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Pretreatment with 1,8-Cineole Potentiates Thioacetamide-Induced Hepatotoxicity and Immunosuppression

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The effect of 1,8-cineole on cytochrome P450 (CYP) expression was investigated in male Sprague Dawley rats and female BALB/c mice. When rats were treated orally with 200, 400 and 800 mg/kg of 1,8-cineole for 3 consecutive days, the liver microsomal activities of benzyloxyresorufin- and pentoxyresorufin-*O*-dealkylases and erythromycin *N*-demethylase were dose-dependently induced. The Western immunoblotting analyses clearly indicated the induction of CYP 2B1/2 and CYP 3A1/2 proteins by 1,8-cineole. At the doses employed, 1,8-cineole did not cause toxicity, including hepatotoxicity. Subsequently, 1,8-cineole was applied to study the role of metabolic activation in thioacetamide-induced hepatotoxicity and/or immunotoxicity in animal models. To investigate a possible role of metabolic activation by CYP enzymes in thioacetamide-induced hepatotoxicity, rats were pre-treated with 800 mg/kg of 1,8-cineole for 3 days, followed by a single intraperitoneal treatment with 50 and 100 mg/kg of thioacetamide in saline. 24 h later, thioacetamide-induced hepatotoxicity was significantly potentiated by the pretreatment with 1,8-cineole. When female BALB/c mice were pretreated with 800 mg/kg of 1,8-cineole for 3 days, followed by a single intraperitoneal treatment with 100 mg/kg of thioacetamide, the antibody response to sheep red blood cells was significantly potentiated. In addition, the liver microsomal activities of CYP 2B enzymes were significantly induced by 1,8-cineole as in rats. Taken together, our results indicated that 1,8-cineole might be a useful CYP modulator in investigating the possible role of metabolic activation in chemical-induced hepatotoxicity and immunotoxicity.

Key words: Cytochrome P450, 1,8-Cineole, Metabolic activation, Thioacetamide, Hepatotoxicity, Immunotoxicity

INTRODUCTION

Following biotransformation, xenobiotics generally become less lipophilic and more polar so that they may leave the body *via* the normal excretion routes. However, biotransformation does not always imply detoxification, because, in certain instances, metabolites that are capable of reacting with tissue macromolecules or acquiring toxic properties different to or greater than those of the parent molecules would be produced (Nebbia, 2001). Among many xenobiotic-biotransforming enzymes, the levels of cytochrome P450s (CYPs) in hepatic microsomes are regulated by several factors, including age, sex, drugs, chemicals and diseases (Bresnick, 1993). Since hepatic monooxygenases play important roles in the biotransformation of endogenous as well as xenobiotic chemicals, metabolic clearance and toxic effects of certain compounds might be modulated by chemical-induced alterations in the activity of these CYP enzymes.

Many compounds in plants have been reported to modulate CYP expression in experimental animals. For example, methoxsalen found in celery, parsnip and fig suppressed CYP 1A1 induction in mouse hepatoma cells (Jeong *et al.*, 1995a) and myristicin found in parsley, carrot, black pepper, nutmeg and flavoring agents induced liver CYP 1A1/2, 2B1/2 and 2E1 in rats (Jeong and Yun, 1995). In addition, some phytochemicals like capsaicin in red peppers and diallyl sulfide in garlic have been characterized to possess chemopreventive effects

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against vinyl carbamate- and dimethylnitrosamine-induced mutagenesis and tumorigenesis by inhibiting CYP 2E1 that is responsible for bioactivating them (Surh *et al.*, 1995). Furthermore, we found that β-ionone in tangerine and tomato clearly induced CYP 2B1 through the accumulation of mRNA in Sprague Dawley rats (Jeong *et al.*, 1995b). Therefore, extensive efforts for evaluating the effects of compounds abundant in plants should be made, with a particular emphasis on CYP expression, because the modulation of CYP by certain compounds in dietary plants may not only interfere with drug action in the body but also play a role in chemical-induced toxicity. In addition, CYP modulators with low toxicity from plant origin are able to be introduced in studies on the possible role of metabolic activation in chemical-induced toxicity in which metabolism by CYP enzymes is required.

The plant volatile oils and their monoterpenoid (C10 compounds) constituents have long been used as ingredients of flavors, preservatives, fragrances, disinfectants, detergents, soaps and medicines. Among them, 1,8-cineole (Fig. 1), also known as eucalyptol, is a monoterpenoid cyclic ether that occurs in wormwood and eucalyptus as well as in many other plant oils. It is one of the components present in essential oils from *Eucalyptus polybractea* (Mitsuo *et al.*, 2000). 1,8-Cineole, possessing characteristic fresh and camphoraceous fragrance and pungent taste, has been used for pharmaceutical preparations as an external applicant, a nasal spray, a disinfectant, an analgesic, or a food flavoring (Huang *et al.*, 1999). It has also been used for cosmetics. Furthermore, it has been reported that 1,8-cineole could be used for treating cough, muscular pain, neurosis, rheumatism, asthma and urinary stone (Juergens *et al.*, 1998; 2003). 1,8-Cineole has been reported to inhibit CYP 2B1 enzymes in liver microsomes, indicating a possible interaction with CYP enzymes (de Oliveira *et al.*, 1999).

