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Comparative liver metabolism and toxicity of carbon disulfide influenced by various inducers of P450 isoenzymes in rats

Ramesh R. Dalvi[*1], Prasad S. Dalvi[2], and Leonard H. Billups[3]

Departments of Biomedical Sciences[1] and Pathobiology[3], College of Veterinary Medicine, Nursing and Allied Health, Tuskegee University, Tuskegee, AL 36088, U.S.A.

[2]Current address: Department of Physiology, University of Toronto, Toronto, ON M4Y1R6, Canada

Abstract

The objective of this study was to examine differential metabolism of CS_2 by various hepatic cytochrome P450 (CYP) isoenzymes including CYP1A1, CYP2B1, CYP2E1 and CYP3A2 induced by their selective inducers and to determine if there is a correlation between its metabolic activation and hepatotoxicity. Because 3-methylcholanthrene (3-MC), phenobarbital (PB), isoniazid (INH), and pregnenolone-16α-carbonitrile (PCN) are typical selective inducers of CYP1A1, CYP2B1, CYP2E1, and CYP3A2, respectively, they were chosen in this study to pretreat male Sprague-Dawley rats for induction of the CYP isoforms. Twenty-four h following the final dose of 3 daily doses of the inducers, rats were treated ip with a single dose of 380 mg/kg of CS_2. Half of the rats in each group were sacrificed at 3 h after CS_2 treatment to identify inhibited isoenzymes since CS_2 is metabolized by them in a suicide substrate manner. The remaining animals in the group were sacrificed at 24 h following CS_2 treatment to determine hepatic damage using serum ALT activity and liver histopathology as the indices of toxicity. At 3 h after treatment, activities of all CYPs (1A1, 2B1, 2E1 and 3A2) were inhibited by CS_2 significantly and differentially. However, the inhibition of CYP1A1 and CYP3A2 was reversed at 24 h post-treatment with no serious liver damage. On the other hand, the inhibition of CYP2B1 and CYP2E1 was irreversible and accompanied by severe liver damage especially in phenobarbital-pretreated rats. The results of this study suggest that induced CYP2B1 and to a lesser extent CYP2E1 are responsible for the bioactivation of CS_2 and the observed liver damage.

Keywords: Carbon disulfide; cytochrome P450 (CYP); CYP isoenzymes; CYP inducers, hepatotoxicity.

Introduction

Carbon disulfide (CS_2) is a widely used, highly volatile industrial solvent which is also used as a fumigant in agriculture. While the most human exposure to CS_2 occurs occupationally in viscose-rayon and rubber industry (Beauchamp *et al.*, 1983), alcoholic patients are directly exposed to CS_2 formed in the body from rapid degradation of disulfiram, a drug most commonly used in the treatment of alcoholism (Rahman *et al.*, 1997; Rabkin *et al.*, 1998). However, the major route of human exposure to CS_2 is inhalation. For example, it is estimated that CS_2 is emitted into the U.S. environment at a rate of approximately 76 million pounds each year by U.S. industries alone. Therefore, industrial discharge of CS_2 into environment remains a potential source for human exposure through inhalation route (Manuel, 1998). Human epidemiological studies have shown that exposure to CS_2 has been associated with increased risk of cardiovascular disease, ischemic heart disease mortality, and adverse effects on the nervous system including parkinsonism and peripheral neuropathy (Price *et al.*, 1997; Godderis *et al.*, 2006). Occupational exposure to CS_2 has also been associated with reproductive effects such as increased spontaneous abortions, premature deliveries, marked degenerative changes in testicular tissue, chromosomal aberrations in sperm and a significant decrease in serum testosterone levels and sperm count (Tepe and Zenick, 1984; Le and Fu, 1996; Patel *et al.*, 1999; Tsai *et al.*, 2000).

In animal studies, although CS_2 has been shown to affect myocardium and kidneys (Morvai

[*]**Corresponding author:**
Tel: (334) 727-8858; Fax: (304) 727-8177
E-mail address: rdalvi@tuskegee.edu

et al., 2005), it also produces hepatic necrosis only in rats pretreated with phenobarbital (Bond *et al.*, 1969). This observation prompted the researchers to postulate that liver toxicity produced by CS_2 requires its metabolism by microsomal enzymes to more toxic metabolite/s rather than CS_2 itself being a toxic agent (Bond *et al.*, 1969; Bond and De Matteis, 1969). Subsequently, a series of in vitro and in vivo experiments revealed that CS_2 is metabolized by P450 enzymes to carbonyl sulfide (COS) and reactive electrophilic sulfur atoms (De Matteis and Seawright, 1973; Dalvi *et al.*, 1974, 1975; Dalvi and Neal, 1978). The electrophilic sulfur has been implicated in both inhibition of the mixed-function oxidase system and the hepatic necrosis observed in phenobarbital-pretreated rats following administration (Dalvi *et al.*, 1974; 1975). Thus, the evidence from both human epidemiological data and animal studies indicated that was a toxic chemical of concern, but the molecular mechanism(s) of its toxicity remained unknown.

