treatment of rats with each of the organosulfur compounds and pyrazine (PZ). Limit of sensitivity for the assay was 0.021–0.042 nmol HCHO/min/mg protein. Values are means ± SE from different microsomal preparations isolated from eight animals. Symbols indicate significant difference from either vehicle (A)- or PZ (B)-treated animals at the respective time, as analysed by Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

that in controls (Fig. 2B). In addition, the hepatic microsomes obtained from the organosulfur-exposed rats exhibited a 40–50% decrease in metabolism of NDMA (Fig. 3A). Administration of each of these organosulfur compounds also completely inhibited the pyrazine-inducible elevation in the rates of NDMA demethylase activity (i.e. by 100–120%) (Fig. 3B). Since decreased rates of metabolic activity could reflect organosulfur-inactivated CYP2E1 and minor catalytic activities of other form(s) [33], as well as possibly reflecting decreased levels of P452E1 protein (i.e. suppression of expression), additional studies were conducted to examine the basis of the altered activities.

CYP2E1 immunoblot analysis

SDS–PAGE of hepatic microsomes isolated from organosulfur-exposed rats showed an apparent decrease in intensity of a band migrating in the region of CYP2E1, relative to that from corn oil-treated animals (data not shown). Immunoblot analyses employing anti-CYP2E1 antibody revealed a significant decrease in hepatic microsomal CYP2E1 apoprotein levels following treatment of rats with either AS, AM or AMS, as compared to those from the corn oil-treated animals (Fig. 4A). In contrast, pyrazine-induced rat hepatic microsomes showed a profound increase in level of CYP2E1 which occurred in a time-dependent manner following treatment. The maximal increase (~6–8-fold) occurred 3 days post-treatment, as assessed by immunoblot analyses (Fig. 4A). Hepatic microsomes, isolated at 24 hr after treatment of rats with both AS and pyrazine, however, exhibited CYP2E1 band intensities more comparable to those in microsomes from corn oil-treated animals (Fig. 4A), indicating that AS is capable of efficiently blocking pyrazine-inducible CYP2E1 expression. The levels of CYP2E1 48 or 72 hr after consecutive daily treatment with both AS and pyrazine were below the limit of detectability in the immunoblot, which demonstrated that AS completely suppressed the expression of CYP2E1 apoprotein throughout the treatment regimen despite the presence of the CYP2E1 inducer. Hepatic microsomes isolated after AM treatment also showed a significant decrease in CYP2E1 levels (Fig. 4B). When animals were treated with both AM and pyrazine, the hepatic CYP2E1 apoprotein levels were comparable to those in microsomes from vehicle-treated rats (Fig. 4B). Less suppression of CYP2E1 expression, however, was noted following AMS treatment (Fig. 4C). The results of metabolic activities and immunoblot analyses suggest that these organosulfur compounds are effective in suppressing both the constitutive and inducible expression of CYP2E1 protein.

RNA blot hybridization analyses of CYP2E1 mRNA

Slot blot hybridization analysis was performed with the poly(A)$^+$ RNA isolated from hepatic tissue of rats treated with each of the organosulfur compounds alone and organosulfur compounds with pyrazine for 1, 2 or 3 days (Fig. 5A). Treatment of rats with each of these organosulfur compounds caused no significant changes in the levels of CYP2E1 mRNA, whereas the levels of CYP2E1 mRNA decreased after treatment with pyrazine as was observed previously [34]. Co-administration of the organosulfur and pyrazine caused no significant changes in CYP2E1 mRNA levels, relative to those in pyrazine-exposed animals (Fig. 5A). Northern blot analysis of samples from AS-, AM- and AMS-treated rats also failed to exhibit notable changes relative to control (Fig. 5B). These results suggest that the organosulfur compounds suppress the expression of CYP2E1 possibly in the absence of transcriptional effects, thereby suggesting post-transcriptional regulatory mechanism(s) in the suppression of CYP2E1 expression.