In many cases, metabolic activation by CYP enzymes to toxic metabolite(s) is required in certain chemical-induced toxicity. Thioacetamide (TA), a potent hepatotoxicant, can be metabolized to more toxic metabolites via thioacetamide S-oxide by CYP enzymes (Mangipudy *et al.*, 1995). In addition, thioacetamide can also stimulate the DNA synthesis and mitosis in livers for hepatic regeneration (Mangipudy *et al.*, 1995). Moreover, thioacetamide has been used to induce immunotoxicity following metabolic activation (Kim *et al.*, 2000). Therefore, thioacetamide was chosen in the present study as a model toxicant requiring metabolic activation by CYP enzymes that might be modulated by the treatment with 1,8-cineole.

The primary objectives of the present study were to characterize effects of 1,8-cineole on CYP enzymes *in vivo* and to apply 1,8-cineole as a CYP modulator in thioacetamide-induced hepatotoxicity and immunosuppression. For hepatotoxicity studies, male Sprague-Dawley rats were employed, because these rats are one of the most useful animal models in studying chemical-induced toxicity. For immunotoxicity studies, female BALB/c mice, a widely used strain for immunotoxicity tests, were employed.

MATERIALS AND METHODS

Animals

Specific pathogen-free male Sprague-Dawley rats (180 to 220 g) and specific pathogen-free female BALB/c mice (16 to 19 g) were obtained from Daehan Experimental Animal Center (Umsong, South Korea). The animals received at 5-6 weeks of age were acclimated for at least 1 week. Upon arrival, animals were randomized and housed five per cage. The animal quarters were strictly maintained at 23±3°C and 50±10% relative humidity. A 12-h light and dark cycle was used with an intensity of 150-300 Lux.

Materials

1,8-Cineole, bovine serum albumin, thioacetamide, DEAE-dextran, ethoxyresorufin, methoxyresorufin, pentoxyresorufin, benzyloxyresorufin, resorufin, *p*-nitrophenol, erythromycin, glucose 6-phosphate, NADPH, the kits for serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) assays and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Company (St. Louis, MO, USA). Earle's balanced salt solution (EBSS) and guinea pig complements were purchased from GIBCO (Grand Island, NY, USA). Sheep red blood cells (SRBCs) were obtained at the College of Natural Resources at Yeungnam University. The primary antibodies against individual CYP proteins were purchased from Easy-bio system (Seoul, Korea). All other chemicals used were of reagent grade commercially available.

Animal treatments

Animal treatment was performed in accordance with our institutional guidelines for care and use of animals for experimental purposes. To investigate the effect of 1,8-cineole on CYP expression, male SD rats were treated orally with 1,8-cineole at 0, 200, 400, and 800 mg/kg/10 mL corn oil once a day for 3 consecutive days. For the hepatotoxicity study, SD rats were pretreated orally with 1,8-cineole in corn oil at 800 mg/kg for 3 consecutive days, followed by a single intraperitoneal administration with thioacetamide in saline at 50 and 100 mg/kg. To investigate the effect of pretreatment with 1,8-cineole in thioacetamide-induced immunotoxicity, female BALB/c mice were pretreated orally with 1,8-cineole in corn oil at 800 mg/kg once a day for 3 consecutive days, followed by

an intraperitoneal treatment with 50 and 100 mg/kg of thioacetamide in saline 24 h after the last treatment with 1,8-cineole. Thirty min later, mice were sensitized intraperitoneally with 5×10^8 SRBCs per mouse in 0.5 mL of EBSS. The antibody-forming cells (AFCs) in spleen were enumerated four days later.

Preparation of liver microsomes

Livers were removed and homogenized with four volumes of ice-cold 0.1 M potassium phosphate buffer, pH 7.4. The liver homogenates were centrifuged at 9000×g for 10 min at 4°C. The resulting post mitochondrial S-9 fractions were centrifuged again at 105,000×g for 60 min at 4°C. The microsomal pellets were resuspended in 0.1 M potassium phosphate buffer, pH 7.4. Aliquots of liver microsomes were stored at 80°C until use. The content of microsomal protein was determined according to the methods of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Hepatotoxicity parameters

For assaying the activities of serum AST and ALT, the serum was prepared by a centrifugation of blood at 2500×g for 10 min at room temperature. The activities were determined according to the instruction manual prepared by the manufacturer.

