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ALTERATION OF CHEMICALLY INDUCED HEPATOTOXICITY BY COPPER (II) (3,5-DIISOPROPYLSALICYLATE)$_2$

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Abstract—The effects of copper (II) (3,5-diisopropylsalicylate)$_2$ (CuDIPS), which is a synthetic superoxide dismutase, on the hepatotoxicity of carbon tetrachloride and acetaminophen in fed and fasted animals were investigated. CuDIPS did not alter the covalent binding of metabolites of either of these chemicals to the hepatic endoplasmic reticulum. However, CuDIPS did inhibit the hepatotoxicity of carbon tetrachloride by inhibiting the induction of lipid peroxidation by carbon tetrachloride. CuDIPS had only a slight, and histologically insignificant, ability to decrease acetaminophen hepatotoxicity which is related to the inability of CuDIPS to prevent depletion of reduced glutathione by acetaminophen. The observation that fasting potentiates the hepatotoxicity of acetaminophen is emphasized, and the mechanism of this potentiation is suggested to be related to the depletion of reduced glutathione.

Copper(II) (3,5-diisopropylsalicylate)$_2$ (CuDIPS) is a copper coordination complex with superoxide dismutase (EC 1.15.1.1) activity [1]. CuDIPS is sufficiently lipid soluble to enter cells and thus act at the site of production of superoxide to catalyze the conversion of superoxide to hydrogen peroxide [2]. Several investigations have indicated that CuDIPS enters cancer cells, damages the cells, and inhibits tumor growth [2, 3]. The investigation by Oberley *et al.* [2] suggested that the antitumor activity is due to hydrogen peroxide generation and accumulation. This work also demonstrated that detoxification of hydrogen peroxide by glutathione peroxidase, using reduced glutathione as an electron donor, inhibits the antitumor activity of CuDIPS.

The antitumor studies with CuDIPS emphasized a dilemma associated with superoxide generation and detoxification that occurs in hepatic cells during metabolism of xenobiotics. The toxicity of a variety of xenobiotics is apparently related, at least in part, to the toxic effects of superoxide, hydrogen peroxide, and hydroxyl radicals which are produced as a result of interaction of superoxide and hydrogen peroxide [4]. One mechanism of toxicity of superoxide and its degradation products is lipid peroxidation [4–6]. Thus, detoxification of these products, which occurs primarily by glutathione peroxidase catalyzing the conversion of hydrogen peroxide to water in hepatocytes [7], is of great importance. However, the detoxification of hydrogen peroxide by glutathione peroxidase requires donation of electrons by reduced glutathione [7, 8]. The detoxification of hydrogen peroxide by glutathione peroxidase apparently proceeds without difficulty in hepatic cells with normal levels of reduced glutathione. However, depletion of reduced glutathione by fasting [9] or by drugs such as acetaminophen [7, 10, 11] may limit the availability of reduced glutathione for hydrogen peroxide detoxification. Also, the use of reduced glutathione in hydrogen peroxide detoxification may further deplete reduced glutathione, and depletion of reduced glutathione results in hepatotoxicity by altering calcium homeostasis [7, 8, 12]. Thus, superoxide and its degradative products may cause lipid peroxidation and hepatic damage if the hydrogen peroxide is not converted to water by glutathione peroxidase but this detoxification process may deplete glutathione and result in altered intracellular calcium levels and hepatotoxicity.

The purpose of this study was to investigate the effect of CuDIPS on the hepatotoxicity of carbon tetrachloride and acetaminophen in fed and fasted rats. Carbon tetrachloride was chosen because it is prominently associated with lipid peroxidation [13, 14], and acetaminophen was chosen because it causes depletion of reduced glutathione [7, 11]. The experiments were performed in fed and fasted animals because the fasting depletes reduced glutathione [9].

MATERIALS AND METHODS

Male Sprague–Dawley rats weighing 200–300 g and male Swiss mice weighing 30–40 g were obtained from Southern Animal Farms in Prattville, AL. Rats were utilized in all studies involving carbon tetrachloride, and mice were utilized in all studies involving acetaminophen. All animals were maintained on water and purina lab chow *ad lib.*, except during periods of fasting. Rats and mice were fasted for 18 hr prior to administration of carbon tetrachloride or acetaminophen respectively. All animals were

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exposed to a 12-hr light–dark cycle with the room being light from 7:00 a.m. until 7:00 p.m.

CuDIPS was synthesized according to the method of Sorenson [15]. Carbon tetrachloride was purchased from Fisher Scientific. Acetaminophen and chemicals used in enzyme assays were purchased from the Sigma Chemical Co. [^{14}C]Carbon tetrachloride and [^{3}H]acetaminophen were purchased from New England Nuclear.