The assays employed in the past to measure the metabolism of CS_2 catalyzed by cytochrome P450-containing drug-metabolizing enzymes have been limited, because they have not been highly CYP-isoenzyme specific. For example, metabolism of CS_2 by P450 enzymes induced by phenobarbital (PB) was often determined by measuring total P450 and dealkylation of aminopyrine or hydroxylation of aniline. Only more recently, the development of monoclonal antibodies, recombinant DNA techniques, and exquisitely specific substrates have made it possible to identify induced CYPs and characterize their role in xenobiotic metabolism. These P450 enzymes utilize molecular oxygen for the oxidation of endogenous as well as exogenous compounds, which makes them a potential source of the generation of reactive oxygen species (ROS). Published reports have indicated that these enzymes can be critical in the production of toxic ROS even in the absence of exogenous substrates as in the case of CYP2E1 (Bondy and Naderi, 1994). Therefore, the following studies were conducted to examine the individual role of induced CYPs in the metabolism of CS_2 and to identify those CYPs most involved in the CS_2 activation and toxicity.

Material and Methods

Test Chemicals

Carbon disulfide was purchased from Fisher Scientific, Atlanta, GA. Phenobarbital (PB), 3-methylcholanthrene (3-MC), isoniazid (INH) and pregnenolone-16α-carbonitrile (PCN) were from Sigma, St. Louis, MO. All other chemicals used in this study were of analytical or reagent grade.

Animals

Male Sprague-Dawley rats weighing 180-200 g were purchased from Harlan (Indianapolis, IN). They were housed in standard polycarbonate cages containing hard wood chip bedding and placed in a temperature-controlled room (25°C) on a 12 h light-dark cycle with free access to water and Purina rat chow. All rats were acclimatized for a minimum of 1 week before the study. All animals received care according to the guidelines approved by the institutional committee for care and use of laboratory animals in research.

Experimental Design

Four groups of 12 rats each were injected ip with 3-MC (20 mg/kg in oil), PB (80 mg/kg in saline), INH (250 mg/kg in saline) or PCN (50 mg/kg in oil) daily for 3 days to induce CYPs 1A1, 2B1, 2E1 and 3A2, respectively. Twenty-four h following the last dose of an inducer, each treatment group was subdivided into 2 groups of 6 rats each. One group was administered 380 mg/kg dose of CS_2 ip in corn oil (2 ml/kg) while the other group received corn oil alone and served as a control for the treated group. This subclinical dose of CS_2 was chosen to be in line with the doses used by other investigators for studying liver toxicity of this chemical (Beauchamp *et al.*, 1983). Half of the animals from each group were sacrificed under carbon dioxide anesthesia at 3 h and the remainder at 24 h. At 3 h sacrifice, only livers were obtained to determine the P450 isoenzymes inhibition while liver and blood samples obtained at 24 h were used to determine the activity of hepatic P450s and serum alanine aminotransferase (ALT),

respectively. Serum samples from blood drawn in vacutainer tubes via cardiac puncture were prepared by centrifugation and used to determine the ALT activity as a marker of liver damage.

Preparation of liver microsomes

Next, the livers were removed, and a small portion of each liver (24-h treatment groups only) was stored in 10% neutral-buffered formalin for hisotopathological examination. The remaining livers from each group were individually homogenized (1:3, w/v) in ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, containing 10 mM EDTA, 150 mM KCl and 0.25 mM PMSF) using a tissue homogenizer (BioSpec Products, Inc., Bartlesville, OK). The homogenate was centrifuged at 9000 x g for 20 min at 4°C, and microsomes from the supernatant were prepared by ultracentrifugation. Portions of microsomal pellet were stored in phosphate buffer (0.1 M potassium phosphate, pH 7.4) containing 20% glycerol at -80°C until analysis within a week. The microsomes were resuspended in the phosphate buffer and used to determine P450 isoenzyme activity. Protein concentration in each sample was determined by the bicinchoninic acid method (Smith *et al.*, 1985).