Assay of microsomal monooxygenase activities

To characterize the effects of 1,8-cineole on specific CYP isozymes, the following monooxygenase activities were determined. Ethoxyresorufin *O*-deethylase (EROD, CYP 1A1-specific) activity was determined as described by Blank *et al.* (1987) with a slight modification. The reaction mixture (2 mL) consisted of 0.1 M potassium phosphate buffer, pH 7.4, containing 2 mg/mL of bovine serum albumin, 5 mM glucose 6-phosphate, 1 U of glucose 6-phosphate dehydrogenase, 5 μM NADPH and 2.5 μM 7-ethoxyresorufin. The formation of resorufin was monitored fluorometrically at an excitation maximum of 550 nm and an emission maximum of 585 nm. Methoxyresorufin *O*-demethylase (MROD, CYP 1A2-specific), pentoxyresorufin *O*-dependentylase (PROD, CYP 2B-specific) and benzyloxyresorufin *O*-debenzylase (BROD, CYP 2B-specific) activities were determined by the method of Lubet *et al.* (1985) with a slight modification. All reaction components and assay procedures were exactly the same as the EROD assay, except that the substrates were 2.0 μM. *p*-Nitrophenol hydroxylase (PNPH, CYP 2E1-specific) activity was determined as described by Koop (1986). The reaction mixture (1.0 mL) was composed of 0.1 M potassium phosphate buffer, pH 7.4, containing 100 μM p-nitrophenol, 1 mM NADPH and the enzyme source. The amount of 4-nitrocatechol formed was measured spectrophotometrically at 512 nm. Erythromycin *N*-demethylase (ERDM, CYP 3A-specific) activity was determined by measuring the amount of formaldehyde formed, as described previously (Nash, 1953). Erythromycin at 400 μM was used as a substrate for assaying ERDM.

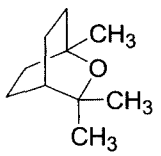

Fig. 1. Structure of 1,8-cineole.

Antibody response to SRBCs

Single cell suspensions of splenocytes were prepared in 3 mL of EBSS, washed and resuspended in 3 mL of EBSS. Spleen cells were then diluted 30-fold by resuspending a 100 μL aliquot of each suspension in 2.9 mL of EBSS. The number of AFCs was determined using a modified Jerne plaque assay, as described previously (Kaminski *et al.*, 1990). Briefly, 0.05% DEAE-dextran was added into melted 0.5% agar in EBSS and maintained at 47°C throughout the assay. Then 400 μL of melted agar was dispensed onto 12×75 mm heated glass tubes (Corning), followed by the addition of 25 μL of indicator SRBCs, 25 μL of guinea pig complement and 50 μL of splenocyte suspension. The SRBCs were washed at least three times with EBSS before use. A 200 μL aliquot from the tube was then immediately pipetted onto a 100×15 mm Petri dish and the agar solution was covered with a 24×40 mm microscopic cover glass. The Petri dishes were placed at room temperature for several minutes to allow the agar to solidify and were, then, incubated at 37°C for 3 h to form hemolytic plaques in a humidified 37°C incubator. Following the incubation, the AFCs were counted. The cell number of each spleen was determined using a Coulter counter. The results were expressed as AFCs/10^6 spleen cells or AFCs/spleen. The body and organ weights of animals used in the antibody response were determined on the day of necropsy.

Western blotting

Microsomal proteins (10 μg/well) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), of which concentrations were 10% for separating and 4% for stacking (Laemmli, 1970). Then the proteins were transferred to nitrocellulose filters. The filters were incubated with 3% gelatin for 1 h to block the non-specific binding, and then were incubated with rabbit polyclonal antibodies against either rat P450 2B1/2, 2E1 or 3A1/2, followed by an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody. For immunostraining, the nitrocellulose filters were developed with a mixture of 5-

bromo-4-chloro 3-indolyl phosphate, nitroblue tetrazolium and 0.1 M Tris buffer (1:1:10) under an instruction by the manufacturer.

Statistics

The mean value ± standard error (S.E.) was determined for each treatment group of a given experiment. Dunnett's *t*-test was used to compare statistical significance of data. The significant values at either P<0.05 (*) or P<0.01 (**) were represented as asterisks.

RESULTS

To investigate the effect of 1,8-cineole on general toxicity, changes of body and organ weights were determined initially (Table I). The body and organ weights were determined following treatment of rats orally with 1,8-cineole in corn oil for 3 consecutive days. At the doses used, the body and organ weights were not changed by 1,8-cineole. Effects of 1,8-cineole on serum AST and ALT activities were also assessed (Table I). 1,8-Cineole did not affect the activities of serum AST and ALT. These results indicated that 1,8-cineole might not be toxic at the doses used.

Table I. Effects of 1,8-cineole on body and liver weights and serum enzymes in male SD rats

Dose, mg/kg	Body weight (g)	Liver weight (g)	AST (IU/mL)	ALT (IU/mL)
Vehicle	197.6±1.2	9.99±0.65	70±3	49±2
200	197.3±2.6	10.07±0.45	68±2	50±4
400	199.5±3.5	10.71±0.43	66±4	49±3
800	190.1±4.5	10.55±0.60	67±4	51±6

Male SD rats were treated orally with 0, 200, 400 and 800 mg/kg of 1,8-cineole in corn oil for 3 consecutive days. All animals were subjected to necropsy 24 h after the last treatment. Each value represents the mean±S.E. of five animals.

