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GADOLINIUM CHLORIDE INHIBITION OF RAT HEPATIC MICROSOMAL EPOXIDE HYDROLASE AND GLUTATHIONE *S*-TRANSFERASE GENE EXPRESSION

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ABSTRACT:

The effects of gadolinium chloride, a Kupffer cell toxicant, on the constitutive and inducible expression of hepatic microsomal epoxide hydrolase (mEH) and glutathione *S*-transferase (GST) genes were examined in rats. Northern blot analysis showed that treatment of rats with $GdCl_3$ caused suppression of mEH and GST gene expression. mEH mRNA levels were decreased in a time-dependent manner after a single injected dose of $GdCl_3$ (10 mg/kg, iv), resulting in 95, 55, 17, 36, and 69% of the levels in untreated animals at 6, 12, 18, 24, and 48 hr after treatment, respectively. A maximal reduction in GST Ya, Yb1/2, and Yc1 mRNA levels was also noted at 18 hr after treatment with $GdCl_3$, followed by a gradual return to levels in untreated rats at later time points. Whereas treatment of rats with thiazole, allyl disulfide, propyl sulfide, oltipraz, or clotrimazole caused 2–13-fold increases in mEH mRNA levels at 18 hr after treatment, concomitant $GdCl_3$ treatment caused 30–70% reductions in the increases in mEH mRNA levels. The chemical-inducible mRNA levels for GST Ya, Yb1/2, and Yc1 were also significantly inhibited by $GdCl_3$ at 18 hr after treatment. Rats treated with $GdCl_3$ (10 mg/kg/day, iv) for 3–5 consecutive days exhibited 40–90% decreases in mEH, GST Ya, and GST Yb1/2 mRNA levels, relative to control, whereas the Yc1 mRNA level was suppressed at early times and returned to levels in untreated animals at day 5 after treatment. The mRNA levels for mEH and GST Ya in rats treated daily with both allyl disulfide (25 mg/kg, po) and $GdCl_3$ for 3 consecutive days were 20–30% of those in rats treated with allyl disulfide alone. Western immunoblotting showed that mEH and GST Ya protein expression was decreased at 1–3 days after consecutive daily treatment with $GdCl_3$. Whereas treatment of rats with $GdCl_3$ at a dose of 1 mg/kg suppressed constitutive hepatic mEH gene expression by 85% at 18 hr, rats treated with $CaCl_2$ (10 mg/kg, iv) in combination with $GdCl_3$ (1 mg/kg, iv) showed 45% suppression of the mEH mRNA level, compared with untreated animals. $GdCl_3$-induced suppression was also significantly reversed for GST Ya mRNA by excessive $CaCl_2$ administration. These results demonstrate that $GdCl_3$ effectively inhibits constitutive and inducible mEH and GST expression, with decreases in their mRNA levels. $GdCl_3$ suppression of detoxifying enzyme expression may be associated with its blocking of intracellular Ca^{2+} influx, which affects signaling pathways for the expression of the genes.

Kupffer cells are involved in the metabolic activation of xenobiotics, as well as in inflammatory injury. Because Kupffer cells are the major source of eicosanoids and cytokines and are associated with increased oxygen uptake after stimulation, pathological alterations induced by toxicants are considered to be associated, at least in part, with Kupffer cells (1–3). Reactive oxygen intermediates derived from redox metabolism in the inflammatory cells may accelerate tissue damage and pathological changes. Gadolinium chloride has been used as an agent that decreases Kupffer cell numbers and function in a number of studies (2, 4, 5). It has been shown that $GdCl_3$ ameliorates allyl alcohol- and CCl_4-induced liver injury, suggesting the possibility that $GdCl_3$ may contribute to improving liver function against hepatotoxicity (6, 7). $GdCl_3$ can prevent the induction of portal venous tolerance, because this agent modulates the function of Kupffer cells (8).

Gadolinium chloride is a potent blocker of voltage-sensitive Ca^{2+} channels, as demonstrated in the dose-dependent inhibition of the rise in intrasynaptosomal free Ca^{2+} levels, as well as depolarization-activated Ba^{2+} current (9). In certain cells, gadolinium ion might act selectively against a slowly inactivating component of the Ca^{2+} current. Injection of $GdCl_3$ causes alterations in the homeostasis of Ca^{2+} metabolism, which is supported by the increases in plasma total calcium and phosphate levels after iv injection of $GdCl_3$ (10).

Qu *et al.* (11) showed that increases in cytochrome P450 monooxygenase activity and glucuronide conjugation produced by liver transplantation were prevented by treatment of rats with gadolinium chloride. In most *in vivo* studies, the effects of Gd^{3+} were studied at early time points (*e.g.* several minutes to 24 hr). Furthermore, the effects were assessed after a single injection of the agent. In view of the wide use and varied applications of $GdCl_3$, the present study was designed to determine single and multiple dosing effects of $GdCl_3$ on mEH[1] and major GST gene expression, information on which would be of assistance in elucidating the mechanism of gene expression in response to xenobiotics, as well as the molecular basis of enzyme expression. We were particularly interested in characterizing the effects of $GdCl_3$ on detoxifying gene expression in response to structurally different chemical inducers and the possible role of Ca^{2+} channel blocking and Kupffer cell inactivation in the regulation of mEH and major GST gene expression.