Carbon tetrachloride studies. The dose of carbon tetrachloride used in all experiments was 1.19 g/kg (0.75 ml/kg). This dose was chosen as the result of preliminary studies that demonstrated a linear increase in lipid peroxidation with doses of carbon tetrachloride from 0.25 ml to 1.0 ml/kg. The carbon tetrachloride and all other chemicals administered to rats were given by intraperitoneal injection. The carbon tetrachloride was diluted with an equal volume of corn oil for injection. CuDIPS was injected 1 hr prior to carbon tetrachloride, or 1 hr prior to corn oil for controls. The CuDIPS was suspended in corn oil.

The same rats were utilized in experiments determining lipid peroxidation and serum glutamate pyruvate transaminase (SGTT) activity. Lipid peroxidation was determined *in vivo* by quantitating the amount of pentane expired [16, 17]. The fact that pentane rather than ethane was primarily expired indicates a low oxygen partial pressure during collection of expired gases from rats [17]. The rats were placed in a two-liter desiccator 30 min after injection of carbon tetrachloride, and a gas sample was taken 30 min later to determine the amount of pentane expired. The animals were then removed from the desiccators, and 24 hr after the carbon tetrachloride injection a blood sample was taken to determine SGPT activity. The SGPT activity was determined by monitoring the decrease in absorbance at 340 nm as described by Wroblewski and LaDue [18]. Covalent binding of [^{14}C] carbon tetrachloride and its metabolites to the hepatic endoplasmic reticulum was determined 3 hr after injection of carbon tetrachloride. In these experiments the animals were decapitated, and the livers were removed and homogenized in 5 vol. of cold 1.15% KCl in 0.05 M Tris buffer, pH 7.4, using a polytron. Microsomes were prepared as previously described [19], and microsomal pellets were resuspended in the 0.05 M Tris–KCl buffer, pH 7.4, using a glass hand homogenizer. Protein was determined by the method of Lowry *et al.* [20]. The amount of [^{14}C]carbon tetrachloride bound to the microsomes was determined by liquid scintillation using a vacuum filtering technique previously described [21]. An aliquot of the microsomal preparation equal to 20 mg of microsomal protein or 800 mg of liver was filtered.

Acetaminophen studies. Acetaminophen was injected intraperitoneally in a dose of 250 mg/kg in all experiments. The acetaminophen was dissolved in saline in a concentration of 12.5 mg/ml and heated to 37° prior to injection. CuDIPS was injected intraperitoneally in a dose of 25 mg/kg to the mice 1 hr prior to acetaminopen. The CuDIPS was suspended in corn oil.

SGPT activity was determined 24 hr after acetaminophen injection as described above in the carbon tetrachloride studies. Mice were decapitated to obtain the blood sample. Covalent binding of [^{3}H]-acetaminophen to microsomes was determined 3 hr after acetaminophen injection, as described in the carbon tetrachloride studies. Hepatic reduced glutathione was determined 1 hr after acetaminophen injection by the method of Ellman [22] as previously described [21]. Histological examination of hepatic damage was performed by preparing paraffin-embedded sections of formalin fixed liver and staining with hematoxylin and eosin.

Table 1. Effect of CuDIPS on carbon tetrachloride induced lipid peroxidation in fed and fasted rats*

	Lipid peroxidation (% of carbon tetrachloride)	
Pretreatment	Fed	Fasted
Corn oil	7.45 ± 2.18†	4.25 ± 0.51†
CuDIPS	10.18 ± 1.70†	2.65 ± 0.17†
Carbon tetrachloride	100.00 ± 10.62	100.00 ± 14.70
CuDIPS plus carbon tetrachloride	60.18 ± 9.33†	49.86 ± 8.83†

* Values are the mean ± S.E. for eight rats. Lipid peroxidation was determined *in vivo* by quantitating the pentane expired between 30 and 60 min after injection of 1.19 g/kg of carbon tetrachloride.

† Significantly different from carbon tetrachloride, $P < 0.05$.

RESULTS

Carbon tetrachloride studies. A single injection of CuDIPS given 1 hr prior to a toxic dose of carbon tetrachloride inhibited lipid peroxidation *in vivo* as measured by the expiration of pentane (Table 1). The CuDIPS decreased the lipid peroxidation by approximately 40% in fed rats and by 50% in fasted rats.

The absolute values for carbon tetrachloride produced lipid peroxidation in the fasted rats and fed rats differed by 12.5%.

