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Original Article

Potentiation of the Hepatic Toxicity of Carbon Disulfide by Chlordane

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ABSTRACT

Objectives: In this study, we investigated whether cytochrome P450s (CYPs) induced by a typical chlorinated hydrocarbon insecticide chlordane (CLD) potentiate hepatic toxicity of carbon disulfide (CS_2). **Materials and Methods:** Male Sprague-Dawley rats were treated with CLD (25 mg/kg, intraperitoneally (i.p.)) daily for 4 days, and 24 h after the final injection the rats were treated with CS_2 (380 mg/kg, i.p.) in corn oil; while controls received the vehicle alone. The rats were then sacrificed at 3, 6, and 24 h following the CS_2 treatment. **Results:** It was found that at 3 h post-treatment, total hepatic glutathione (GSH) decreased modestly, but lipid peroxidation increased markedly, while all CLD-inducible CYPs (1A1, 2B1, 2E1, and 3A2) were inhibited by CS_2 variably but significantly. On the other hand, samples taken at 24 h following the CS2 treatment showed a significant increase in relative liver weights, hepatic GSH and lipid peroxidation, microsomal reactive oxygen species (ROS), and serum alanine transaminase (ALT) level. Activity of the CYPs was also increased, but remained significantly depressed, especially that of CYP2B1. Livers removed at 3 and 6 h after CS_2 treatment showed subtle to distinct apoptotic changes, while a severe lesion of hydropic degeneration of the centrilobular cells with apoptosis was microscopically distinguishable in samples taken at 24 h. **Conclusions:** These results suggest that the metabolism of CS_2 by CLD-induced CYPs and the generation of lipid peroxides may have in concert contributed to the distinct hepatocellular damage.

Key words: Carbon disulfide, cytochrome P450s, chlordane, glutathione, lipid peroxidation, oxidative stress, reactive oxygen species

INTRODUCTION

Carbon disulfide (CS_2) is an industrial solvent extensively used in the production of rubber, rayon, and cellophane. While persons working in these industries are at direct risk of exposure to CS_2, other populations can also be occupationally exposed to CS_2. For example, workers who apply pesticides or harvest agricultural produce sprayed with the pesticides may also be at risk, because dithiocarbamates and their disulfides, the chemicals that are widely used in agricultural pesticides, spontaneously liberate CS_2 upon their decomposition in the environment.[1,2] Further, in addition to industrial and agricultural exposure of humans to CS_2, therapeutic agents such as diethyldithiocarbamates (DDCs) and disulfiram (antabuse) are a potential source for direct human exposure to CS_2. While they are used as antidotes in metal poisoning, DDCs are also receiving more attention in the treatment of acquired immunodeficiency syndrome (AIDS) due to their immune modulating action and antiviral activity against AIDS virus.[3] These drugs rapidly break down to CS_2 in the body which then can target vital organs such as heart, brain, and liver.[4,5] From clinical point of view, it is worth noting that because of its early

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use as only available drug for alcoholism, disulfiram has not undergone rigorous preclinical and clinical evaluations that are required for drug approval today.[6,7] The ability of disulfiram and other dithiocarbamates used in a variety of clinical applications to generate CS_2 *in vivo* suggests that CS_2 may be a contributing factor to their toxic effects. Human epidemiological studies have shown that exposure to CS_2 has been associated with increased risk of liver damage, ischemic heart disease, and adverse effects on the nervous and reproductive systems.[8,9]

In animal studies; CS_2 has been shown to produce hepatic necrosis in rats pretreated with phenobarbital (PB), but not in normal rats.[10] Studies reported by Wronska-Nofer *et al.,* indicate that simultaneous exposure to ethanol over a long period results in potentiation of CS_2 hepatotoxicity, probably as a result of CYP2E1 induction by ethanol.[11] Even though these studies implicate CYP2E1 and PB-inducible CYPs in the metabolism and toxicity of CS_2, it is not known whether many other CYP inducers may have a potentiating effect on the hepatotoxicity of CS_2. Studies in our laboratory and those by others have shown that even at very low levels, chlordane (CLD) is a powerful inducer of various CYPs, especially CYP2B1 which is also induced by PB.[12] Widespread usage of CLD, which has an environmental half-life of 10-20 years, has resulted in its accumulation in the food chain.[12] Induction of hepatic xenobiotic-metabolizing enzymes by chlorinated hydrocarbons including CLD has been linked to multiple effects on endocrine organs such as thyroid and adrenal gland.[13] This prompted us to use CLD as a typical inducer of hepatic CYP enzymes to examine if CLD-inducible CYPs can potentiate the hepatic toxicity of CS_2 in the rats exposed to CS_2.