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[1] Abbreviations used are: mEH, microsomal epoxide hydrolase; ADS, allyl disulfide; CL, clotrimazole; GST, glutathione *S*-transferase; OZ, oltipraz; PKC, protein kinase C; PS, propyl sulfide; PZ, pyrazine; TH, thiazole; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

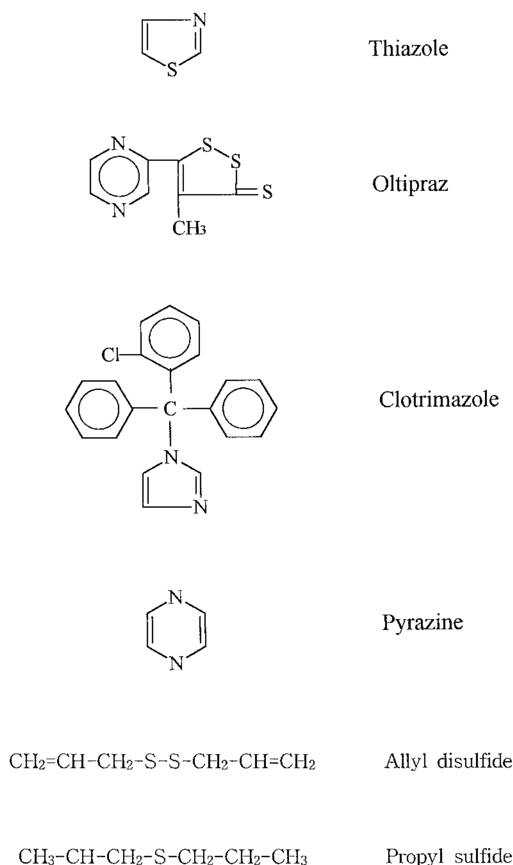


FIG. 1. *Chemical inducers of mEH and GSTs.*

Materials and Methods

Materials. TH, CL, ADS, PS, and PZ were purchased from Aldrich Chemical Co. (Milwaukee, WI). [α-^{32}P]dATP (3000 mCi/mmol) and [γ-^{32}P]ATP (3000 mCi/mmol) were purchased from Amersham (Arlington Heights, IL). Random prime labeling and 5′-end labeling kits, biotinylated goat anti-rabbit IgG, and streptavidin-conjugated horseradish peroxidase were purchased from Life Technologies (Gaithersburg, MD). Form-specific, polyclonal, rabbit anti-rat GST Ya antibody was purchased from Biotrin International (Dublin, Ireland). OZ was a gift from Rhône-Poulenc Rorer (Virtry-sur-Seine, France).

Animal Treatment. Male Sprague-Dawley rats (200–250 g) were obtained from the Korea Food and Drug Administration (Seoul, Korea) and maintained at a temperature of 20–23°C, with a relative humidity of 50%. Animals were caged under a supply of filtered, pathogen-free air. Cheiljedang rodent chow (Seoul, Korea) and water were available *ad libitum* unless specified. Rats (200–300 g) were treated with each of the inducing agents (25 and/or 50 mg/kg body weight/day, 1–3 days) before gadolinium chloride injection and were fasted 16 hr before sacrifice. Gadolinium chloride was injected through the tail vein at a dose of 10 or 1 mg/kg. To determine the effects of gadolinium chloride on inducible expression, the compounds, including TH, OZ, CL, ADS, PS, and PZ, were administered at a daily dose of 25 and/or 50 mg/kg. $GdCl_3$ was given at the same time as the xenobiotics. Chemical structures of the agents used in this study are shown in fig. 1. TH and PZ were administered ip as aqueous solutions, whereas ADS and PS were administered by gavage, using corn oil as a vehicle. OZ and CL were administered by gavage as suspensions in a 0.1% carboxymethylcellulose solution. Northern blot analysis was carried out with liver samples pooled from at least two animals. Each data point represents the mean ± SD from three independent experiments.

Subcellular Fractionation. Hepatic microsomal and cytosolic fractions were prepared by differential centrifugation. Microsomes were washed in pyrophosphate buffer and stored in 50 mM Tris acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol. The hepatic cytosol was 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 then at 100,000*g* for 90 min. Microsomal and cytosolic preparations were stored at −70°C until use.

Immunoblot Analysis. Immunoblot analysis was performed according to previously published procedures (12–14). Microsomal and cytosolic proteins were separated by 8% and 11% SDS-polyacrylamide gel electrophoresis, respectively, and electrophoretically transferred to nitrocellulose paper (15). The nitrocellulose paper was incubated with either rabbit anti-rat mEH or rabbit anti-rat GST antibodies, as described previously (13, 14). Immunoreactive protein was visualized by incubation with streptavidin-horseradish peroxidase, followed by addition of both 4-chloro-1-naphthol and hydrogen peroxide.

cDNA Synthesis and Polymerase Chain Reaction Amplification. Specific cDNA probes for GST genes Ya, Yb1, Yb2, and Yc1[2] were amplified by reverse transcription-polymerase chain reaction using selective primers for each gene, as described previously (12–14). 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.

RNA Blot Analysis. Northern blot analysis was carried out according to the procedures described previously (12–14). Total RNA (20 μg) isolated from rat livers was resolved by electrophoresis in an 1% agarose gel containing 2.2 M formaldehyde and was then transferred to nitrocellulose paper by capillary transfer, followed by hybridization (16, 17). The nitrocellulose paper was baked in a vacuum oven at 80°C for 2 hr. Blots were incubated in hybridization buffer, containing 6× standard saline/phosphate/EDTA (1× standard saline/phosphate/EDTA contains 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 mM Na_2EDTA, pH 7.4), 200 μg/ml sonicated salmon sperm DNA, 0.1% SDS, and 5× Denhardt's solution [0.1% Ficoll, 0.1% polyvinylpyrrolidine, 0.1% bovine serum albumin (Pentex fraction V)], at 53°C for 1 hr without probe. Hybridization was performed at 42°C for 18 hr with a heat-denatured probe, which had been random prime labeled with [α-^{32}P]dATP, as described previously (12–14). Filters were washed in 2× SSC (1×SSC contains 0.15M NaCl and 0.015M sodium citrate pH 7.0)/0.1% SDS for 10 min at room temperature twice and in 0.1× SSC 0.1% SDS for 10 min at room temperature twice. Filters were finally washed in 0.1× SSC/0.1% SDS for 60 min at 60°C. The stripped membranes were hybridized with poly(dT)$_{16}$, which had been end labeled with [γ-^{32}P]ATP, to quantify the amount of mRNA loaded onto the agarose gel and transferred to the nitrocellulose paper. Films were exposed at −70°C for 12–48 hr, using intensifying screens.

