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Induction of Microsomal Epoxide Hydrolase, rGSTA2, rGSTA3/5, and rGSTM1 by Disulfiram, but not by Diethyldithiocarbamate, a Reduced Form of Disulfiram

Sang Geon Kim and Hye Jung Kim

College of Pharmacy, Duksung Women's University,
419 Ssangmoon-dong, Dobong-gu Seoul 132-714, Korea
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ABSTRACT *: Disulfiram (DSF) and diethyldithiocarbamate (DDC), a reduced form of DSF, protect the liver against toxicant-induced injury through inhibition of cytochrome P450 2E1. The effect of DSF and DDC on the levels of major hepatic microsomal epoxide hydrolase (mEH) and glutathione S-transferase (GST) expression was comparatively studied, given the view that these enzymes are involved in terminal detoxification events for high energy intermediates of xenobiotics. Treatment of rats with a single dose of DSF (20-200 mg/kg, po) resulted in 2- to 15-fold increases in the mEH mRNA level at 24 hr with the ED50 value being noted as 60 mg/kg. The mEH mRNA level was elevated ~15-fold at 24 hr after treatment at the dose of 100 mg/kg, whereas the hepatic mRNA level was rather decreased from the maximum at the dose of 200 mg/kg, indicating that DSF might cause cytotoxicity at the dose. In contrast to the effect of DSF, DDC only minimally elevated the mEH mRNA level at the doses employed. DSF moderately increased the major GST mRNA levels in the liver as a function of dose, resulting in rGSTA2, rGSTA3/5 or rGSTM1 mRNA levels being elevated 3- to 4-fold at 24 hr post-treatment, whereas the rGSTM2 mRNA level was not altered. DDC, however, failed to stimulate the mRNA levels for major GST subunits, indicating that the reduced form of DSF was ineffective in stimulating the GST the expression. The effect of other organosulfides including aldrithiol, 2,2'-dithiobis(benzothiazole) (DTB), tetramethylthiouram disulfide (TMTD) and allyl disulfide (ADS) on the hepatic mEH and GST mRNA expression was assessed in rats in order to further confirm the increase in the gene expression by other disulfides. Treatment of rats with aldrithiol (100 mg/kg, po) resulted in a 16-fold increase in the mEH mRNA level at 24 hr post-treatment. DTB, TMTD and ADS also caused 5-, 9- and 12-fold increases in the mRNA level, respectively, as compared to control. Thus, all of the disulfides examined were active in stimulating the mEH gene in the liver. The organosulfides significantly increased the rGSTA2, rGSTA3, rGSTA5 and rGSTM1 mRNA levels at 24 hr after administration. In particular, aldrithiol was very efficient in stimulating the rGSTA and rGSTM genes among the disulfides examined. These results provide evidence that DSF and other sulfides effectively stimulate the mEH and major GST gene expression at early times in the liver and that DDC, a reduced form of DSF, was ineffective in stimulating the expression of the genes, supporting the conclusion that reduced form(s) of organosulfur compound(s) might be less effective in inducing the mEH and GST genes through the antioxidant responsive element(s).*

Key Words *: Glutathione S-transferase, Microsomal epoxide hydrolase, Disulfiram, Diethyldithiocarbamate, Induction, Gene expression*

I. INTRODUCTION

Studies have shown that many organosulfur compounds inhibit the enzymatic activity of cytochrome P450 2E1 and suppress the expression of the enzyme through the formation of metabolite intermediates (Kim *et al*, 1993, 1996; Kim and Cho, 1996). Disulfiram (DSF) prevents N-nitrosodimethylamine- and carbon tetrachloride-induced hepatotoxicity through inhibition of cytochrome P450 2E1 (Brady *et al*, 1991). DSF also induces P450 2B 1/2 in rats (Pan *et al*, 1993). Several organosulfur compounds are efficacious in blocking the toxicities induced by the substrates catalyzed by cytochrome P450 2E1 such as acetaminophen, isoniazid and carbon tetrachloride, which are primarily meta-