Table 2. Effect of CuDIPS on covalent binding of carbon tetrachloride metabolites to the hepatic endoplasmic reticulum in fed and fasted rats*

	Covalent binding (nmoles/100 mg protein)	
Pretreatment	Fed	Fasted
Carbon tetrachloride	30.66 ± 3.02	87.75 ± 12.86†
CuDIPS plus carbon tetrachloride	25.19 ± 1.27	89.20 ± 8.30†

* Values are the mean ± S.E. for six rats. The covalent binding of [^{14}C]carbon tetrachloride metabolites to the hepatic endoplasmic reticulum was determined 3 hr after injection of 1.19 g/kg of [^{14}C]carbon tetrachloride by quantitating bound isotope using a standard vacuum filtration technique. The endoplasmic reticulum was isolated using a standard differential centrifugation technique.

† Significantly different from corresponding treatment in fed rats, $P < 0.05$.

Table 3. Effect of CuDIPS on elevation of SGPT levels by carbon tetrachloride in fed and fasted rats*

Pretreatment	SGPT activity (μmoles/min/ml serum)	
	Fed	Fasted
Corn oil	16.14 ± 0.65†	12.00 ± 3.21†
CuDIPS	14.90 ± 3.07†	33.75 ± 5.50†‡
Carbon tetrachloride	708.08 ± 135.47	1481.14 ± 281.07‡
CuDIPS plus carbon tetrachloride	247.20 ± 77.64†	666.00 ± 109.77†‡

* Values are the mean ± S.E. for eight rats. The SGPT activity was determined 24 hr after injection of 1.19 g/kg of carbon tetrachloride by a coupled enzyme assay utilizing change in absorbance at 340 nm as a measure of SGPT activity.

† Significantly different from carbon tetrachloride, $P < 0.05$.

‡ Significantly different from corresponding treatment in fed rats, $P < 0.05$.

In contrast to the decrease in lipid peroxidation produced by CuDIPS pretreatment, CuDIPS did not alter significantly covalent binding of carbon tetrachloride and its metabolites to the endoplasmic reticulum (Table 2). Also, the binding of carbon tetrachloride was increased approximately 3-fold in the fasted rats regardless of whether they received CuDIPS pretreatment.

The CuDIPS pretreatment did decrease the hepatotoxicity of carbon tetrachloride in fed and fasted rats as measured by the SGPT levels (Table 3). The carbon tetrachloride induced elevation of SGPT activity after CuDIPS pretreatment was only 34 and 45% of the SGPT activity in fed and fasted rats, respectively, that received only carbon tetrachloride.

Acetaminophen studies. CuDIPS pretreatment did not prevent the depletion of hepatic reduced glutathione by acetaminophen in fasted mice (Table 4). The depletion of reduced glutathione caused by fasting and the depletion caused by acetaminophen were additive. The fasting caused a 23% depletion, and the fasted mice treated with acetaminophen had 53% depletion as compared to fed controls. This additive effect of fasting and acetaminophen is apparently very important since the acetaminophen pretreatment was not toxic in fed mice.

In agreement with the finding that CuDIPS pretreatment did not prevent depletion of reduced glutathione depletion by acetaminophen was the observation that CuDIPS had no significant effect on [^{3}H]-acetaminophen binding to the hepatic endoplasmic reticulum in fed or fasted mice (Table 5). However, fasting did increase significantly the binding of acetaminophen to the hepatic endoplasmic reticulum.

In spite of the finding that CuDIPS pretreatment did not alter acetaminophen binding or the acetaminophen induced depletion of reduced glutathione, the CuDIPS did inhibit the elevation of SGPT caused by acetaminophen in fasted mice, thus indicating that CuDIPS may provide some protection from hepatic damage induced by acetaminophen (Table 6). However, the SGPT levels were still very high in the fasted mice, and histological analysis

Table 4. Effect of CuDIPS on the depletion of reduced glutathione by acetaminophen in mice*

Pretreatment	Diet	Reduced glutathione (μmoles GSH/g liver)	
		Control	Acetaminophen
Corn oil	Fed	15.90 ± 0.19	12.55 ± 0.52†
Corn oil	Fasted	12.18 ± 0.30†	7.54 ± 0.19†‡
CuDIPS	Fasted	11.55 ± 0.26†	6.84 ± 0.35†‡

* Values are the mean ± S.E. of six mice. The reduced glutathione concentration was determined 1 hr after the injection of 250 mg/kg of acetaminophen. Livers were homogenized in 0.1 M metaphosphoric acid, and an aliquot of a 10,000 *g* supernatant fraction was assayed for reduced glutathione using Ellman's Reagent and relating the absorbance at 412 nm to the concentration of sulhydryl groups.

† Significantly different from fed control, $P < 0.05$.

‡ Significantly different from appropriate control, $P < 0.05$.