DISCUSSION

Studies showed that a number of compounds modulate 2E1-catalytic activity mostly through competitive or non-competitive mechanisms, and that certain compounds which serve as specific substrates or inhibitors for CYP2E1 also enhance its expression. For example, organic agents such as ethanol and pyridine, which are substrates for P450, serve as CYP2E1 inducers [8–10]. Previous studies have also shown that pyrazine, which forms part of the molecular structure of a number of natural products and drugs (e.g. pyrazinamide and oltipraz), increases the levels of CYP2E1, glutathione *S*-transferases (GSTs) and microsomal epoxide hydrolase (mEH) enzymes concomitantly and that the increases in CYP2E1 levels are associated with a significant decrease in CYP2E1 poly(A)$^+$ RNA levels which was observed at early times of induction [34, 35].

Brady *et al.* [20, 36, 37] showed that some organosulfur compounds including disulfiram and diallylsulfide caused selective inhibition of CYP2E1-catalysed NDMA activity and suppression of CYP2E1 protein levels in hepatic microsomes. The selective suppression of CYP2E1 levels may provide a mechanism for the anticarcinogenic properties of these compounds. Thus, diallylsulfide may serve as a chemoprotective agent through the inhibition of CYP2E1. Given the potential use of AS or its derivatives as a chemopreventive agent(s) and the observation that AS suppresses constitutive expression of CYP2E1, organosulfur compound including AS, AM and AMS were employed in the present study to examine the structure–activity relationship for CYP2E1 suppression and to establish whether these agents have the capacity to prevent the inducible expression of CYP2E1 in the presence of an organic CYP2E1 inducer (i.e. pyrazine).

Substantial elevation of 2E1 metabolic activities was noted 3 days post-treatment with pyrazine, which is consistent with previous observations [35]. Concomitant treatment of rats with each of these organosulfur compounds and pyrazine completely blocked pyrazine-mediated elevation of 2E1-catalysed metabolic activities. The metabolic data were in good agreement with CYP2E1 protein levels, as evidenced by immunoblot analyses. Although all three organosulfur compounds employed in this study were effective in inhibiting both constitutive and inducible CYP2E1 protein expression, AM or AMS were slightly less effective in suppressing