The effects of 1,8-cineole on the activities of CYP-associated monooxygenase enzymes in rat liver microsomes prepared from the animals were also elucidated (Fig. 2). The BROD, PROD and ERDM activities were dose-dependently and significantly induced by treatment with 1,8-cineole when compared to the vehicle-treated control. Meanwhile the PNPH activity was not changed by 1,8-cineole in male SD rats.

The liver microsomes prepared from 1,8-cineole-treated rats in Table I were electrophoresed and immunoblotted

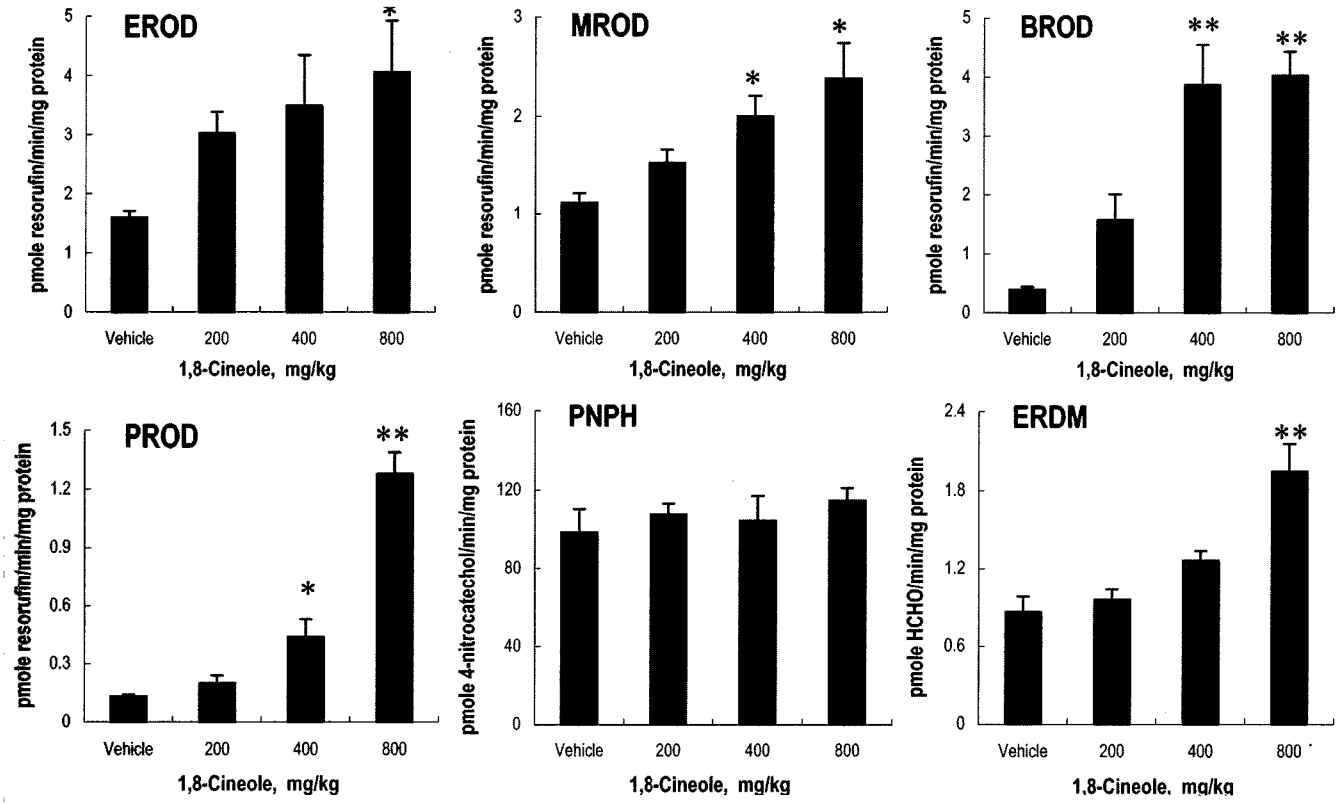

Fig. 2. Effects of 1,8-cineole on CYP-associated monooxygenase activities in male SD rats. Rats were treated orally with 0, 200, 400 and 800 mg/kg of 1,8-cineole in corn oil for 3 consecutive days. Twenty four hr after the last treatment, livers were removed to prepare the microsomes. Each bar represents the mean activity±S.E. of five animals. The asterisks indicate the values significantly different from the vehicle-treated control at either P<0.05 (*) or P<0.01 (**).

with polyclonal antibodies against either one of the CYP 2B1/2, 3A1/2 or 2E1 (Fig. 3). 1,8-Cineole clearly induced CYP 2B1/2 and CYP 3A1/2 proteins in liver microsomes. Meanwhile, CYP 2E1 proteins were not changed by the treatment with 1,8-cineole. CYP 1A proteins were not changed by 1,8-cineole (data not shown). Except the CYP 1A, these results were consistent with the increases of microsomal monooxygenase activities.