Table 5. Effect of CuDIPS on covalent binding of acetaminophen metabolites to the hepatic endoplasmic reticulum in fed and fasted mice*

Pretreatment	Covalent binding (nmoles/g liver)	
	Fed	Fasted
Acetaminophen	109.85 ± 14.32	185.07 ± 15.48†
CuDIPS plus acetaminophen	89.71 ± 18.37	207.98 ± 8.00†

* Values are the mean ± S.E. of six mice. The covalent binding of [^{3}H]acetaminophen metabolites to the hepatic endoplasmic reticulum was determined 3 hr after the injection of 250 mg/kg of [^{3}H]acetaminophen by quantitating bound isotope using a standard vacuum filtration technique. The endoplasmic reticulum was isolated using a standard differential centrifugation technique.

† Significantly different from corresponding treatment in fed mice, $P < 0.05$.

Table 6. Effect of CuDIPS on elevation of SGPT levels by acetaminophen in fed and fasted mice*

Pretreatment	SGPT activity (μmoles/min/ml serum)	
	Fed	Fasted
Corn oil	57.11 ± 2.71	71.81 ± 6.43†
CuDIPS	57.77 ± 6.72	104.53 ± 5.88†‡
Acetaminophen	50.35 ± 3.40	2997.33 ± 200.49‡
CuDIPS plus acetaminophen	57.00 ± 3.83	2097.60 ± 135.92†‡

* Values are the mean ± S.E. of ten mice. The SGPT activity was determined 24 hr after the injection of 250 mg/kg of acetaminophen by a coupled enzyme assay utilizing change in absorbance at 340 nm as a measure of SGPT activity.

† Significantly different from acetaminophen, $P < 0.05$.

‡ Significantly different from corresponding treatment in fed mice, $P < 0.05$.

indicated no alteration of damage to the liver by CuDIPS pretreatment. Interestingly, the 250 mg/kg dose of acetaminophen was non-toxic in the fed mice and did not elevate SGPT activity, but it was very toxic in the fasted mice. This observation was also confirmed by histological analysis.

DISCUSSION

Carbon tetrachloride and acetaminophen are two of the most extensively investigated hepatotoxic agents [10, 11, 23–27]. Early actions in the sequence of events by which each of these agents produce hepatic damage include: (a) both agents are metabolized by the hepatic mixed-function oxidase system to form toxic metabolites [11, 28, 29], and (b) the toxic metabolites bind hepatic cell components including the endoplasmic reticulum [30, 31]. As expected, pretreatment with CuDIPS did not alter these initial events in the production of hepatic damage, as demonstrated by the lack of effect of CuDIPS on covalent binding of these agents and their toxic metabolites to the hepatic endoplasmic reticulum.

The observation that CuDIPS inhibited the hepatotoxicity of carbon tetrachloride more effectively than it alleviated the hepatotoxicity of acetaminophen indicates that these two agents produce hepatotoxicity by slightly different mechanisms. Carbon tetrachloride toxicity is dependent on its metabolism by the hepatic MFO system to toxic metabolites including a trichloromethyl free radical, an electrophilic chlorine and phosgene [17, 28, 32]. These toxic metabolites are thought to promote lipid peroxidation and hepatotoxicity by binding cell components and interacting with cell lipids [17, 28, 32]. The possibility that superoxide generated during the metabolism of carbon tetrachloride may contribute, at least in part, to the lipid peroxidation might be proposed as a result of the observation that CuDIPS did inhibit the carbon tetrachloride induced lipid peroxidation. Such a proposal is controversial since recent work by Morehouse *et al.* [33] indicates that minimal superoxide is generated by the hepatic MFO system. However, Ekstrom and Ingelman-Sundberg [34] have indicated that cytochrome P-450 may generate superoxide to promote lipid peroxidation. Also of interest is the observation that ethanol metabolism by cytochrome P-450 generates superoxide and hydroxyl radicals [35], and ethanol does potentiate the hepatotoxicity of carbon tetrachloride [36, 37].

Acetaminophen hepatotoxicity also depends on metabolism of the drug to toxic metabolites by the hepatic MFO system [11] and the binding of these toxic metabolites to hepatic cell components [30]. Our data do not support the idea that superoxide makes an important contribution to the hepatotoxicity of acetaminophen, since CuDIPS pretreatment did not greatly alter acetaminophen hepatotoxicity. However, Wendel and Feuerstein [38] have investigated the relationship of acetaminophen induced hepatotoxicity to superoxide generation and have hypothesized that superoxide generation by the hepatic MFO systems, at least in part, contributes to the resulting lipid peroxidation and cell damage. However, they do emphasize that acetaminophen induced superoxide accumulation and hepatotoxicity is dependent on glutathione depletion [38].

In conclusion, the observation that CuDIPS, which is known to have superoxide dismutase activity, particularly decreased the hepatotoxicity of carbon tetrachloride suggests the possibility that superoxide generation may contribute to the production of lipid peroxidation and hepatotoxicity produced by carbon tetrachloride. An alternate explanation of the action of CuDIPS would be the possibility that CuDIPS acts as a radical scavenger.

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