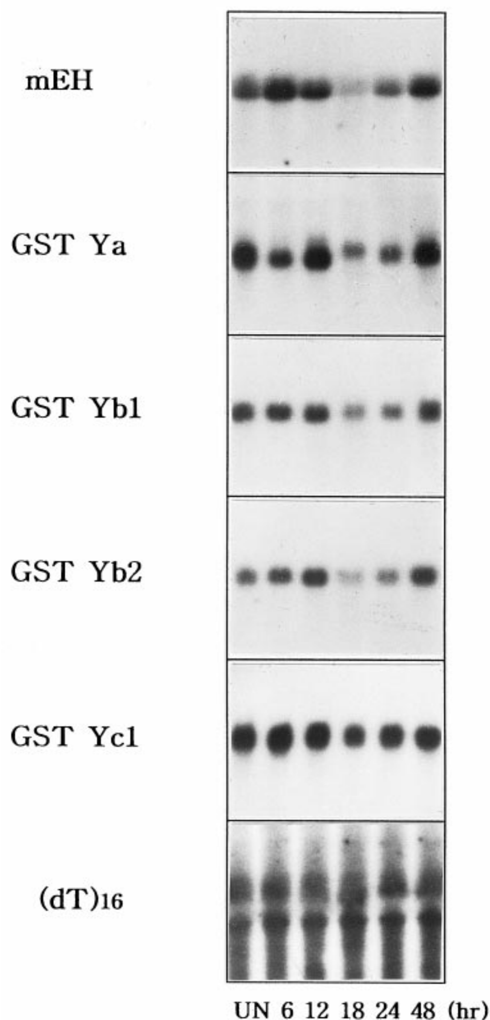
Scanning Densitometry. Scanning densitometry was performed with a Microcomputer Imaging Device, model M1 (Imaging Research, St. Catharines, Ontario, Canada). The area of each lane was integrated using MCID software version 4.20, revision 1.0, followed by background subtraction.

Data Analysis. Data were analyzed using computer programs for pharmacological calculations (18). One-way analysis of variance procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman-Keuls test was used for comparisons of multiple group means. Student's *t* test was used to determine whether two population means differed significantly. The criterion for statistical significance was set at $\alpha = 0.05$.

Results

Effects of a Single Injected Dose of $GdCl_3$ on mEH and GST Gene Expression. The effect of $GdCl_3$ on the constitutive expression of hepatic mEH and GST genes was examined (fig. 2). Northern blot analyses revealed that $GdCl_3$ suppressed the expression of mEH and GST genes in the liver. mEH mRNA levels were decreased in a time-dependent manner at early times after $GdCl_3$ treatment. The relative mEH mRNA levels were suppressed to 95, 55, 17, 36, and 69% of those in untreated animals at 6, 12, 18, 25, and 48 hr, respectively, after a single dose of $GdCl_3$ injection (10 mg/kg, iv). Thus, a maximal reduction was noted at 18 hr after treatment, followed by a gradual rebound at 24 and 48 hr. Expression of GST Ya,

[2] The class-based subunit nomenclature for rat GST Ya, Yb1, Yb2, and Yc1 is rGSTA2, rGSTM1, rGSTM2, and rGSTA3, respectively (32).



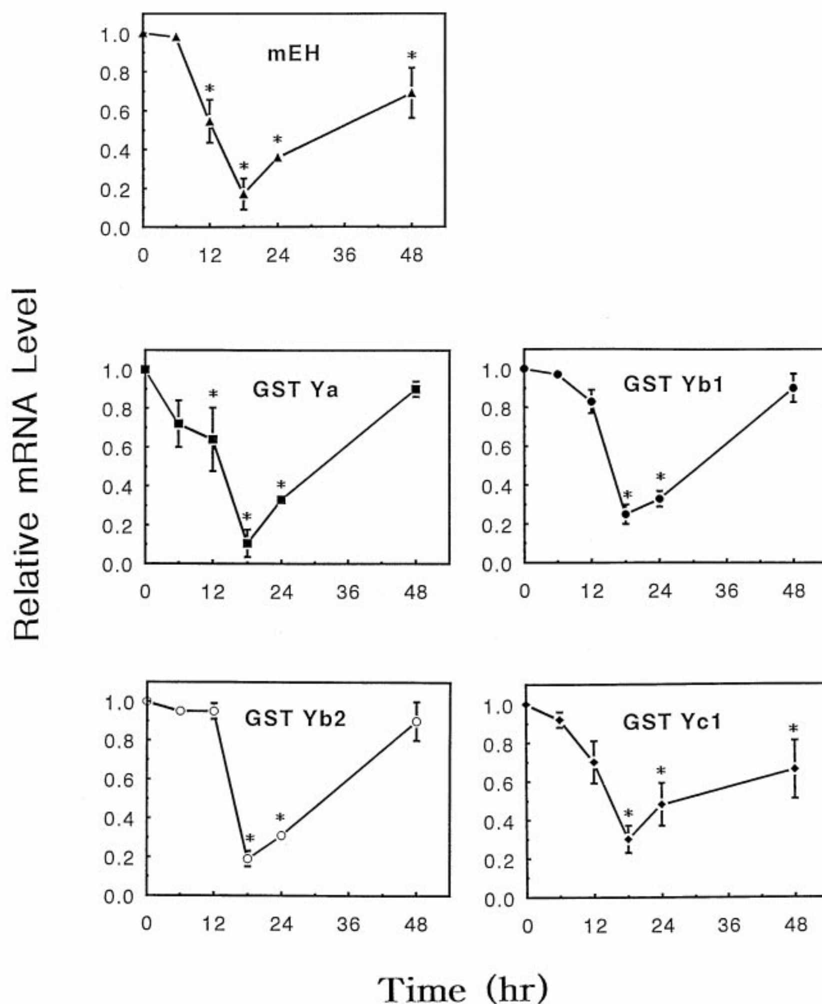


FIG. 2. *Effects of $GdCl_3$ on the levels of constitutive mEH and GST mRNA in the liver.*