bolized by P450 2E1 (Kim *et al*, 1996, 1997a). Protective effects of certain organosulfur compounds including DSF and allylsulfur-containing natural and synthetic agents against toxicant-induced liver injury appeared to be associated with the suppression in P450 2E1 expression in the liver and the induction of conjugating enzymes such as glutathione S-transferase (GSTs) (Brady *et al*, 1991; Ansher *et al*, 1983). Previous studies in this laboratory have shown that alkyl sulfides differentially modulate the expression of cytochrome P 450 2E1 and that P450 2E1 suppression is not necessarily correlated with the transcriptional activation of microsomal epoxide hydrolase (mEH) and GSTs in the liver (Kim *et al*, 1996). The enhanced expression of hepatic mEH and GST enzymes in the liver by oltipraz and 2-(allylthio)pyrazine also appeared to contribute to the protective effects against ionizing radiation as well as against toxicant-induced injury (Kim *et al*, 1997b).

The intracellular redox potential appeared to be responsible for the transcriptional activation of mEH and certain GSTs (e.g. rGSTA2) through the antioxidant responsive elements (AREs) on the genes. Michael acceptors and reactive oxygen species are considered to be involved in the ARE-mediated transcriptional activation of the genes (Hayes and Pulford, 1995). Thus, we were interested in comparatively evaluating the capability of disulfiram (DSF) and diethyldithiocarbamate (DDC), the reduced form of disulfiram, on the expression of hepatic mEH and major GSTs. This study was designed with the aim of characterizing the differential effect of the agents on the expression of mEH and GSTs in association with the possible change of redox-potential in the cells. The present study demonstrates the selective effect of DSF on the expression of mEH and major GST subunits in the liver. The effects of other sulfides including aldrithiol (ALD), 2,2'-dithiobis(benzothiazole) (DTB), tetramethylthiouram disulfide (TMTD) and allyl disulfide (ADS) were comparatively assessed toward the end of this study.

II. MATERIALS AND METHODS

1. Materials

[α-^{32}P]dATP (185 TBq/mmol) and [γ-^{32}P]ATP (185 TBq/mmol) were purchased from NEN Research Products (Arlington Heights, IL, U.S.A.). DSF and the other organosulfur compounds were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Most of the reagents in the molecular studies were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2. Animal treatment

Male Sprague-Dawley rats (180-220 g) were treated with DSF and DDC, as suspended in 0.1% carboxymethylcellulose solution and dissolved in an aqueous solution, respectively (20-200 mg/kg body weight, po). ALD, DTB, TMTD and ADS were administered, as dissolved in corn oil. Animals were maintained at a temperature between 20 and 23°C with a relative humidity of 50%. Rats were killed at 24 hr after treatment. Animals were fasted 16 hr prior to killing. Three separate animal experiments were carried out for each treatment group.

3. Isolation of total RNA

Total RNA was isolated using the improved single-step method of thiocyanate-phenol-chloroform RNA extraction according to the method of Puissant and Houdebine (1990).

4. Preparation of cDNA probes for mEH and GST

A specific cDNA probe for mEH was amplified by reverse transcriptase-polymerase chain reaction using the selective primers for each gene, as published previously (Kim *et al*, 1993). Specific cDNA probes for GST genes rGSTA2, rGSTA3, rGSTA5, rGSTM1 and rGSTM2 were amplified by reverse transcriptase-polymerase chain reaction using the selective primers for each gene, as described previously (Kim *et al*, 1996, 1997b). Polymerase chain reactions were performed for 40 cycles using the following parameters : denaturing at 94°C for 1 min, annealing at 50°C for 1 min, and elongation at 72°C for 3 min.

5. Northern blot hybridization

Northern blot was carried out according to the procedures described previously (Kim 1992, Kim *et al*, 1996). Briefly, total RNA isolated from rat livers was resolved by electrophoresis in an 1% agarose gel containing 2.2M 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, 5x Denhardt's solution [0.1% Ficoll, 0.1% polyvinylpyrrolidine and 0.1% bovine serum albumin (Pentex Fraction V)], 0.1% sodium dodecylsulfate (SDS), 200 μg/mL of sonicated salmon sperm DNA and 5x SSPE (1x 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 2x standard saline citrate (SSC) and 0.1% SDS for 10 min at room temperature and in 0.1x SSC and 0.1% SDS for 10 min at room temperature twice as well. The filters were finally washed in the solution containing 0.1x 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)$_{16}$.

