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EFFECT OF AGENTS KNOWN TO ALTER CARBON TETRACHLORIDE HEPATOTOXICITY AND CYTOCHROME P-450 LEVELS ON CARBON TETRACHLORIDE-STIMULATED LIPID PEROXIDATION AND ETHANE EXPIRATION IN THE INTACT RAT

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Abstract—Administration of carbon tetrachloride (CCl_4) to rats led to an increase in expired ethane by 15 min. Prior treatment of rats with phenobarbital led to a significant increase in both CCl_4-stimulated ethane expiration and hepatic microsomal lipid diene conjugation, while prior treatment with 3-methylcholanthrene or CCl_4 led to a decrease in both parameters. Treatment with isopropanol increased CCl_4-stimulated ethane expiration, while neither ethanol nor diethyl maleate treatment altered the response to CCl_4. Cobaltous chloride treatment significantly decreased CCl_4-stimulated ethane expiration. A strong correlation was found between CCl_4-stimulated hepatic microsomal lipid diene conjugation and ethane expiration.

Recent investigations have suggested the feasibility of monitoring ethane expiration as an index of lipid peroxidation *in vivo*. Riely and Cohen [1] have shown that spontaneously peroxidizing mouse tissues evolve ethane *in vitro* and that carbon tetrachloride stimulates ethane production *in vivo*. This result is in accord with the finding that CCl_4 administration leads to an increase in hepatic lipid peroxidation within 15 min, as indicated by an increase in lipid diene conjugation [2]. The antioxidant, α-tocopherol, decreases ethane expiration associated with CCl_4 administration in mice [1], and rats fed a diet deficient in α-tocopherol spontaneously evolve ethane [3].

Carbon tetrachloride is believed to exert its hepatotoxic effects by inducing a peroxidative degeneration of cellular lipids [4]. However, much of the evidence for this hypothesis is indirect. Such a hypothesis could be better tested with an appropriate assay *in vivo* for lipid peroxidation. Ethane expiration appears to be a suitable index of lipid peroxidation *in vivo* associated with peroxidative decomposition of ω-3 fatty acids [3, 5]. Therefore, it was of interest to determine the effect of various agents known to alter CCl_4-induced hepatotoxicity on CCl_4-stimulated ethane expiration and diene conjugation. We report herein the effects of phenobarbital (PB), 3-methylcholanthrene (3-MC), cobaltous chloride ($CoCl_2$), diethyl maleate (DEM), CCl_4, ethanol and isopropanol on CCl_4-stimulated ethane expiration in the intact rat.

METHODS

Male Sprague–Dawley rats weighing 300–350 g were maintained on Purina Lab Chow and kept on a 12-hr light and 12-hr dark cycle. Where indicated, rats were injected i.p. with phenobarbital sodium (50 mg/kg) or 3-MC in corn oil (20 mg/kg) daily for 4 days; 24 hr after the last dose, 1 ml CCl_4/kg was injected i.p. as a 50% (v/v) solution in light mineral oil. Cobaltous chloride (60 mg/kg) was administered s.c. both 24 and 48 hr prior to dosing with CCl_4. Diethyl maleate (0.6 ml/kg) was given s.c. 30 min prior to CCl_4 administration. Ethanol (5 ml/kg) or isopropanol (2.5 ml/kg) were administered orally 18 hr prior to CCl_4 injection as a 50% and 25% (v/v) solution in water respectively. Rats were also injected i.p. with 0.1 ml CCl_4/kg as a 10% (v/v) solution in light mineral oil 24 hr prior to a challenging dose of 1 ml CCl_4/kg; controls received saline or corn oil prior to the challenging dose of CCl_4. All rats were tested for ethane expiration for a control period of 90 min at 15-min intervals. Ninety min after completion of the control experiment, the rats were injected with 1 ml CCl_4/kg as described above. The animals were immediately returned to the chamber and ethane expiration was again measured for 90 min at 15-min intervals.