MATERIALS AND METHODS

Test chemicals

Carbon disulfide (99.9% purity) and CLD (technical grade) were purchased from Aldrich (Allentown, PA). All other chemicals used in this study were of analytical or reagent grade and were purchased from Sigma, St. Louis, MO.

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 Tuskegee University institutional committee for care and use of laboratory animals in research.

Experimental design

The rats were treated intraperitoneally (i.p.) with 25 mg/kg dose of CLD daily for 4 days, and 24 h after the final injection, the animals were administered i.p. 380 mg/kg dose of CS_2 in corn oil (2 ml/kg), while an induced control group received corn oil alone and served as a control for the CS_2-treated group. This subclinical dose of CS_2 was selected to be in line with the doses used in our previous studies and that used by other investigators for studying liver toxicity of this chemical.[14] The CS_2-treated animals ($n = 3$) were sacrificed under light carbon dioxide anesthesia at 3, 6, and 24 h after CS_2 dosing. Liver samples were removed and weighed at each time point, while blood samples were drawn via cardiac puncture only at 24 h after dosing for the determination of serum alanine transaminase (ALT) activity as a marker of liver damage. Pieces of liver from each sample were fixed in 10% neutral buffered formalin for histopathological evaluations. After collecting liver samples for the determination of glutathione (GSH) and lipid peroxidation, remaining liver tissue samples were flash-frozen in liquid nitrogen and stored at −80°C for microsomal preparations later within a week.

Preparation of liver microsomes

To isolate microsomes from the liver samples, a small portion of each liver was homogenized (1:3, w/v) individually in an ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, containing 10 mM ethylenediaminetetraacetic acid (EDTA), 150 mM KCl, and 0.25 mM phenylmethylsulfonyl fluoride (PMSF)) with a tissue homogenizer (BioSpec Products, Inc., Bartlesville, OK). The homogenate was centrifuged at 9000 × g for 20 min at 4°C, and microsomes were prepared by centrifuging the supernatant in an ultracentrifuge at 105,000 × g for 1 h. 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. These microsomes were resuspended in the phosphate buffer and used to determine P450 isoenzymes activity. Portions of microsomes from each sample were used for the determination of reactive oxygen species (ROS) as markers of liver damage.

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.[14] The CYP2E1 assay was based on the hydroxylation of p-nitrophenol to 4-nitrocatechol which was quantified spectrophotometrically; while CYP3A2 activity was determined following N-demethylation of erythromycin as the substrate.[14]

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Assessment of hepatotoxicity

GSH content in the liver samples was measured using Ellman reagent, while lipid peroxidation in the samples was estimated by the thiobarbituric acid reactive substances (TBARS) procedure. Serum ALT activity was measured spectrophotometrically with commercial reagent kit (No. 505) from Sigma, St. Louis, MO. Generation of ROS in liver microsomes was assessed employing 2',7'-dichlorofluoresein diacetate (DCFDA) as an index of ROS production. Isolated microsomes (0.2-0.3 mg/ml) were incubated for 10 min in 40 mM potassium phosphate buffer (pH 7.4) in the presence of 5 μM DCFDA and 0.5 mM NADPH at 37°C in final volume of 2 ml as described previously.[15] After the incubation, sample containers were placed in ice and centrifuged at 600 × g for 5 min. The fluorescence in the supernatant was monitored in a Shimadzu RF-1501 spectorfluorometer with excitation at 488 nm and emission at 525 nm. ROS formation was quantitated from a DCF standard curve and results are expressed as nmoles of DCF formed per min per mg of protein. Protein concentration in each sample was measured by the bicinchoninic acid method as described previously.[15] 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 and E). Slides were evaluated by light microscopy for necrosis, swelling, vacuolation, and other lesions of the liver.