Enzyme Assays

The activities of ethoxyresorufin O-dealkylase (CYP1A1) and pentoxyresorufin O-dealkylase (CYP2B1) were measured fluorometrically using ethoxyresorufin and pentoxyresorufin as their specific substrates, respectively (Burke *et al.*, 1985). CYP2E1 assay was based on the hydroxylation of p-nitrophenol to 4-nitrocatechol which was quantified spectrophotometrically (Koop, 1992) while CYP3A2 activity was determined following N-demethylation of erythromycin as the substrate (Arlotto *et al.*, 1987).

Assessment of Hepatotoxicity

Serum ALT activity was measured spectrophotometrically with commercial reagent kit (No. 505) from Sigma, (St. Louis, MO). For histopathological study, transverse sections from the middle of each left lateral liver lobe collected at necropsy were fixed in 10% neutral buffered formalin and processed for histological examination. Formalin-fixed pieces of the liver were embedded in paraffin and 5-μm thick sections cut and stained with hematoxylin and eosin (H & E). Slides were evaluated by light microscopy for necrosis, swelling, vacuolation and other lesions of the liver.

Statistical Analysis

The data were analyzed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). Statistical analysis was performed by t test or analysis of variance (ANOVA) followed by Bonferroni multiple comparison test, and $p < 0.05$ was considered statistically significant.

Results and Discussion

The present study was conducted to compare the metabolism of CS_2 by various inducible CYPs including CYPs 1A1, 2B1, 2E1 and 3A2, and to determine if there is a relationship between the CYP-mediated metabolism and hepatotoxicity of CS_2. Majority of the studies previously conducted on the metabolism and hepatotoxicity of CS_2 had been performed without evaluating CS_2 effect on individual CYP isoenzymes (Beauchamp *et al.*, 1983). In recent years, following the identification of several different CYPs, metabolism of CS_2 mediated by inducible P450s has received a great deal of attention of investigarors. As a result, CYP1A1, CYP2B1, CYP2E1 and CYP3A2 have been implicated in CS_2 metabolism using their inhibition by CS_2 as its metabolism indicator since CS_2 acts as a suicide substrate (Snyderwine *et al.*, 1988; Kivisto *et al.*, 1995; Dalvi *et al.*, 2003). However, a direct linkage between metabolic activation of CS_2 by individual P450s and CS_2 hepatotoxicity has not been fully established. Availability of selective inducers of individual CYP isoenzymes has made the identification of individual isoforms involved in xenobiotic metabolism much easier. Carbon disulfide has long been shown to be a substrate of cytochrome P450 enzymes that is oxidatively metabolized by them to reactive sulfur atoms shown to be responsible for its toxicity (Dalvi and Neal, 1978). It is also known that the hepatotoxicity of CS_2 characterized by extensive

centrilobular hepatic necrosis has been observed only in rats pretreated with phenobarbital (PB), but not in normal rats (Bond *et al.*, 1969; Dalvi *et al.*, 1974, 1975). This suggests that the hepatotoxicity of CS_2 is related to its metabolism by inducible P450s.

Despite these findings, it is not known which inducible CYPs are actually significantly involved in the oxidative metabolism of CS_2 and subsequent hepatocellular damage. To address this point, we pretreated rats with various CYP inducers for the induction of CYPs, and then they were injected with a moderately toxic dose of CS_2. Rats sacrificed at 3 h after CS_2 adminstration were used to determine only the inhibition of CYP activity whereas both the CYP activity and liver damage were measured in rats sacrificed at 24 h. As can be seen from Fig. 1, the rats pretreated with 3-MC showed significant inhibition of CYP1A1 at 3 h, but the inhibition was significantly reversed at 24 h following the CS_2 treatment. On the other hand, the inhibition of PB-induced CYP2B1 caused by CS_2 at 3 h was not reversed at 24 h but remained significantly suppressed. It should be noted that although the loss of CYP1A1 and CYP2B1 inflicted by CS_2 at 3 h was nearly identical, interestingly the recovery of the two isoenzymes at 24 h was markedly different. This means CS_2-mediated inhibition of 3-MC-inducible CYP1A1 is reversible whereas that of PB-inducible CYP2B1 is irreversible. This reversibility of CYP1A1 activity is consistent with the observation made by Masuda and Yasoshima (1988) who have reported that ethoxyresorufin O-dealkylase (CYP1A1) induced by 3-MC in mice was much less affected by CS_2 as compared to normal and PB-pretreated mice. It is very likely that 3-MC-inducible CYP1A1