Ethane expiration was quantitated by placing a rat into a sealed chamber (desiccator) with an air environment. The rat was placed on a wire screen above a layer of Baralyme and Drierite granules which removed respiratory CO_2 and water vapor respectively. Removal of CO_2 and consumption of O_2 created a partial vacuum in the chamber which drew in oxygen from an outside source. The total volume of the chamber was 1.22 liters. At 15-min intervals, a 4-ml sample of chamber atmosphere was removed by means of a 3-way stopcock and syringe. One ml of this sample was injected by means of a gas sampling valve into a Varian model 1400 gas chromatograph equipped with a flame ionization detector. A 2 mm i.d. × 6 ft glass column containing Carbosieve-B, 60/80 mesh, and maintained at 150° was used to separate ethane from other respiratory gases. A

100 ppm standard of ethane in nitrogen (Alltech Associates) eluted at 2 min. The injector and detector temperatures were 205° and 260° respectively.

Lipid conjugated dienes were determined 90 min after administration of CCl_4, according to the method of Klaassen and Plaa [6]. The results are expressed as the absorbance at 243 nm of a solution containing 1 mg of microsomal lipid/ml. In order to test the significance of difference between means, a Mann-Whitney non-parametric U test [7] was used for ethane measurements and a Student's *t*-test was used for conjugated diene measurements.

RESULTS

Figures 1–4 show the CCl_4-stimulated ethane production in rats treated with several agents known to alter CCl_4 hepatotoxicity. It can be seen that control rats exhibited a low basal production of ethane in the absence of CCl_4 administration. Injection of 1 ml CCl_4/kg body weight significantly increased ethane production within 15 min and resulted in a 3-fold increase above the control level by 90 min.

PB and 3-MC treatment. Ethane production in rats treated with PB alone did not differ from control ethane production in the absence of CCl_4 (data not shown); however, CCl_4-stimulated ethane expiration was significantly increased in these rats by 15 min (Fig. 1). Prior treatment with PB produced a 110 per cent increase in CCl_4-stimulated ethane production by 90 min. In contrast, 3-MC treatment led to a 55 per cent decrease in CCl_4-stimulated ethane production by 90 min. Ethane production in rats treated with 3-MC alone was not significantly different from control levels (data not shown).

Table 1. Microsomal lipid diene conjugation 90 min after CCl_4 administration to rats*

Treatment	CCl_4	N	E at 243 nm†
Saline	–	6	0.254 ± 0.011
	+	9	0.380 ± 0.018‡
Corn oil	+	5	0.382 ± 0.021‡
PB	–	3	0.241 ± 0.021
	+	5	0.565 ± 0.053§
3-MC	–	5	0.280 ± 0.023
	+	5	0.295 ± 0.026‖
CCl_4¶	–	3	0.323 ± 0.036‡
	+	5	0.307 ± 0.024§
DEM	–	3	0.253 ± 0.007
	+	3	0.490 ± 0.019§

* CCl_4 was administered i.p. 1 ml/kg, as a 50% (v/v) solution in light mineral oil.

† Absorbance of 1 mg of microsomal lipid/ml of methanol. Values are the mean ± S. E.

‡ Significantly different from saline alone, $P < 0.05$.

§ Significantly different from saline + CCl_4, $P < 0.05$.

‖ Significantly different from corn oil + CCl_4, $P < 0.05$.

¶ CCl_4 was administered i.p., 0.1 ml/kg, as a 10% (v/v) solution in light mineral oil.

Table 1 shows that microsomal diene conjugation paralleled the production of ethane in PB- and 3-MC-treated rats. It can be seen that CCl_4 alone increased the production of conjugated dienes by about 50 per cent. Furthermore, PB treatment led to a 50 per cent increase in CCl_4-stimulated diene conjugation, while treatment with 3-MC decreased CCl_4-stimulated diene conjugation by 22 per cent.