Statistical analysis

All the samples were collected from three mice in each group. Each sample was analyzed in triplicate, and the mean of the three values was reported. 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 objective of this study was to examine the impact of CLD-induced toxicologically important cytochrome P450s 1A1, 2B1, 2E1 and 3A2 on the metabolism and hepatic toxicity of CS_2 in Sprague-Dawley rats. CS_2 has long been known to be a suicide substrate for CYP enzymes which is oxidatively metabolized by CYPs to reactive sulfur atoms that are, at least in part, responsible for the toxicity of CS_2.[14] It is also known that the hepatotoxicity of CS_2 characterized by extensive centrilobular hepatic necrosis has been observed only in rats pretreated with PB, but not in normal rats.[10,14] This suggests that the necrogenic activity of CS_2 is related to its metabolism in the liver by induced P450s. Since there is increasing evidence to suggest that in many cases of liver damage induced by toxic chemicals, apoptosis and necrosis coexist in hepatocytes;[16,17] this study was designed to monitor toxic changes occurring in the livers of CLD-pretreated rats as early as 3-24 h after CS_2 treatment.

In the rats pretreated with CLD and sacrificed at 3 h following treatment with CS_2, it was observed that the body weights and liver weights of the treated and untreated animals did not change significantly as compared to their corresponding controls. Biochemical data on hepatic levels of GSH and lipid peroxidation measured as TBARS at 3 h after CS_2 dosing indicated that there was a considerable decrease in GSH levels (21%) with marked increase in TBARS (474%) accompanied by a significant increase in serum ALT [Figure 1a]. Examination of the histopathological changes at 3, 6, and 12 h in the liver showed an incidence of preapoptotic to apoptotic changes characterized by cell shrinkage, nuclear fragmentation and chromatin condensation in the hepatocytes [Figure 2a-d].

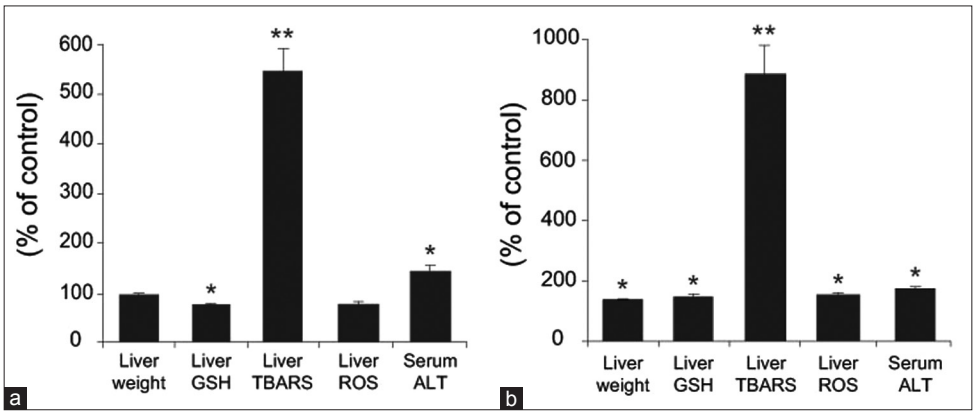


Figure 1: Effect of carbon disulfide treatment on the liver weight relative to body weight (g), liver glutathione (GSH; μg/g liver), liver thiobarbituric acid reactive substances (TBARS; nmol/g liver), liver reactive oxygen species (ROS; nmol/min/mg protein), and serum alanine transaminase activity (U/l) at 3 h (a) and 24 h (b) after carbon disulfide administration in chlordane (CLD)-induced rats. Each bar represents the mean percent of control ± standard error of the mean of three individual animals (*$P < 0.05$, **$P < 0.001$ vs corresponding control)

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The histopathological changes occurring at 3 and 24 h coincided well with the significant inhibition of CYP1A1, CYP2B1, CYP2E1, and CYP3A2 [Figure 3] caused by CS_2 suggesting that the observed histopathological and biochemical changes were related to the metabolism of CS_2 resulting in oxidative stress/damage. In view of our previous studies with CS_2 metabolism and toxicity, these changes may be attributed to direct action of CS_2 metabolites on hepatocytes,[14] and secondarily to the induction of CYP enzymes by CLD.