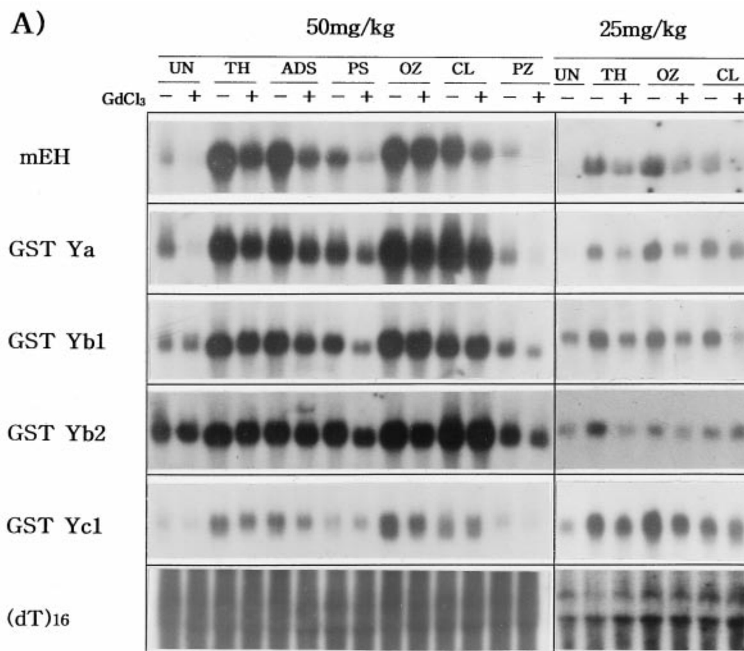
A, RNA blot analyses of hepatic mEH and GST Ya, Yb1, Yb2, and Yc1 mRNA. Northern blot analysis was performed to examine mRNA levels in total RNA fractions (20 μg each) isolated from rats at 6, 12, 18, 24, and 48 hr after an iv injection of $GdCl_3$ at a dose of 10 mg/kg (*UN*, untreated animals). The RNA was fractionated in a 1% agarose gel containing 2.2 M formaldehyde, transferred to nitrocellulose paper, and hybridized with a ^{32}P-labeled cDNA probe. The amount of RNA loaded in each lane was assessed by rehybridization of the stripped membranes with ^{32}P-labeled poly$(dT)_{16}$. *B*, Relative changes in the levels of mRNA for mEH and GST Ya, Yb1, Yb2, and Yc1, compared with those in untreated rats. The mRNA levels were assessed by scanning densitometry of the blots, followed by normalization. Each point represents the mean ± SD of three experiments. Data were analyzed with one-way analysis of variance followed by Newman-Keuls test for comparison with untreated animals ($*p < 0.05$).

Yb1/2, and Yc1 was also suppressed by 70–90% at 18 hr after $GdCl_3$ treatment (fig. 2). Thus, $GdCl_3$ was effective in substantially inhibiting mEH and GST gene expression at 18 hr after treatment.

Structurally different organic inducers were used in subsequent experiments to determine whether $GdCl_3$ was capable of inhibiting the inducible expression of the genes. Preliminary experiments showed that TH, OZ, and CL were more potent than ADS, PS, and PZ in elevating mEH and GST Ya mRNA levels and that the greatest increase was noted at 18–24 hr after treatment. Thus, rats were treated with TH, OZ, or CL at a dose of 25 mg/kg, with or without $GdCl_3$, whereas ADS, PS, and PZ were given at doses of 50 mg/kg. $GdCl_3$ effects on inducible mRNA levels were assessed at 18 hr after treatment.

Whereas treatment of rats with TH, ADS, PS, OZ, CL, or PZ caused 7-, 13-, 4-, 9-, 4-, and 1.1-fold increases, respectively, in mEH mRNA levels at 18 hr after treatment, a concomitant $GdCl_3$ injection resulted in 4.5-, 4-, 1-, 4-, 1.3-, and 0.1-fold relative changes, compared with levels in untreated animals (fig. 3). Fig. 3*B* depicts a comparative evaluation of $GdCl_3$ effects on mEH gene expression. Thus, $GdCl_3$-mediated inhibition of gene expression was a common phenomenon in response to structurally diverse chemical inducers, indicating that $GdCl_3$ may not affect the metabolism of the compounds but, rather, may act on a common step in the pathway(s) of gene expression.

Studies were extended to establish whether $GdCl_3$ suppresses the expression of major GST genes. Northern blot analysis revealed that GST Ya mRNA levels were increased by 3.5-, 10-, 5.5-, 7-, 7-, and 1.6-fold at 18 hr after treatment with TH, ADS, PS, OZ, CL, or PZ, respectively, whereas a single-dose, concomitant, $GdCl_3$ injection inhibited the chemical-inducible increases in GST Ya mRNA levels by 10–90% (fig. 3*B*). The inducible mRNA levels of GST Yb1 and