From these results, it was concluded that 1,8-cineole might be useful to modulate CYP 2B and 3A enzymes in rats.

Subsequently, the role of metabolic activation in thioacetamide-induced hepatotoxicity was investigated in male SD rats using 1,8-cineole as a CYP modulator. The doses of thioacetamide studied were moderate to elevate serum enzymes and to elicit hepatic injury, because the objective of the present study was to demonstrate the potentiation of thioacetamide-induced hepatotoxicity by the pretreatment of rats with the CYP inducer, 1,8-cineole.

The effects of pretreatment with 1,8-cineole on thioacetamide-induced hepatotoxicity in rats were shown in Fig. 4. When male rats were pretreated orally with 800 mg/kg of 1,8-cineole for 3 consecutive days before the treatment with thioacetamide, the elevation of serum ALT activity induced by thioacetamide was significantly potentiated, particularly when 100 mg/kg of thioacetamide was treated. To determine whether CYP 2B enzyme activity was induced by the pretreatment with 1,8-cineole, the liver microsomal activities of BROD and PROD were assayed using the liver microsomes prepared from the animals used in Fig. 4 (Fig. 5). The activities of CYP 2B-specific two monooxygenase activities were significantly induced by the pretreatment with 1,8-cineole, indicating that more thioacetamide might be biotransformed to its hepatotoxic metabolite(s).

In the present studies, the effects of pretreatment with 1,8-cineole on thioacetamide-induced immunosuppression were also studied (Fig. 6). Following pretreatments of

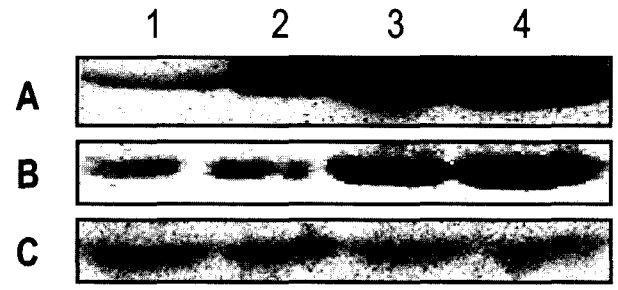

Fig. 3. Western immunoblotting analyses for CYPs 2B1/2, 3A1/2 and 2E1 with 1,8-cineole-treated rat liver microsomes. The liver microsomal proteins (10 μg/well) prepared from rats administered orally with 1,8-cineole at 0, 200, 400 and 800 mg/kg for 3 days were resolved on 10% SDS-PAGE. The results represent a typical experiment conducted at least twice for individual isozymes with the pooled microsomes used in Fig. 1. A, CYP 2B1/2; B, CYP 3A1/2; C, CYP 2E1. Lane 1, vehicle control; lane 2, 1,8-cineole at 200 mg/kg; lane 3, 1,8-cineole at 400 mg/kg; lane 4, 1,8-cineole at 800 mg/kg.

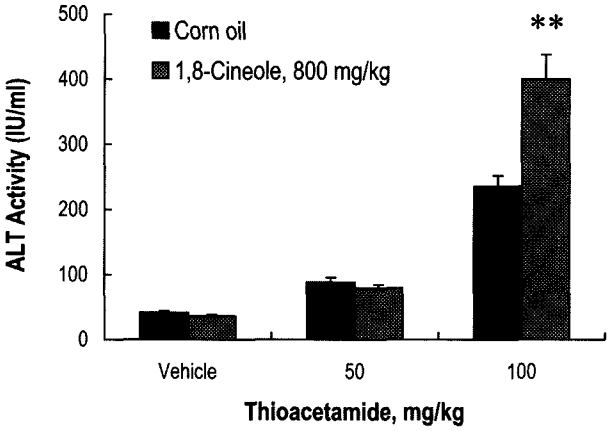

Fig. 4. Effects of pretreatment with 1,8-cineole on thioacetamide-induced hepatotoxicity. Male SD rats were pretreated with 800 mg/kg of 1,8-cineole for 3 consecutive days, followed by an intraperitoneal treatment with thioacetamide. 24 h later, the serum was prepared for ALT assay. Each bar represents the mean activity±S.E. of five animals. The asterisks indicate the values significantly different from corn oil-pretreated control at P<0.01 (**).