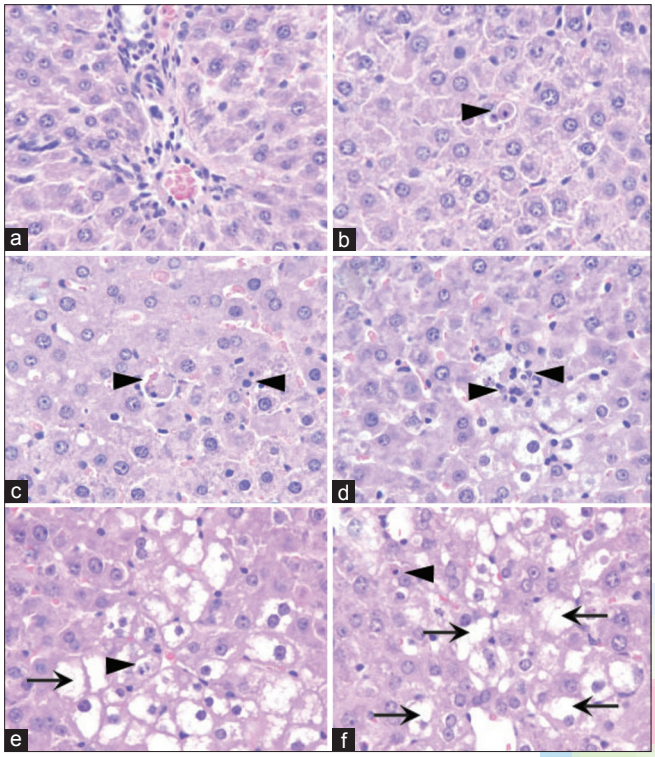


Figure 2: Photomicrographs of paraffin-embedded liver from CLD-pretreated rats (magnification, ×400). (a) Representative section from rat treated with vehicle showing normal histology. (b-d) Representative sections from rat sacrificed 3, 6, and 12 h after CS_2 treatment, respectively; apoptotic hepatocytes (arrowheads) show cell shrinkage, nuclear fragmentation, chromatin condensation, and round shape. (e and f) Representative sections from rat sacrificed 16 and 24 h after CS_2 treatment, respectively; showing severe hydropic degeneration of hepatocytes (arrows) with apoptotic cells (arrowheads)

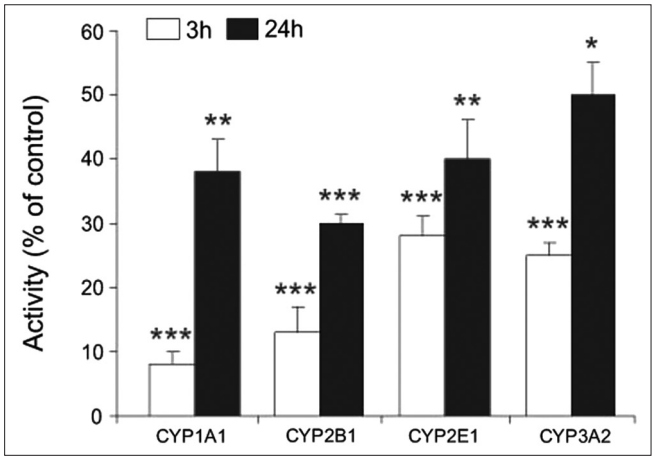


Figure 3: Effect of CS_2 treatment on cytochrome P450 (CYP) 1A1, 2B1, 2E1, and 3A2 at 3 and 24 h after CS_2 administration in CLD-pretreated rats. Each bar represents the mean percent of control ± SEM of three individual animals (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs corresponding control)