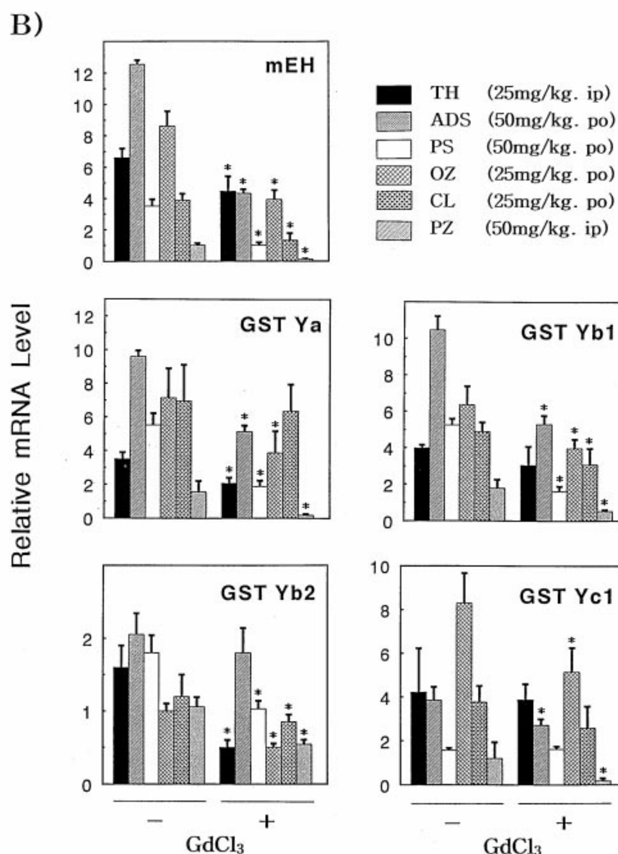


FIG. 3. *Effects of GdCl$_3$ on inducible mRNA levels for mEH and GST major subunits.*

A, Northern blot analyses for mEH and GST Ya, Yb1, Yb2, and Yc1 mRNA levels. Twenty micrograms of hepatic total RNA were isolated at 18 hr after a single dose of TH (25 mg/kg, ip), ADS (50 mg/kg, po), PS (50 mg/kg, po), OZ (25 mg/kg, po), CL (25 mg/kg, po), or PZ (50 mg/kg, ip), with or without a GdCl$_3$ injection (10 mg/kg, iv) (*UN*, untreated animals). *B*, Relative changes in mEH and GST Ya, Yb1, Yb2, and Yc1 mRNA levels in response to TH, ADS, PS, OZ, CL, or PZ, with or without a GdCl$_3$ injection, compared with levels in untreated rats (relative mRNA for untreated animals = 1). Relative changes in the levels of mRNA were assessed by scanning densitometry of the blots, followed by normalization. Each point represents the mean ± SD of three experiments. Data were analyzed with one-way analysis of variance followed by Newman-Keuls test for comparison with the respective xenobiotic without GdCl$_3$ (*$p < 0.05$).

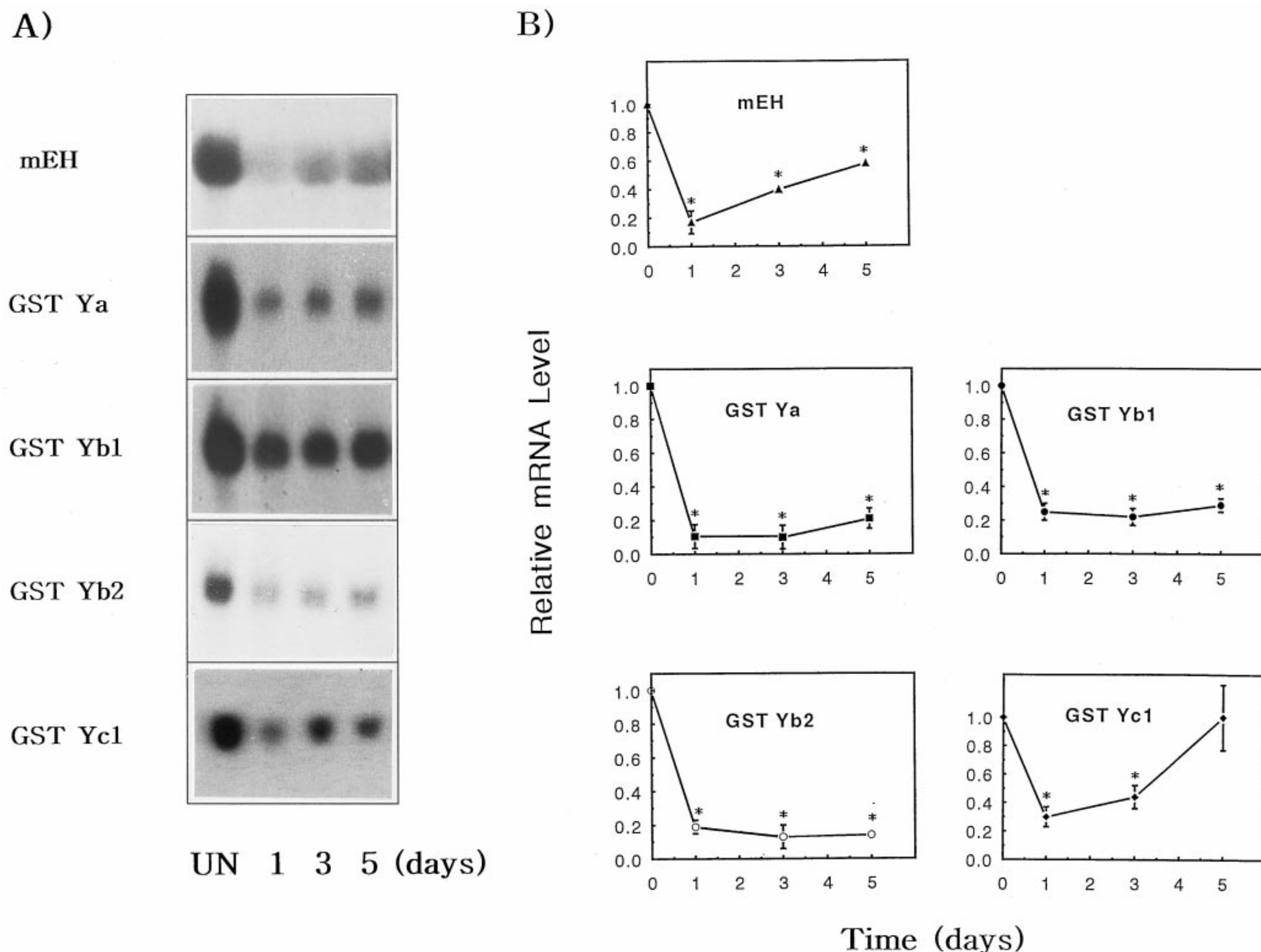


FIG. 4. *Constitutive mEH and GST mRNA levels after consecutive $GdCl_3$ treatment.*