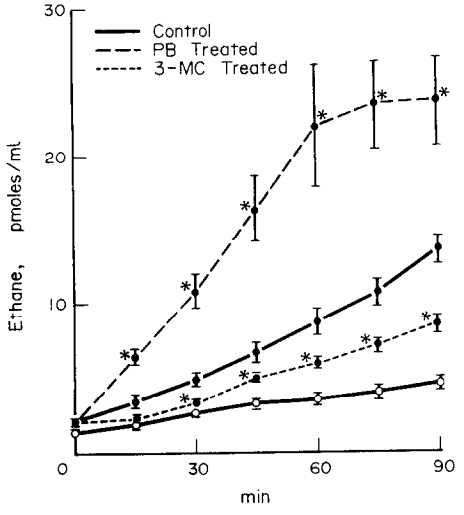

Fig. 1. Ethane production in rats treated with PB or 3-MC. Animals were treated as described in Methods. Rats were injected with light mineral oil alone (○) or with 1 ml CCl_4/kg in light mineral oil (●) and immediately placed in a sealed chamber; ethane concentrations were periodically monitored. The data are the means and the standard errors for at least four rats/group, expressed as pmoles ethane/ml of chamber gas. Ethane production by rats treated with PB or 3-MC alone was not significantly different from control (data not shown). Values marked with an asterisk (*) were significantly different from control given 1 ml CCl_4/kg, $P < 0.05$.

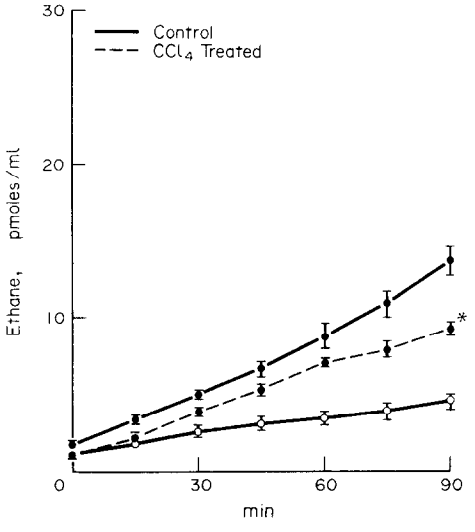

Fig. 2. Ethane production in rats treated with a prior dose of CCl_4. Animals were treated as described in Methods. Rats were injected with light mineral oil alone (○) or with 1 ml CCl_4/kg in light mineral oil (●) and immediately placed in a sealed chamber; ethane concentrations were periodically monitored. The data are the means and the standard errors for at least five rats/group, expressed as pmoles ethane/ml of chamber gas. Ethane production by rats treated with a prior dose of CCl_4 only was not significantly different from control (data not shown). The value marked with an asterisk (*) was significantly different from control given 1 ml CCl_4/kg, $P < 0.05$.

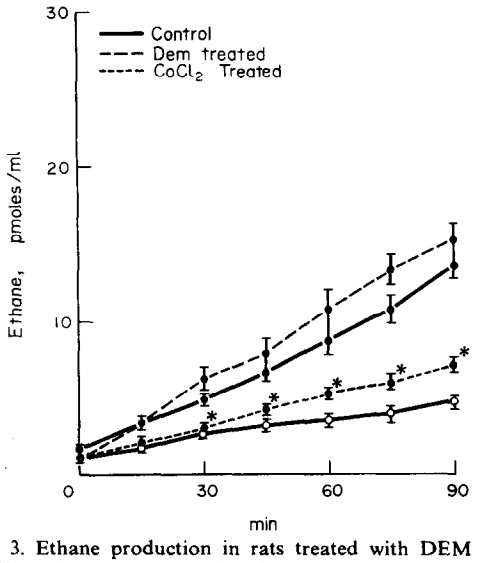

Fig. 3. Ethane production in rats treated with DEM or $CoCl_2$. Animals were treated as described in Methods. Rats were injected with light mineral oil alone (○) or with 1 ml CCl_4/kg in light mineral oil (●) and immediately placed in a sealed chamber; ethane concentrations were periodically monitored. The data are the means and the standard errors for at least five rats/group, expressed as pmoles ethane/ml of chamber gas. Ethane production by rats treated with DEM or $CoCl_2$ alone was not significantly different from control (data not shown). Values marked with an asterisk (*) were significantly different from control given 1 ml CCl_4/kg, $P < 0.05$.