6. 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 using MCID software version 4.20, rev 1.0, followed by background subtraction.

III. RESULTS

1. Hepatic mEH gene expression

Northern blot analysis was performed to examine the mEH mRNA level in total RNA fractions at 24 hr after DSF or DDC treatment. The chemical structures of DSF and DDC were shown in Fig. 1. Treatment of rats with DSF at the dose of 20, 50, 100 and 200 mg/kg resulted in 2-, 6-, 15- and 8-fold increases in the mEH mRNA level, respectively (Fig. 2). The ED50 value for the increase in mEH mRNA after DSF treatment was ~60 mg/kg. The mEH mRNA level was increased less at the dose of 200 mg/kg probably because of its cytotoxicity. In constrast to the effect of DSF, DDC was ineffective in stimulating the mEH gene in the liver (Fig. 2).

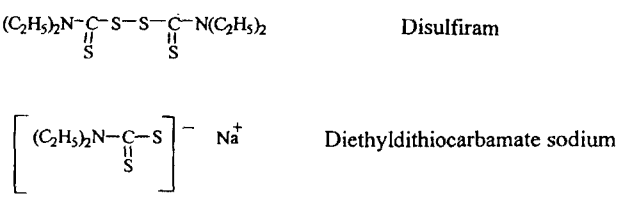

Fig. 1. Chemical structures of disulfiram and diethyldithiocarbamate sodium.

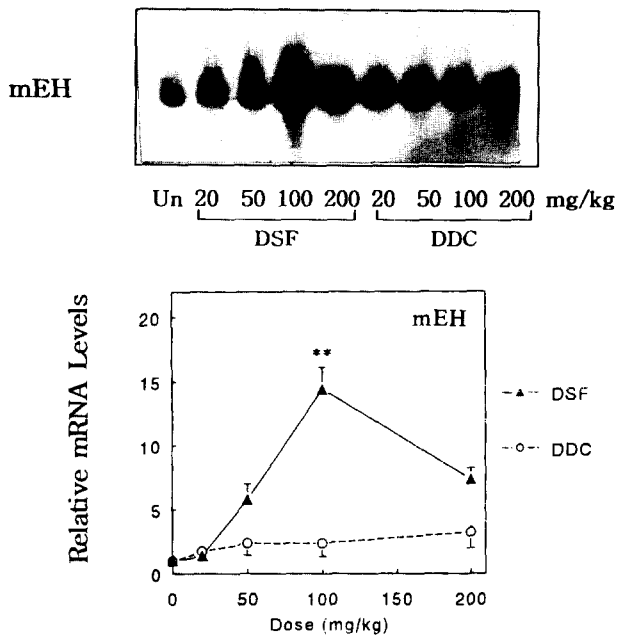

Fig. 2. RNA blot analyses of hepatic mEH mRNA level in rats after DSF or DDC treatment. Northern blot analysis was performed to examine the mRNA levels in total RNA fractions (20 μg each) isolated from rats at 24 hr after treatment with either DSF or DDC at the dose of 20, 50, 100 or 200 mg/kg. Un represents untreated animals. Relative increases in the mRNA level were depicted as a function of dose, as assessed by scanning densitometry of the Northern blots. Each point represents the mean±S.D. with N=3. The amount of RNA loaded in each lane was assessed by re-hybridization of the stripped membrane with ^{32}P-labeled poly(dT)$_{16}$. Significantly different from control (** $p<0.01$).