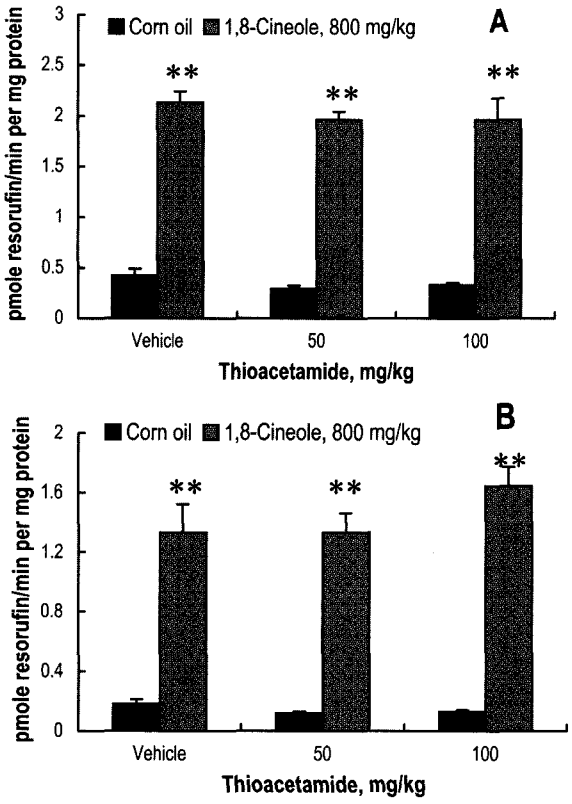

Fig. 5. Effects of 1,8-cineole on CYP enzyme activities in hepatotoxicity studies. Livers were removed from the same animals used in Fig. 4 to prepare the microsomes. Each bar represents the mean activity±S.E. of five animals. The asterisks indicate the values significantly different from corn oil pretreated controls at P<0.01 (**). A, BROD; B, PROD

female BALB/c mice with 800 mg/kg of 1,8-cineole for 3 days, mice were treated intraperitoneally with 100 mg/kg of thioacetamide. 30 min after the thioacetamide treatment, mice were immunized with SRBCs. The antibody response to SRBCs was enumerated 4 days later.

Thioacetamide at 100 mg/kg suppressed the antibody response to SRBCs, which was more potentiated by the pretreatment with 1,8-cineole. When mice were treated orally with 1,8-cineole for 3 consecutive days, CYP 2B-specific BROD and PROD activities were dose-dependently and significantly induced in liver microsomes (Fig. 7).

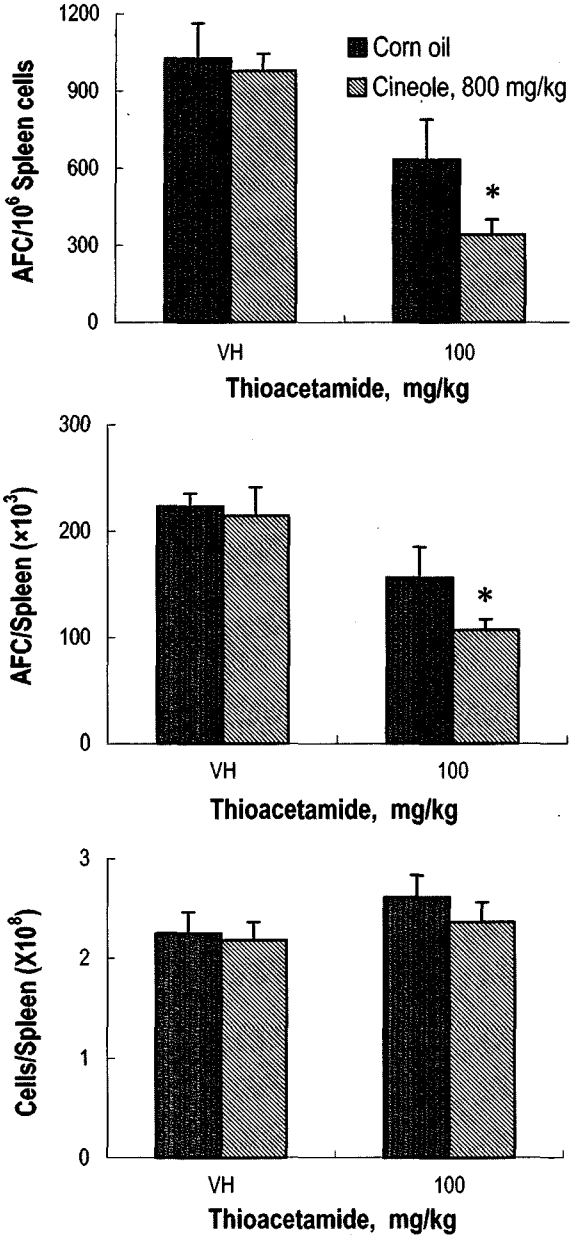

Fig. 6. Effects of pretreatment with 1,8-cineole on thioacetamide-induced suppression of antibody response to SRBCs. Female BALB/c mice were pretreated with 800 mg/kg of 1,8-cineole for 3 consecutive days, followed by an intraperitoneal treatment with thioacetamide. Thirty minutes after the treatment with thioacetamide, mice were immunized with SRBCs. Four days later, the number of antibody-forming cells (AFCs) was enumerated. Each bar represents the mean ±S.E. of five animals. The asterisks indicate the values significantly different from the vehicle-treated (VH) control at $P<0.05$ (*).