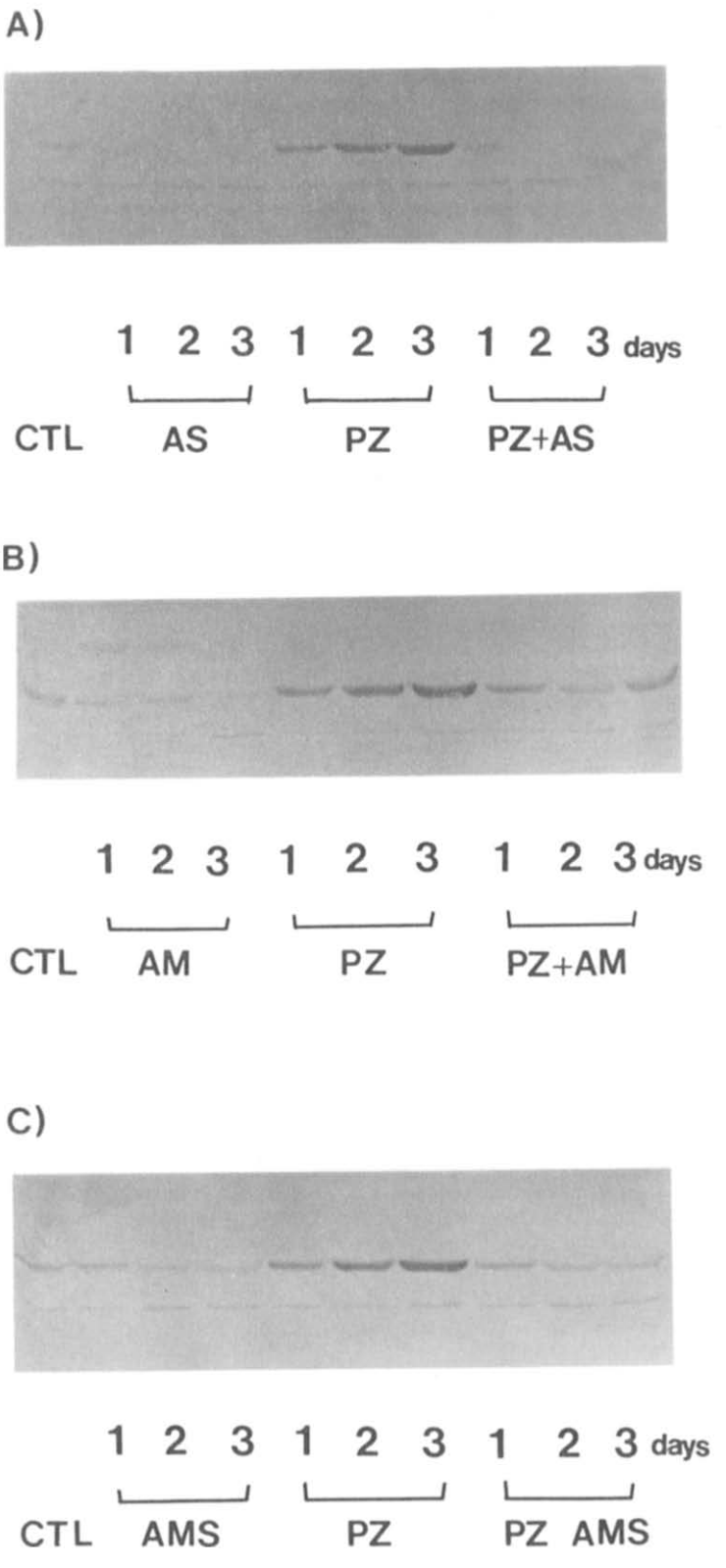

Fig. 4. Immunoblot analysis of rat hepatic microsomes with anti-CYP2E1 antibody. (A) Each lane was loaded with 20 μg of rat liver microsomes isolated from rats treated with corn oil (CTL), AS, pyrazine (PZ) or PZ + AS for 1–3 consecutive days. (B) Each lane (20 μg) contains rat liver microsomes isolated from rats treated with corn oil, AM, PZ or PZ + AM for 1–3 consecutive days. (C) Lanes are associated with rat hepatic microsomes isolated from rats treated with corn oil, AMS, PZ or PZ + AMS for 1–3 consecutive days (20 μg).