2. Hepatic rGSTA gene expression

The mRNA levels for rGSTA subunits including

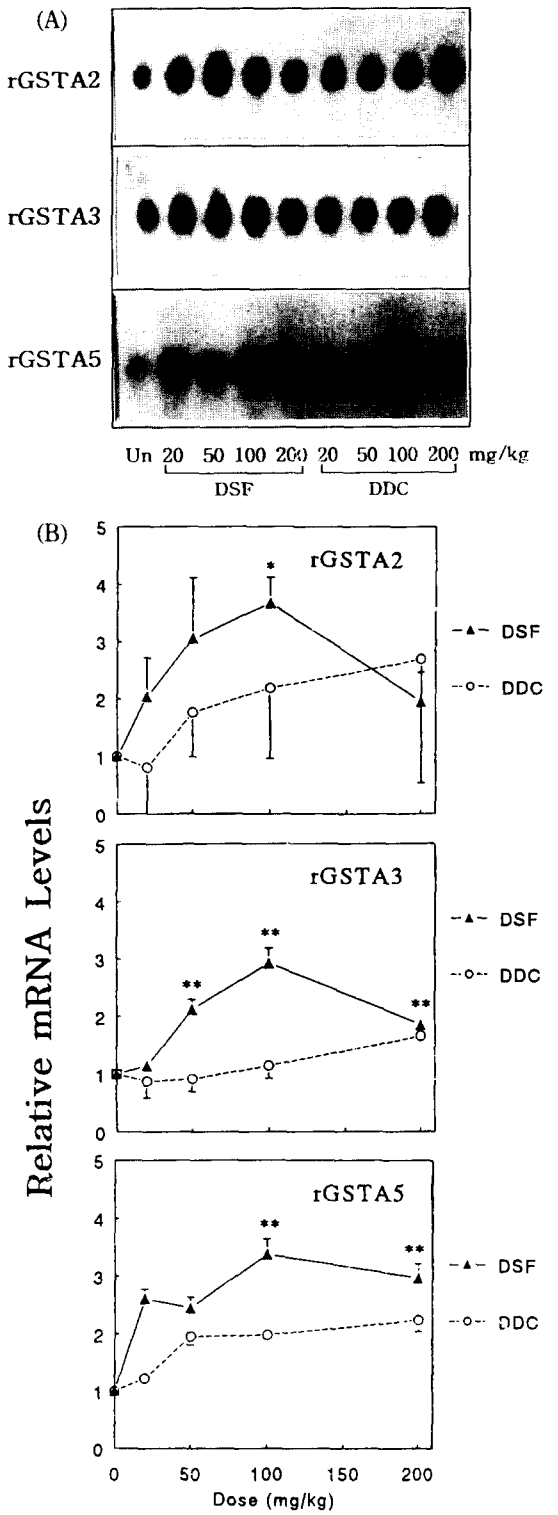

Fig. 3. RNA blot analyses of mRNA levels for rGSTA subunits in the rat liver. Panel A) Northern blot analysis was performed as described in Fig. 2. Panel B) Relative increases in the mRNA levels for rGSTA2, rGSTA3 and rGSTA5 were depicted as a function of dose following scanning densitometry of the Northern blots, as described in Fig. 2. Significantly different from control (*p<0.05; ** p<0.01).

rGSTA2, rGSTA3 and rGSTA5 were elevated after DSF treatment, as observed in mEH mRNA expression (Fig. 3A and 3B). A dose-dependent increase in rGSTA2 mRNA was observed at the doses of 20, 50 and 100 mg/kg, whereas the rGSTA2 mRNA level was rather decreased from the maximum at the dose of 200 mg/kg. Comparable increases were noted in rGSTA3 and rGSTA5 in