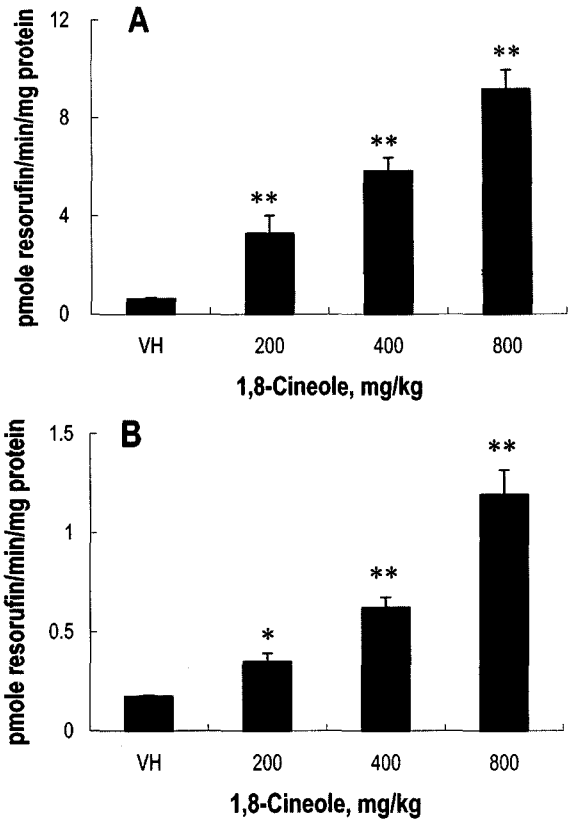

Fig. 7. Effects of 1,8-cineole on BROD (A) and PROD (B) activities in female BALB/c mice. Mice were treated orally with 0, 200, 400, and 800 mg/kg of 1,8-cineole for 3 consecutive days. Twenty four h after the last treatment, all animals were subjected to necropsy. Each bar represents mean activity±S.E. of five animals. The asterisks indicate the values significantly different from the vehicle (VH)-treated control at either $P<0.05$ (*) or $P<0.01$ (**).

Taken together, the present results indicated that 1,8-cineole dose-dependently induces CYP 2B enzymes without hepatotoxicity and immunotoxicity, and that 1,8-cineole pretreatment might potentiate thioacetamide-induced hepatotoxicity and immunotoxicity. Therefore, 1,8-cineole would be a useful CYP inducer in studying chemical-induced hepatotoxicity and immunotoxicity in which metabolic activation by CYP enzymes is required.

DISCUSSION

Many toxicants undergo metabolic activation by CYP enzymes to exert their toxicity (Guengerich and Shimada, 1991). To investigate toxic actions following metabolic

activation, it is very useful to introduce nontoxic model inducers and inhibitors for CYP enzymes *in vivo*. Although many inducers and inhibitors for CYP enzymes have been known to date, nontoxic CYP modulators are very limited. For examples, 3-methylcholanthrene and β-naphthoflavone, well-known inducers of CYP 1A enzymes, can not be used in such purposes, due to their immunotoxicity at the doses for CYP induction (White *et al.*, 1985). Phenobarbital and β-ionone induce CYP 2B enzymes (Jeong *et al.*, 1995). Ethanol and dexamethasone can induce CYP 2E1 and CYP 3A enzymes, respectively, but they have been turned out immunosuppressive (Holsapple *et al.*, 1993; Sabbele *et al.*, 1987). So only phenobarbital and β-ionone could be useful for the purpose of investigating the possible role of metabolic activation in chemical-induced immunosuppression, because these two inducers were not immunosuppressive at the doses for CYP induction. Therefore, it is of importance to develop new model inducers of CYP enzymes which may be able to induce specific CYP enzyme(s) without any toxicity *in vivo* at the dose for induction. In this regard, our research was focused on natural products because many compounds from natural products are reportedly capable of modulating CYP enzymes, and because natural products have long been used, at least in part, without severe toxicity.

It has been reported that 1,8-cineole might interact with CYP enzymes. For example, 1,8-cineole could be metabolized by CYP 3A enzymes in rat and human liver microsomes (Mitsuo *et al.*, 2000). Some data also showed that 1,8-cineole has a potent inhibitory effect on CYP 2B1 enzymes in rat liver microsomes (de Oliveira *et al.*, 1999). In addition, many monoterpenes, such as β-myrcene, β-pinene and camphor, could inhibit CYP 2B1 enzymes *in vitro* (de Oliveira *et al.*, 1997a). The same authors found that β-myrcene could induce CYP-associated mononxygenase activities *in vivo* (de Oliveira *et al.*, 1997b). These findings support an idea that monoterpenoid compounds may modulate the expression of CYP enzymes *in vivo*.