In contrast to the cellular changes at 3 h, the results obtained at 24 h on the liver weights (relative to body weights), TBARS, ROS, ALT levels, and liver histopathology were indicative of severe hepatic damage. The increased levels of GSH observed at 24 h may be a reflection of increased rate of GSH synthesis in the liver as an adaptive response to low GSH levels seen at early times (3 h) due to the onset and progression of oxidative stress conditions [Figure 1b]. Similar temporal changes in GSH levels and lipid peroxidation in the liver caused by lindane have been reported.[18] On the other hand, the body weights of the animals after CS_2 treatment did not change significantly; however, there was a significant increase in their absolute liver weights as compared to the corresponding controls [Figure 1b]. Gross examination of the livers of CS_2-treated animals showed marked hydropic liver enlargement, while microscopic examination of the hepatocytes (16 and 24 h) revealed a significant centrilobular hypertrophy and cytoplasmic vacuolation [Figure 2e and f]. These CS_2 treatment-related microscopic lesions were limited to centrilobular region of the liver, where CYP enzymes are abundant suggesting the involvement of induced P450 enzymes and CS_2 metabolism in the hepatic damage. In this respect, it should be noted that CLD has been shown to produce lipid peroxidation and ROS in the liver which have been implicated in DNA damage and cell death by several investigators.[19,20] Similarly, trans-nonachlor (an analog of CLD) has been reported to increase hepatic lipid peroxidation via CYP induction, resulting in the release of activated toxic oxygen species.[13] Other chlorinated hydrocarbons such as polychlorinated biphenyls that are inducers of CYP enzymes have also been linked to the production of lipid peroxidation in the liver.[21]

It has been previously reported that induced P450s produce active oxygen species without a substrate that may also contribute to liver damage. Thus, Imaoka *et al.,* have reported that PB-induced P450s produced active oxygen species without a substrate and PB treatment along with induction of microsomes also induced lipid peroxidation via production of hydroxyl radicals as ROS.[22] Furthermore, these investigators found that among the induced P450s, CYP2B1 produced hydroxyl radicals much higher than CYP2E1 which is known to contribute to liver damage via lipid peroxidation. This observation that CYP2B1 produces higher amount of hydroxyl radicals is particularly significant in light of the fact that CS_2 produces liver damage only in PB-treated rats but not in normal rats.[10] This may also explain why CS_2 metabolized by induced CYP 2B1 and 2E1 produced more liver damage than the induced

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CYP 1A1 and 3A2 as reported in our previous studies.[14] Taken together, it is not untenable to hypothesize that the induction of CYP isoforms by CLD and the metabolism of CS_2 by the induced CYPs, especially by CYP2B1, in concert may have contributed to the production of reactive oxygen species (ROS) and increased lipid peroxidation leading to subsequent liver damage as manifested by increased ALT activity [Figure 1a and b].

In addition to the liver damage caused by CYP induction and CS_2 metabolism, several other mechanisms of CS_2-induced hepatotoxicity have been proposed. For example, the P450-mediated metabolism of CS_2 in isolated hepatocytes produces an unstable intermediate carbonyl sulfide (COS) which is readily metabolized to CO_2 and hydrogen sulfide.[23] It is possible that H_2S can inhibit cellular respiration and create mitochondrial oxidative stress. The other metabolite, reactive sulfur species, reacts readily with sulfhydryl groups of cysteine residues of microsomal proteins to form a hydrodisulfide and damages microsomal membranes resulting in liver injury. Other studies have suggested that this microsomal membrane damage may result in inhibition of the microsomal calcium pump leading to hepatic damage.[24] Alternatively, it is proposed that CS_2-induced hepatotoxicity may be caused partly by destruction of CYP protein through heme oxygenase induced by CS_2 in rats pretreated with PB.[25] Thus, even though it is widely reported that metabolism of CS_2 by hepatic microsomes results in liver toxicity, the precise mechanism of CS_2 hepatotoxicity still remains obscure.

CONCLUSIONS

The endpoint parameters used in this communication support the hypothesis that both CLD-induction of CYPs and CS_2 metabolism mediated by the CYPs appear to be responsible for the observed liver damage. Nevertheless, it may therefore be assumed that several complementary mechanisms may be involved in the hepatic damage after exposure to CS_2.

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