A, RNA blot analyses of hepatic mEH and GST Ya, Yb1, Yb2, and Yc1 mRNA (*UN*, untreated animals). Northern blot analysis was performed to determine mRNA levels in total RNA fractions (20 μg each) isolated from rats at days 1, 3, and 5 after $GdCl_3$ treatment (10 mg/kg/day, iv). *B*, Relative changes in the levels of mRNA, compared with those in untreated animals. The mRNA levels were assessed by scanning densitometry of the blots, followed by normalization. Each point represents the mean $\pm$ SD of three experiments. Data were analyzed with one-way analysis of variance followed by Newman-Keuls test for comparison with untreated animals (*$p < 0.05$).

Yb2 were also decreased by 20–80% at 18 hr after $GdCl_3$ treatment. Inducible expression of GST Yc1 was suppressed by 10–40% (fig. 3*B*). The relative $GdCl_3$-inducible changes in GST Ya, Yb1, and Yb2 mRNA levels in response to various chemical inducers were quite comparable to those for mEH. Statistical analysis showed that suppression of mEH mRNA levels by $GdCl_3$ was correlated with that of GST Ya, Yb1, or Yc1 to a certain extent ($r = 0.6$).

Expression of mEH and GST Genes after Consecutive $GdCl_3$ Treatment. Because the maximal suppression of mEH and major GST mRNA levels was detected at 18 hr after a single dose of $GdCl_3$, we were interested in the time courses of constitutive gene expression after consecutive 3–5-day treatment with $GdCl_3$ (fig. 4*A*). Fig. 4*B* illustrates the relative changes in mEH mRNA levels in rats after consecutive daily treatment with $GdCl_3$. mEH mRNA levels slightly rebounded at later time points after the early maximal decrease, whereas GST Ya, Yb1, and Yb2 mRNA levels were suppressed by ~80% throughout the treatment period. Yc1 gene expression was suppressed at early times, although suppressed Yc1 mRNA levels gradually returned to levels observed in untreated animals, at 5 days after treatment (fig. 4).

The effect of multiple injections of $GdCl_3$ on ADS-inducible mEH expression was also determined. Whereas rats treated with ADS at a daily dose of 25 mg/kg showed 1.2–1.5-fold increases in mEH and GST Ya mRNA levels at days 1 and 3 after treatment, concomitant $GdCl_3$ treatment with ADS resulted in only 40% of levels in untreated animals (fig. 5). Thus, mEH and GST Ya mRNA levels inducible by the low dose of ADS were suppressed after consecutive $GdCl_3$ treatment for 3 days. The extent of inhibition by $GdCl_3$ of the inducible expression of mEH and GST mRNA at 3 days after multiple injections of 50 or 100 mg/kg ADS, however, was less than that after daily treatment with 25 mg/kg (data not shown), indicating that inducible expression produced by higher doses of ADS might not be effectively suppressed after multiple $GdCl_3$ injections.

Immunoblot Analysis of mEH and GST Ya. mEH and GST Ya protein levels, as assessed by immunoblot analysis, were determined after daily $GdCl_3$ treatment of animals (fig. 6). Scanning densitometric values for mEH protein levels at 1, 3, and 5 days after daily $GdCl_3$ treatment were 0.87 $\pm$ 0.13, 0.33 $\pm$ 0.21, and 0.63 $\pm$ 0.31, respectively, compared with control (mean $\pm$ SD, $N = 3$). Relative GST Ya levels at the aforementioned time points were 0.33 $\pm$ 0.41, 0.20 $\pm$ 0.22, and 0.6 $\pm$ 0.31, respectively (mean $\pm$ SD, $N = 3$). Thus, treatment with $GdCl_3$ for 1 or 3 days suppressed the expression of

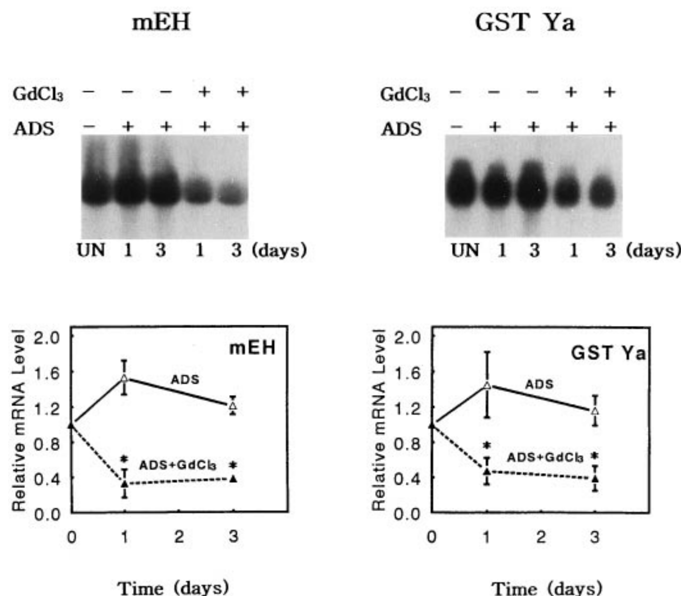


FIG. 5. *Representative Northern blot analyses of ADS-inducible mRNA levels for mEH and GST Ya after consecutive $GdCl_3$ treatment.*

Hepatic total RNA was isolated after ADS treatment at a daily dose of 25 mg/kg (po) for 1 or 3 days, with or without concomitant $GdCl_3$ daily injection (10 mg/kg, iv) for 1 or 3 days (*UN*, untreated animals). The relative changes in mEH and GST Ya mRNA levels, compared with those in untreated animals, are depicted. Each point represents the mean ± SD of three experiments. Data were analyzed with one-way analysis of variance followed by Newman-Keuls test for comparison with ADS alone ($*p < 0.05$).