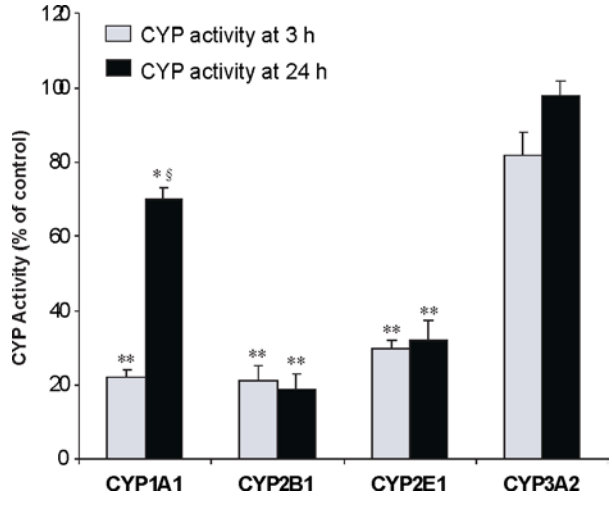


Fig.1. Effect of carbon disulfide on the activities of CYP 1A1, 2B1, 2E1 and 3A2 in 3-MC-, PB-, INH- and PCN-induced rats, respectively, at 3 and 24 h following carbon disulfide treatment. Each bar represents the mean percent of control + SE of three individual animals (*$p < 0.05$, **$p < 0.01$ vs. corresponding control, §$p < 0.01$ vs. corresponding 3 h treatment group).

inhibition may not be related to CS_2 metabolism whereas irreversible inhibition of PB-induced CYP2B1 could have been caused by toxic, reactive sulfur atoms generated during CS_2 metabolism. If this hypothesis is true, then in the 3-MC-pretreated rats there should be no hepatotoxicity whereas PB-pretreated rats should exhibit liver toxicity. In fact, the determination of serum ALT activity as a marker of liver damage indicated that ALT activity remained unchanged in 3-MC-treated rats while in PB-treated rats it increased 2-fold (Table 1). Histopathologically, in PB-pretreated group, exposure to CS_2 resulted in extensive vacuolation (ballooning degeneration) and swelling of hepatocytes with occluded sinuses ultimately resulting in severe necrotic changes in the

Table 1: Effect of carbon disulfide on serum alanine aminotransferase (ALT) activity in the rats pre-treated with CYP inducers at 24-h following CS_2 treatment

Dose of CS_2	ALT Activity (Sigma-Frankel Units/ml)			
	3-MC	PB	INH	PCN
Control	22±1	20±1	20±1	23±2
380 mg/kg	28±1	49±8*	34±2*	29±4

Results are expressed as the mean+S.E. *Significantly different from the corresponding control value ($P < 0.05$)

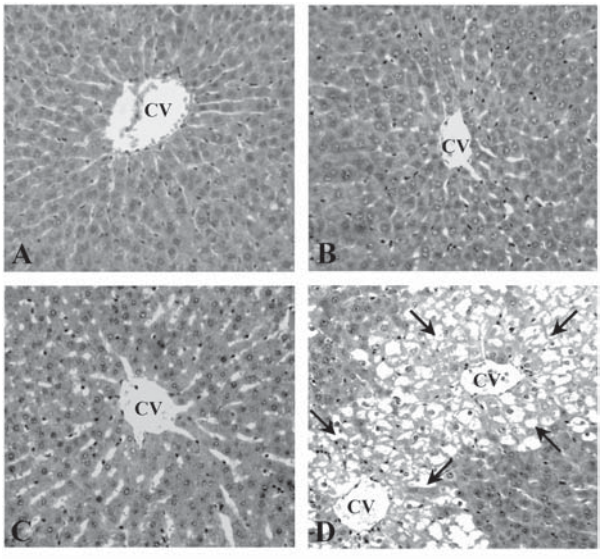


Fig.2. Liver histology from 3MC- and PB-pretreated rats with or without CS_2 treatment at 24 h time-point. A and C are liver sections from 3MC- and PB-induced plus vehicle-treated control rats, respectively, showing normal cell architecture and centrilobular pattern. B and D are liver sections from 3-MC- and PB-induced plus CS_2-treated rats. In 3-MC-induced rat CS_2 treatment did not result in any noticeable hepatocellular degenerative changes, while in PB-pretreated rat it caused extensive vacuolation (ballooning degeneration) and swelling of hepatocytes resulting in severe necrotic changes (arrows) in the area surrounding central vein (CV). Sections were stained with hematoxylin-eosin. Magnifications, X400.