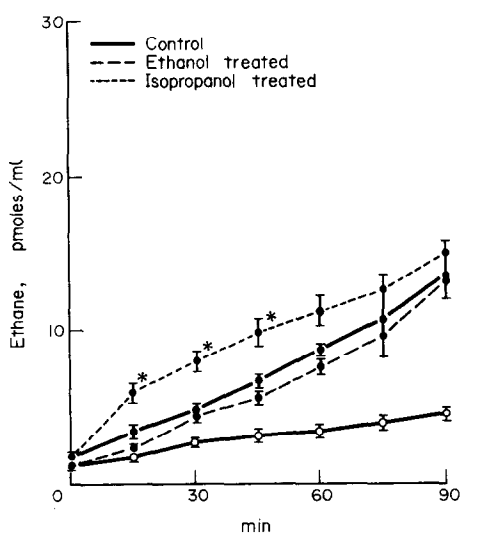

Fig. 4. Ethane production in rats treated with ethanol or isopropanol. Animals were treated as described in Methods. Rats were injected with light mineral oil alone (○) or with 1 ml CCl_4/kg in light mineral (●) and immediately placed in a sealed chamber; ethane concentrations were periodically monitored. The data are the means and the standard error for at least four rats/group, expressed as pmoles ethane/ml of chamber gas. Ethane production by rats treated with ethanol or isopropanol alone was not significantly different from control (data not shown). Values marked with an asterisk (*) were significantly different from control given 1 ml CCl_4/kg, $P < 0.05$.

Prior CCl_4 treatment. Prior treatment of rats with a small dose of CCl_4 (0.1 ml/kg) led to a 30 per cent decrease in CCl_4-stimulated ethane production by 90 min (Fig. 2). Diene conjugation stimulated by 1 ml CCl_4/kg was decreased approximately 20 per cent by prior treatment with CCl_4 (Table 1). Although there was a significant increase in conjugated dienes 24 hr after treatment with 0.1 ml CCl_4/kg, ethane production was not significantly different from control (data not shown).

DEM treatment. Treatment with DEM did not significantly increase CCl_4-stimulated ethane production (Fig. 3); in contrast, diene conjugation was increased by 30 per cent (Table 1). Ethane production and diene conjugation in rats treated with DEM alone were not significantly different from control.

$CoCl_2$ treatment. Figure 3 shows that, after 30 min, CCl_4-stimulated ethane production was significantly reduced in $CoCl_2$-treated rats, resulting in a 70 per cent decrease by 90 min. Ethane production in rats treated with $CoCl_2$ alone was not significantly different from control (data not shown).

Ethanol or isopropanol treatment. No significant effect on CCl_4-stimulated ethane production was observed in ethanol-treated rats (Fig. 4). Isopropanol treatment resulted in an early increase in ethane production which, after 60 min, was no longer significantly different from the control group which received 1 ml CCl_4/kg. Ethane production in rats treated with ethanol or isopropanol alone was not significantly different from control (data not shown).

DISCUSSION

Riely and Cohen [1] showed that ethane production in mice was stimulated by CCl_4, a known prooxidant. Furthermore, Hafeman and Hoekstra [8] found that dietary supplementation with vitamin E or selenium decreased ethane production in CCl_4-treated rats. It has been suggested that the exhaled ethane results from the peroxidative decomposition of ω-3 fatty acids [3, 5]. This is supported by the finding that in a non-enzymatic system containing linolenic acid, an ω-3 fatty acid, ethane is produced only under conditions which stimulate the formation of hydroperoxides [9]. However, before ethane expiration can be accepted as a valid measure of lipid peroxidation *in vivo*, it is important to correlate this phenomenon with a known index of lipid peroxidation. This investigation has shown that a strong correlation exists between ethane production *in vivo* and lipid peroxidation in rats treated with CCl_4. As can be seen in Fig. 5, treatment with drugs which altered the extent of conjugated diene formation in the liver of CCl_4-poisoned rats also altered the ethane expiration in an analogous manner. These results validate ethane expiration as an accurate indicator lipid peroxidation *in vivo*.