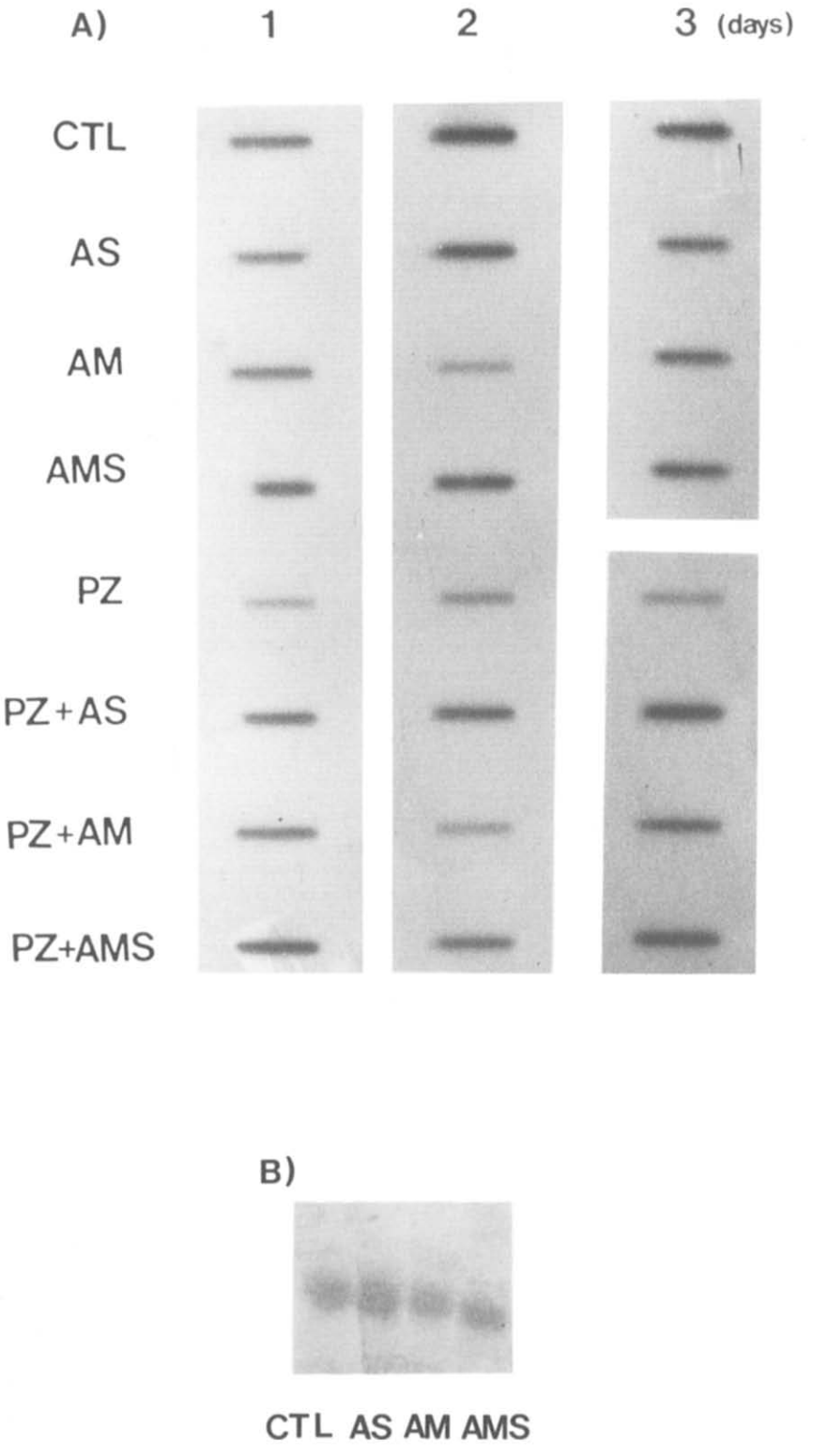

Fig. 5. RNA hybridization analyses. (A) Slot blot analyses of CYP2E1 poly(A)$^+$ mRNA in rat liver after treatment with each of the organosulfur compounds. Poly(A)$^+$ mRNA (1 μg) isolated from rats treated with corn oil (CTL), AS, AM, AMS, pyrazine (PZ), PZ + AS, PZ + AM, PZ + AMS for 1, 2 or 3 consecutive days was blotted to nitrocellulose membrane and hybridized with ^{32}P-end-labeled CYP2E1 oligonucleotide probe. (B) Northern blot analyses of hepatic CYP2E1 poly(A)$^+$ RNA fractionated after treatment of rats with each of the organosulfur compounds. Each lane contained 2 μg of poly(A)$^+$ RNA isolated from rats treated with either corn oil, AS, AM or AMS for 1–3 consecutive days (lane 1 through 4, respectively).

inducible CYP2E1 expression caused by pyrazine, as monitored by immunoblot analyses. Thus, AS appeared to be the most effective suppressor of CYP2E1 on the basis of both metabolic activities and immunoblot analyses.

It has been reported previously that elevation of CYP2E1 protein levels following treatment with organic inducers including pyridine, ethanol and acetone involves protein synthesis in the absence of transcriptional activation [31, 38]. The absence of significant changes in CYP2E1 mRNA levels following treatment with the organosulfurs, as observed in the present study, is in agreement with the previous reports on the role of protein synthesis in the absence of transcriptional activation, implying that suppression of CYP2E1 protein levels by these organosulfur compounds may be associated with regulation at post-transcriptional levels.

A number of compounds are active against chemical-induced carcinogenesis through the mechanism of elevation of Phase II detoxifying enzymes. Studies from Kensler's group [39, 40] strongly support the role of enhanced expression of Phase II detoxifying enzymes including GSTs in the protective effect of Oltipraz against Aflatoxin B1-induced hepatocarcinogenesis. The studies showed that Oltipraz, a chemopreventive agent, causes an initial induction of hepatic GST activity with changes in steady-state levels of GST mRNA and rates of GST gene transcription. Furthermore, the three compounds used in this study were also effective in modulating the expression of Phase II enzymes including GST and mEH. Efficacy of the three organosulfur compounds on the expression of GST and mEH was consistent with that of CYP2E1 expression, with AMS being less active in inducing the enzymes. These results provide evidence that these organosulfur compounds serve as selective modulators in the expression of xenobiotic-metabolizing enzymes.

In summary, treatment of rats with AS, AM or AMS causes suppression of CYP2E1 expression and blocks inducible expression of the enzyme. The results of this research may be of assistance in the design and development of agents active in protecting organs from reactive toxicants which are produced by CYP2E1-mediated catalysis.

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