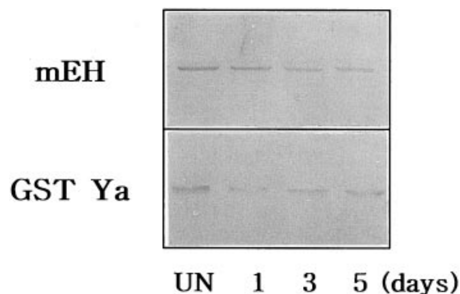


FIG. 6. *Representative immunoblot analyses of mEH and GST in the liver.*

These blots show hepatic mEH and GST Ya protein levels in untreated rats and rats treated with $GdCl_3$ (10 mg/kg/day, iv) for 1, 3, or 5 consecutive days (*UN*, untreated animals). These changes were confirmed by multiple immunoblottings.

mEH or GST Ya proteins levels, whereas the protein levels rebounded to certain extents at 5 days after consecutive treatment with $GdCl_3$. These data showed that $GdCl_3$-induced changes in the mRNA levels were closely related to those in protein expression.

Reversal of $GdCl_3$ Suppression of mEH and GST mRNA Levels by $CaCl_2$. The possibility of $GdCl_3$ blocking of intracelluar calcium influx through Ca^{2+} channels was examined to elucidate the mechanism of $GdCl_3$ inhibition of mEH and GST gene expression. Whereas treatment of rats with $GdCl_3$ at a dose of 1 mg/kg resulted in suppression of constitutive hepatic mEH expression by 85% at 18 hr after treatment, rats treated with $CaCl_2$ at a dose of 10 mg/kg in combination with $GdCl_3$ (1 mg/kg) showed 45% suppression of the mEH mRNA level, compared with untreated animals (*i.e.* ~4-fold increase in the mRNA level, compared with $GdCl_3$ alone) (fig. 7). $GdCl_3$-induced suppression of GST Ya mRNA levels was also significantly

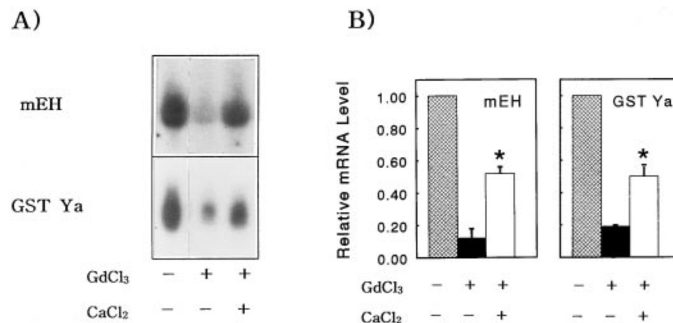


FIG. 7. *$CaCl_2$ reversal of $GdCl_3$ inhibition of mEH and GST mRNA levels.*

A, Northern blot analyses of mEH and GST Ya mRNA levels after treatment with $GdCl_3$ in combination with excessive $CaCl_2$. Lanes are associated with hepatic total RNA isolated from untreated rats (*UN*) or from rats at 18 hr after an injection of $GdCl_3$ (1 mg/kg, iv), with or without $CaCl_2$ (10 mg/kg, iv). Treatment of rats with $CaCl_2$ alone did not alter the mRNA levels for mEH and GST Ya. *B*, Relative changes in mEH and GST Ya mRNA levels, compared with those in untreated animals. Each point represents the mean ± SD of three experiments. Data were analyzed by Student's *t* test for comparison with $GdCl_3$ alone ($*p < 0.05$).

reversed by excessive $CaCl_2$. Although GST Ya mRNA levels were decreased by 80% at 18 hr after 1 mg/kg $GdCl_3$, concomitant administration of $CaCl_2$ with $GdCl_3$ resulted in 50% of levels in untreated rats. Treatment of rats with $CaCl_2$ alone did not alter the mRNA levels for mEH and GST Ya (data not shown). These results provided strong evidence that the suppressive effect of $GdCl_3$ was at least in part due to the blocking of Ca^{2+} channels.

Discussion

Because $GdCl_3$ is widely used as an agent that suppresses the activity and function of Kupffer cells, the regulation of hepatic mEH and GST expression in response to single or consecutive $GdCl_3$ treatment was studied in this research. The present study demonstrated for the first time that major GST mRNA levels were notably altered by $GdCl_3$, which was consistent with effects on mEH mRNA levels. We used structurally diverse chemical inducers to determine possible differential effects of $GdCl_3$ on detoxifying enzyme expression. Interestingly, however, $GdCl_3$ inhibition of the inducible expression of the detoxifying genes was a common phenomenon, irrespective of the chemical structures of the inducers. The time course of suppression of inducible expression was also consistent with that of constitutive expression. A single injected dose of $GdCl_3$ suppressed mEH or GST Ya expression to a maximal extent at 18 hr after treatment, with a return to 70–90% of the constitutive level at 48 hr. The expression of mEH and major GST genes challenged by structurally diverse chemical inducers was also inhibited in common at 18 hr after a single dose of $GdCl_3$. This marked suppression of mEH and GST expression by $GdCl_3$ was also observed after multiple daily treatments, except for GST Yc1 expression at a later time point. The comparable extents of inhibition of constitutive and inducible expression of mEH and GST, irrespective of the chemical structures of the inducers, and the similar time points for maximal inhibition indicate that $GdCl_3$ might block certain common steps in the expression of the detoxifying enzymes and that $GdCl_3$ may affect the activity of upstream enzymes involved in the signaling pathway.