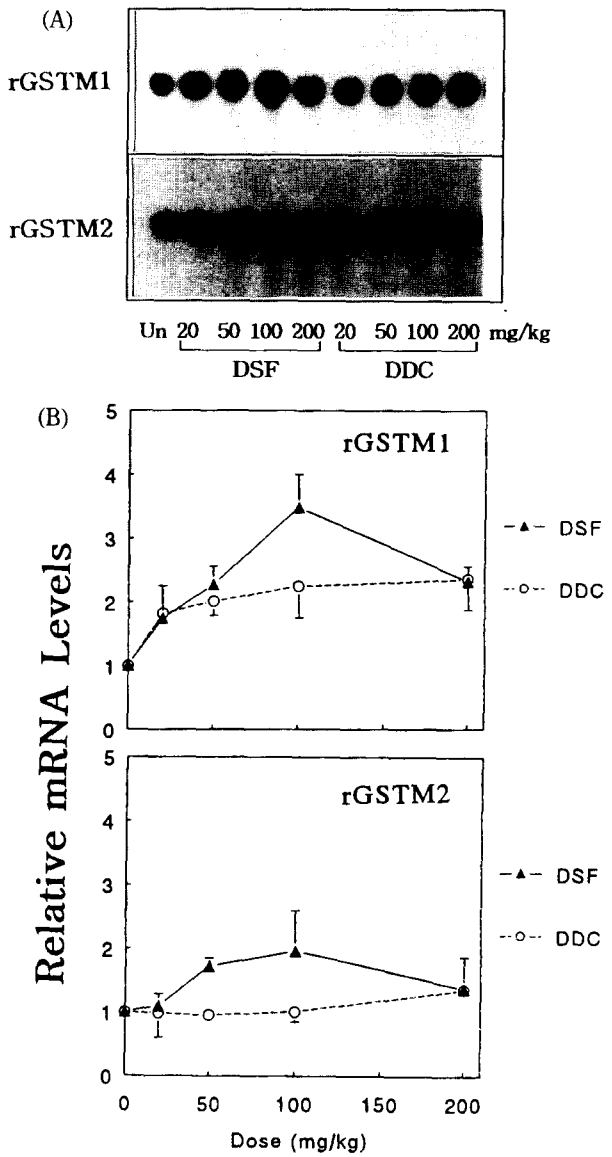

Fig. 4. RNA blot analyses of mRNA levels for rGSTM subunits in the rat liver. Panel A) Northern blot analysis was performed as described in Fig. 2. Panel B) Relative increases in the mRNA levels for rGSTM1 and rGSTM2 were depicted as a function of dose following scanning densitometry of the Northern blots.

response to DSF. These Northern blot analyses revealed that DSF increased the mRNA levels for rGSTA subunits with the ED50 values being noted as ~50 mg/kg. However, DDC was less effective in increasing the mRNA levels at the doses of 20, 50 or 100 mg/kg, although minimal 2- to 3-fold increases in rGSTA2, rGSTA3 and rGSTA5 were detected at the dose of 200 mg/kg.

3. Hepatic rGSTM gene expression

The mRNA levels for class mu GST genes were assessed after DSF or DDC treatment (Fig. 4A and 4B). Whereas the rGSTM1 mRNA level was increased 2-, 2.5- and 3.5-fold at 24 hr following a single dose of 20, 50 and 100 mg/kg of DSF, as compared to that in vehicle-treated animals, the mRNA level of rGSTM2 was not significantly altered. DDC was ineffective in elevating the rGSTM1 and rGSTM2 mRNA levels. Thus, changes in the class α GST mRNA levels were greater after DSF treatment than those in class μ. DSF was less active at the dose of 200 mg/kg.

These results showed that DSF increased the mRNA levels for mEH and major GSTs, whereas DDC was minimally active in stimulating the mRNA expression.

4. Effects of other organosulfides on the mEH mRNA expression

The effects of organosulfides including ALD, DTB, TMTD and ADS on the mEH mRNA expression was assessed in rats in order to further confirm the enhancing effects of other disulfides on the gene expression (Fig. 5). Treatment of rats with ALD at the dose of 100 mg/kg resulted in a 16-fold increase in the mEH mRNA level at 24 hr post-treatment (Fig. 6). DTB, TMTD and ADS also caused 5-, 9- and 12-fold increases in the mRNA level, respectively, as compared to control. ADS was used for comparative purpose. Thus, all of the disulfides examined were capable of stimulating the hepatic mEH gene.

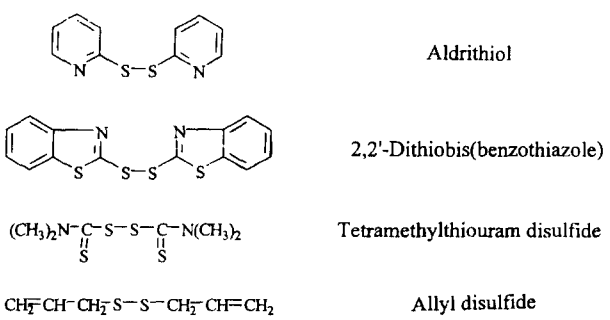

Fig. 5. Chemical structures of other disulfides including aldrithiol, 2,2'-dithiobis(benzothiazole), tetramethylthiouram disulfide and allyl disulfide.