The objectives of our present studies were to characterize the modulatory effects of 1,8-cineole on CYP enzymes *in vivo* and to apply 1,8-cineole as a CYP modulator in thioacetmiade-induced hepatotoxicity and immunotoxicity. We selected male SD rats for CYP expression and hepatotoxicity studies for its wide use in toxicity studies. Likewise, female BALB/c mice were employed in the immunotoxicity studies due to its wide use in immunotoxicology studies. Because the model CYP modulators should not be toxic at the doses for CYP induction, general toxicological signs and hepatotoxic parameters were also determined. As a protoxicant, thioacetamide was selected, because it is metabolized to more toxic S-oxide metabolite by CYP enzymes (Hunter *et al.*, 1977). Thioactamide has been reported to cause hepatotoxicity and immunotoxicity following metabolic activation by CYP enzymes (Kim *et al.*, 2000; Lee *et al.*, 2003).

As shown in Figs. 2 and 3, 1,8-cineole clearly induced CYP 2B1/2 proteins and the microsomal activities of BROD and PROD. At the doses for CYP induction, 1,8-cineole did not show any signs of toxicity, indicating that 1,8-cineole might be a useful inducer of CYP enzymes in rats. In Fig. 2, CYP 1A-specific EROD and MROD activities were significantly induced. However, the induction of CYP 1A proteins by 1,8-cineole was not observed in the Western immunoblotting studies (data not shown). These results indicated that the induced EROD and MROD activities might result from the induction of other CYP isozymes by 1,8-cineole. In fact, we have recently observed that ethoxyresorufin, a CYP 1A-selective substrate, could be metabolized by CYP 2B (manuscript submitted). Subsequently, to show its applicability in studying the role of metabolic activation by CYP enzymes in chemical-induced toxicity, the effects of pretreatment of animals with 1,8-cineole on thioacetamide-induced hepatotoxicity and immunotoxicity were studied. Pretreatment of male SD rats with 1,8-cineole at 800 mg/kg for induction of CYP 2B enzymes potentiated thioacetamide-induced hepatotoxicity with significant inductions of CYP 2B-specific monooxygenases (Figs. 4 and 5). These results indicated that 1,8-cineole could increase in the metabolic activation of thioacetamide to its hepatotoxic metabolite(s). In addition, thioacetamide-induced suppression of antibody response to SRBCs was also potentiated by pretreatment of mice with 1,8-cineole at the doses for CYP induction (Figs. 6 and 7). Moreover, 1,8-cineole at 800 mg/kg did not alter the antibody response, indicating that 1,8-cineole can also be used in the immunotoxicology studies in which metabolic activation by CYP enzymes is required. To confirm this hypothesis whether the enhanced hepatotoxicity of thioacetamide is *via* CYP 2B-mediated mechanism, specific inhibitors of CYP 2B might be also introduced to see decreases in thioacetamide-induced hepatotoxicity and immunotoxicity.

To date, CYP 2B and flavin-containing monooxygenases have been implicated to metabolize thioacetamide to its toxic metabolites (Hunter *et al.*, 1977; Lee *et al.*, 2003; Jeong *et al.*, 1999). Our present results that the induction of CYP 2B by 1,8-cineole could potentiate thioacetamide-induced toxicity are consistent with the previous reports. Meanwhile, recent literatures also implied that CYP 2E1 might be another important enzyme capable of activating thioacetamide to its toxic metabolites (Wang *et al.*, 2000; 2001). The authors reported that the induction of CYP 2E1 by diet restriction and in streptozotocin-induced diabetic rats could enhance thioacetamide-induced hepatotoxicity, and that flavin-containing monooxygenase might not be involved in the metabolism of thioacetamide. Our present

results, however, showed that the pretreatment with 1,8-cineole did not affect CYP 2E1-specific PNPH activity and the level of hepatic CYP 2E1 protein. Although we do not have any reasonable explanation on a possible role of CYP 2E1 in the metabolism of thioacetamide at the present time, this discrepancy might be resulted from the effects of diet restriction and streptozotocin on other xenobiotic-metabolizing enzymes including CYP enzymes. In this regard, a possible role of CYP 3A in thioacetamide activation should be investigated further, because our present results showed a clear induction of CYP 3A proteins by 1,8-cineole in the Western immunoblotting and because suppression of CYP enzymes including CYP 3A protected mice against thioacetamide-induced hepatic injury (Liu *et al.*, 1995). The reaction phenotyping of CYP enzyme(s) involved in the metabolism of thioacetamide may clearly explain the role of individual CYP enzymes and flavin-containing monooxygenase.

In conclusion, the present results indicated that 1,8-cineole might be a useful CYP modulator in investigating a possible role of metabolic activation in chemical-induced hepatotoxicity and immunotoxicity.

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