Previous studies showed that a single injected dose of $GdCl_3$ caused a substantial decrease in hepatic triglyceride levels elevated by pyridine pretreatment (13). The effect of $GdCl_3$ seemed to be associated with a decrease in the production of oxygen or drug free radicals from

endogenous or exogenous substances, which was also supported by the improvement in liver function. This phenomenon, as well as Kupffer cell inhibition, with $GdCl_3$ might explain $GdCl_3$-induced amelioration of toxicant-induced liver injury. Induction of certain cytochromes P450 results in a pronounced increase in the rate of NADPH-dependent microsomal lipid peroxidation (19, 20). Lipid peroxide levels are also elevated in plasma and red blood cells of animals and humans during intake of large amounts of ethanol. It has been proposed that the formation of cytochrome P450 2E1-dependent lipid peroxides is involved in ethanol-dependent liver damage. These effects also seem to be associated with Ito cells and Kupffer cells in conjunction with cytokines and collagen production (20).

The induction of mEH and GST isoforms was comparatively evaluated. The inducible expression of GST Ya gene was quite comparable to that of the mEH gene after treatment with the compounds. It was shown that mEH expression in response to xenobiotics, including azole heterocycles and organosulfurs, accompanied the induction of GSTs including Ya, Yb1, Yb2, Yc1, and Yc2 subunits (13, 14). Certain GSTs and possibly mEH gene expression appeared to be mediated through the common *cis*-acting element(s) present in the genes (*i.e.* XRE or ARE) (21–23). Previous studies have shown that nitrogen- or sulfur-containing heterocycles such as PZ and TH induce mEH and GSTs, as well as inducing cytochrome P450 2E1 concomitantly (24). Other studies in this laboratory have shown that azole antimycotic agents, nitrogen-substituted imidazoles, and a few azole prototypic heterocycles affect mEH gene expression, showing that nitrogen- or sulfur-containing heterocycles induce mEH with 15–20-fold elevation of its mRNA levels (25–27). Because TH and CL were potent in stimulating mEH and GST genes (12, 24), the inhibitory effects of $GdCl_3$ were minimal at doses of 50 mg/kg or greater.

$GdCl_3$-induced suppression of mEH and GST mRNA levels appeared to be mediated by the competitive inhibition of intracellular calcium influx. The present study clearly shows that treatment of rats with an excessive amount of $CaCl_2$ (10 mg/kg) in combination with $GdCl_3$ (1 mg/kg) significantly reversed $GdCl_3$-induced suppression of mEH and GST mRNA levels, providing strong evidence that $GdCl_3$ suppression is at least partly due to blocking of intracellular Ca^{2+} influx. The comparable extents of inhibition of constitutive and inducible expression of mEH and GST, irrespective of the chemical structures of the inducers, and the similar time points for maximal inhibition after $GdCl_3$ treatment are also supportive of the role of changes in intracellular Ca^{2+} levels after $GdCl_3$ treatment. A preliminary study also showed that treatment of animals with verapamil at a dose of 10 mg/kg suppressed the expression of detoxifying genes. ADS- or PS-inducible increases in mEH and GST Ya genes were also substantially blocked by verapamil.

The effects of gadolinium ions might be due to differences in PKC-dependent sensitivity in the induction pathway(s), in association with intracellular Ca^{2+} levels. PKC is involved in the induction of certain enzymes and in the up-regulation of c-*jun* after certain drugs or UV irradiation (28, 29). The suppression of mEH and GST gene expression by $GdCl_3$ observed in this study is likely to be associated with the activity of PKC. Because PKC activity is dependent on the intracellular Ca^{2+} level, $GdCl_3$ blocking of Ca^{2+} influx into hepatocytes might affect the activity of PKC. Hence, $GdCl_3$-induced changes in the expression of the xenobiotic-metabolizing enzymes might be associated with modulation of intracellular Ca^{2+} levels. Mobilization of Ca^{2+} from other intracellular storage sites, as well as increases in plasma Ca^{2+} levels, would adjust the intracellular Ca^{2+} levels, which might provide the ability to overcome the effect of $GdCl_3$ inhibition, to a certain extent, after multiple treatments. Other studies in this laboratory showed that acriflavine, a PKC inhibitor, potently suppressed mEH and GST gene expression (30). Concomitant treatment of rats with both acriflavine and $GdCl_3$ completely prevented the ADS-inducible expression of the enzymes and shifted the dose-inhibitory response curves for acriflavine to the left, with a 15-fold increase in the relative inhibitory potency. These results also support the conclusion that the site of action for $GdCl_3$ is not in common with that of PKC inhibitors. Because the catalytic activity of PKC is mediated through Ca^{2+} mobilization, sequential blocking of Ca^{2+} influx and PKC activity by the two agents would produce synergistic suppression of mEH and GST gene expression.

The effect of $GdCl_3$, a known Kupffer cell toxicant, may be associated with the inactivation of Kupffer cells. Because Kupffer cells have a role in secreting cytokines that might modulate the expression of hepatic enzymes, changes in mEH and GST expression might be mediated by changes in the secretion of certain cytokines after $GdCl_3$ inactivation of Kupffer cells. It has been shown that the expression of drug-metabolizing enzymes is modulated by cytokines. In particular, the levels of cytochromes P450 are altered in the presence of cytokines such as interleukins and interferons (31). These inflammatory mediators are also involved in tissue injury, such as hepatic ischemia and reperfusion injury. Hepatic expression of a number of cytochromes P450 is suppressed during inflammatory responses. For example, the specific expression of cytochrome P450 2C11 in male rat liver is transcriptionally decreased by endotoxin treatment, through inflammatory cytokines such as interleukin-1, interleukin-6, tumor necrosis factor, and interferons (31). However, it is unlikely that $GdCl_3$ produces inhibitory effects on mEH and GST expression through changes in the levels of cytokine mediators in the absence of inflammatory responses, although it should be further established whether cytokines would alter mEH and GST expression. Cytokines would exert their effects through membrane-associated receptors. Rather, the present experimental results support the conclusion that the modulation of Ca^{2+} levels by $GdCl_3$ affects downstream events under membrane-associated ligand binding, irrespective of the potential positive or negative actions of cytokines.

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