5. Hepatic rGSTA gene expression by disulfides

Treatment of rats with ALD, DTB and TMTD at the dose of 100 mg/kg resulted in 7-, 2- and 3.5-fold increases in the rGSTA2 mRNA levels at 24 hr

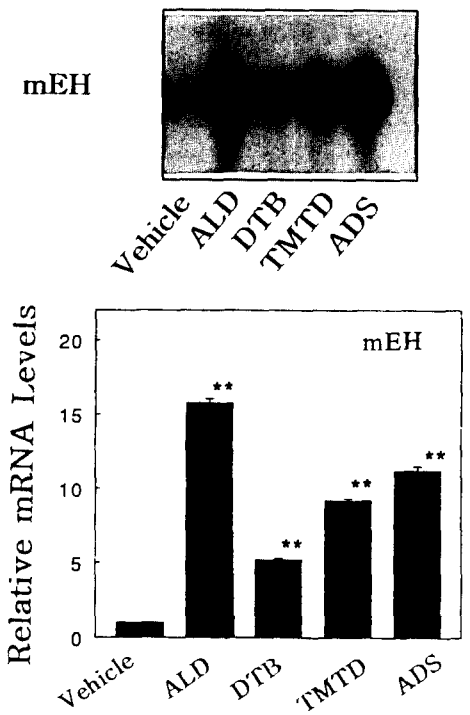

Fig. 6. RNA blot analyses of mRNA levels for mEH in the rat liver at 24 hr after treatment with ALD, DTB, TMTD or ADS. Northern blot analysis was performed to examine the mRNA levels in total RNA fractions (20 μg each) obtained from rat liver at 24 hr after gavaging with corn oil (vehicle), ALD (100 mg/kg), DTB (100 mg/kg), TMTD (100 mg/kg) or ADS (50 mg/kg). Relative increases in the mRNA levels for mEH were depicted following scanning densitometry of the Northern blots. Each point represents the mean ± S.D. with N=3. The amount of RNA loaded in each lane was assessed by re-hybridization of the stripped membrane with ^{32}P-labeled poly(dT)$_{16}$. Significantly different from control (** $p<0.01$).