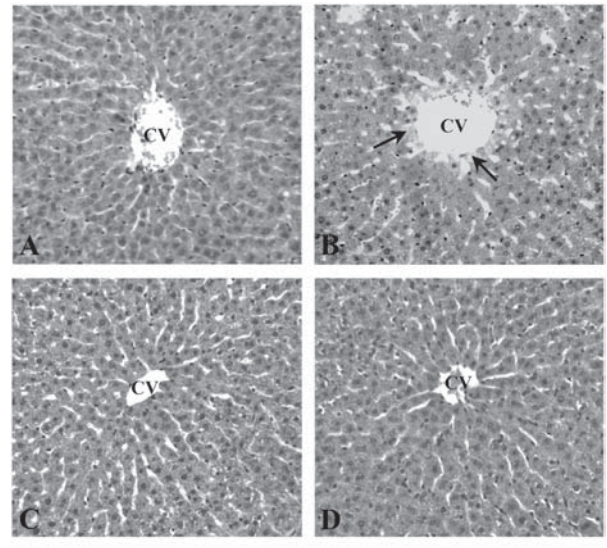


Fig.3. Liver histology from INH- and PCN-pretreated rats with or without CS_2 treatment at 24 h time-point. A and C are liver sections from INH- and PCN-induced plus vehicle-treated control rats, respectively, showing normal cell architecture and centrilobular pattern. B and D are liver sections from INH- and PCN-induced plus CS_2-treated rats. CS_2 treatment of INH-induced rat resulted in mild vacuolation with slight hepatocellular degeneration (arrows) in the area surrounding central vein (CV). PCN-induced plus CS_2-treated rat show normal liver tissue. Sections were stained with hematoxylin-eosin. Magnifications, X400.

centrilobular region (Figs. 2C and 2D). On the other hand, 3-MC plus CS_2-treated group showed normal liver cells with open sinusoids and clear veins (Figs. 2A and 2B). Thus, the biochemical observations are in tandem with the histopathological evaluation of the liver supporting the involvement of CYP2B1 in CS_2 metabolism and hepatotoxicity.

In the INH-pretreated rats also CS_2 produced a significant depression of induced CYP2E1 at 3 h that remained suppressed even at 24 h indicating that metabolism of CS_2 by CYP2E1 is similar to CYP2B1 induced by PB (Fig. 1). In contrast, Fig. 1 shows that CS2 had no effect on the PCN-induced CYP3A2 at 3 or 24 h following the CS_2 administration suggesting that CS_2 was not metabolized by CYP3A2 and therefore it does not appear to be a substrate for this isoform. These results are consistent with the activity of serum ALT in these rats (Table 1). Furthermore, liver sections from INH plus CS_2-treated rats showed evidence of centrilobular damage such as swollen hepatocytes with condensed nuclei, mild vacuolation and occluded sinuses (Figs. 3A and 3B). The histology of liver sections from PCN-treated rats that received CS_2 showed slightly swollen sinusoids, but was otherwise normal in appearance (Figs. 3C and 3D). Compared to PB plus CS_2-treated rats, the intensity of liver damage in INH plus CS_2-treated rats as evaluated histopathologically was not as severe as in PB-treated rats (Fig. 2 D). It is well known that CS_2 is an inhibitor of CYP2E1 and inducers of this isoenzyme show potentiating effect on the CS_2 hepatotoxicity (Wronska-Nofer *et al.*,1986).

Taken together, these findings on CS_2 hepatotoxicity corroborate the studies reported by other investigators. For example, a potentiation effect similar to that of phenobarbital on the liver

toxicity of CS_2 has been reported in sheep after pretreatment of the animals with DDT (Wilkie *et al.*, 1985). Studies conducted by Wronska-Nofer *et al.* (1986) indicate that simultaneous exposure to ethanol over a long period results in the potentiation of CS_2 hepatotoxicity, probably due to CYP2E1 induction by ethanol. However, a subsequent report suggests that CS_2 metabolism associated with CYP induction by ethanol is not sufficient to result in CS_2-induced hepatic damage (Snydewine *et al.*, 1988). According to these investigators, phenobarbital-induced isoenzymes may have a higher capacity to metabolize CS_2 to toxic intermediates than ethanol. In summary, the results of the present study indicate that CYP2B1 and CYP2E1 are involved in the metabolism of CS_2 since they are significantly inhibited at 3 h following the treatment with CS_2, but the irreversible inhibition of PB-inducible CYP2B1 and INH-inducible 2E1 associated with subsequent hepatic damage due possibly to increased production of reactive sulfur suggest that CS_2 is predominantly metabolized by CYP2B1 and CYP2E1 in descending order of metabolism and toxicity.

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