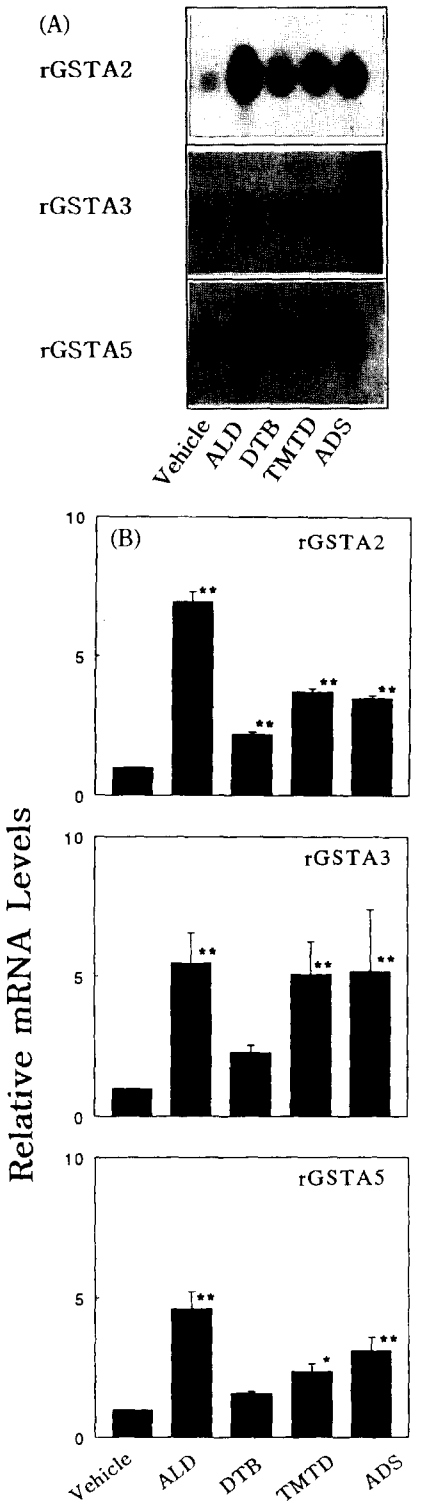

Fig. 7. RNA blot analyses of mRNA levels for rGSTA subunits in the rat liver. Panel A) Northern blot analysis was performed as described in Fig. 6. Panel B) Relative increases in the mRNA levels for rGSTA2, rGSTA3 and rGSTA5 were depicted following scanning densitometry of the Northern blots. Significantly different from control (*p <0.05; ** p<0.01).

post-treatment (Fig. 7A and 7B). In particular, ALD was very effective in stimulating the rGSTA2, rGSTA3 and rGSTA5 genes in common. Time- and dose-dependent increases in the mRNA levels by ALD were also noted (data not shown). DTB caused 2- to 3-fold elevation in the mRNA levels, as compared to control. TMTD treatment resulted in greater increases in the mRNA levels than DTB (i.e. 3- to 5-fold increases).

6. Hepatic rGSTM gene expression by disulfides

The mRNA levels for class μ GST genes were

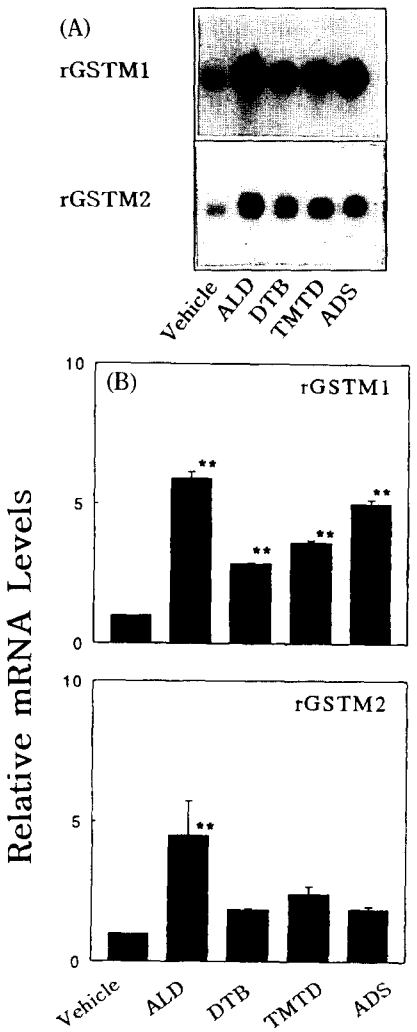

Fig. 8. RNA blot analyses of mRNA levels for rGSTM subunits in the rat liver. Panel A) Northern blot analysis was performed as described in Fig. 6. Panel B) Relative increases in the mRNA levels for rGSTM1 and rGSTM2 were depicted following scanning densitometry of the Northern blots. Significantly different from control (** p<0.01).

monitored (Fig. 8A and 8B). All of the disulfides increased the rGSTM1 mRNA level at 24 hr after administration. Interestingly, ALD was again very efficient in stimulating both rGSTM1 and rGSTM2 genes among the disulfides examined. Changes in rGSTM2 mRNA were less than those in rGSTM1 by the disulfides. Taken together, these results showed that DSF and other disulfides were active in increasing the mRNA levels for hepatic mEH and major GSTs, whereas DDC, a reduced form of DSF, was ineffective.

IV. DISCUSSION

Disulfiram has been used as a alcohol deterrent drug because the agent nonspecifically inhibits aldehyde dehydrogenase (Gilman *et al*, 1990). It has been shown that intoxication of DSF and DDC may acutely disrupt dopamine balance, which underlys some of the central neurotoxic, extrapyramidal symptoms associated with dithiocarbamate overdose (Vaccari *et al*, 1996). These sulfur-containing chelating agents mobilize metals from blood and tissues, and reverse the metal-induced biochemical alterations (Tandon *et al*, 1996). For example, DDC chelates heavy metals including lead, cadminium, mercury and arsenic and effectively scavenges the metals.

We have shown that alkyl sulfide-modulation in carbon tetrachloride-induced liver toxicity is related with differential induction of rGST subunits in association with suppression in the expression of cytochrome P450 2E1 (Kim *et al*, 1996). It has been shown that DSF, as one of sulfur-containing hepatoprotective agents, minimizes the liver necrosis induced by N-nitrosodimethylamine and carbon tetrachloride, indicating that DSF was effective in reducing cytochrome P450 2E1-mediated N-nitrosodimethylamine-induced liver toxicity, which was supported by the inhibition of cytochrome P450 activity as well as by cytochrome P450 suppression in response to DSF (Brady *et al*, 1991).

GST and mEH catalyze the detoxication of epoxide reactive intermediates. Transcriptional activation of rGST is coupled to the redox potential in the hepatocytes, which is associated with the reduced glutathione levels in tissues (Rushmore *et al*, 1991). Previous studies have shown that suppression in cytochrome P450 2E1 by allylsulfide as well as induction of rGSTA, in particular rGSTA5, was highly correlated with the hepatoprotective effects of allylsulfide and allyldisulfide against toxicant-induced liver injury (Kim *et al*, 1996). Given the potential protective role of enhanced GST expression in association with changes in certain cytochrome P450 expression, the present study was designed to examine the major GST gene expression in response to DSF and DDC and the structure-activity basis for the expression of the enzymes. mEH, rGSTA2, rGSTA3 and rGSTA5 mRNA levels were elevated in rats after treatment with DSF. DSF induced rGSTM1 in a dose-related manner, although DSF minimally altered the rGSTM2 mRNA level. DSF, however, rather exhibited suppression in the expression of mEH and major GST mRNA levels from the maximal elevation at the dose of 200 mg/kg. Furthermore, the extents of GST induction by DSF was only moderate, not like other hepatoprotective agents such as oltipraz (Pearson *et al*, 1988; Buetler, 1995). Thus, the induction by DSF was different from that observed with other typical antioxidants. This discrepancy may be related with the potent inhibition of cytochrome P450 2E1 expression through the formation of reactive metabolite intermediate and possibly with the lack of increase in the levels of hepatic reduced glutathione by DSF. Thus, it is likely that DSF might cause tissue injury at the high dose, following production of reactive metabolic intermediate.

GST is induced as part of adaptive responses because the enzyme is capable of reducing organic hydroperoxides or unsaturated aldehydes. Thus, GST is involved in protecting the tissue against the cytotoxicity of chemotherapeutic agents including adriamycin and bleomycin, and other oxidative stress (Daniel, 1993). Interestingly, however, the present study demonstrated that DDC was ineffective in stimulating the mEH and GST mRNA levels, providing evidence that the redox-state of sulfur atom in the chemicals is important in stimulating the mEH and rGST genes. It has been shown that GSTs at high enzyme concentrations catalyze the formation of dithiocarbamate from benzyl, allyl and phenethyl isothiocyanates (Hayes

and Pulford, 1995). These dithiocarbamate compounds appeared to be uncapable of stimulating the transcriptional activation of the genes, probably because dithiocarbamates are the conjugate forms of isothiocynates. This was consistent with the result in the present study.

Studies in this laboratory have shown that $GdCl_3$ effectively inhibits the constitutive and inducible mEH and GST expression with decreases in their mRNA levels (Kim and Choi, 1997). $GdCl_3$ suppression in the expression of the detoxifying enzymes seems to be associated with its blocking of intracellular Ca^{++} influx, which affects signaling pathway(s) for the expression of the genes. Thus, the intracellular calcium level is highly associated with the expression of mEH and GST genes. Because DDC chelates divalent cations, the differential effect of DSF and DDC might in part result from the change in the intracellular Ca^{++} level after treatment with the agents.

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