It has been suggested that the interaction of CCl_4 with NADPH-cytochrome *c* reductase leads to lipid peroxidation in CCl_4 poisoning [10]. This hypothesis is supported by the observation that cytochrome *c* decreases both the hepatic necrosis [11] and the lipid peroxidation [10] stimulated by CCl_4. However, cytochrome *c* may also serve to decrease the flux of electrons from NADPH to cytochrome P-450, an alter-

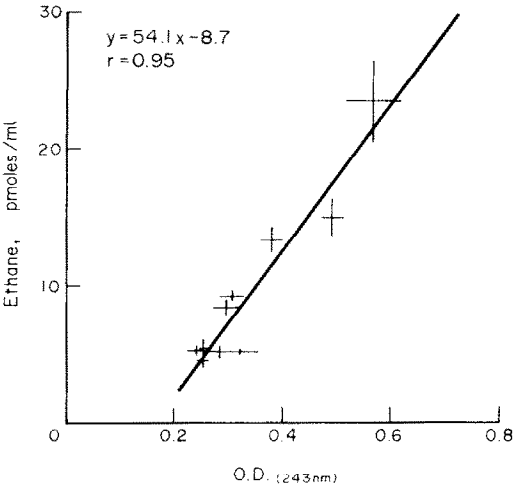

Fig. 5. Correlation between diene conjugation and ethane production. The data are the means and the standard errors of each treatment group at 90 min after administration of CCl_4 and their respective controls; $r = 0.95$ ($P < 0.001$).

nate site of CCl_4 bioactivation, thereby preventing the cytochrome P-450-mediated activation of CCl_4. Consequently, the results obtained with cytochrome *c* do not clearly support the hypothesis that NADPH-cytochrome *c* reductase is the site of CCl_4 activation.

Drugs which alter the content of microsomal cytochrome P-450 are known to modify the toxicity of CCl_4. Phenobarbital, a known inducer of the cytochrome P-450 drug-metabolizing system [12], has been shown to increase the toxicity of CCl_4 [13]. Suarez *et al.* [14] have shown that PB treatment increased CCl_4-stimulated diene conjugation in rats, which the present investigation confirms (Table 1). Furthermore, CCl_4-stimulated ethane expiration was also increased by PB treatment (Fig. 1).

Treatment with 3-MC has been shown to decrease centrolobular necrosis [15], enzyme release [16] and [^{14}C]CCl_4 binding to microsomal lipid [17] in CCl_4-poisoned rats. Our results suggest that CCl_4-stimulated lipid peroxidation, as indicated by diene conjugation and ethane expiration, is significantly reduced by 3-MC treatment. Since 3-MC treatment does not decrease the level of hepatic cytochrome *c* reductase [18] but does induce the synthesis of an altered form of cytochrome P-450 [19], these results suggest that cytochrome P-450 is the site of reactive metabolite formation leading to lipid peroxidation. In addition, microsomes from 3-MC-treated rats exhibited a decreased capacity to bind type I compounds to cytochrome P_1-450 [20]. Since CCl_4 is a type I compound [21], a reduced capacity to both bind CCl_4 to cytochrome P_1-450 and metabolize it to a reactive intermediate in 3-MC-treated rats may account for the decreased toxicity of CCl_4.

Further support for the involvement of cytochrome P-450 in CCl_4-stimulated lipid peroxidation comes from experiments utilizing $CoCl_2$-treated rats. Cobaltous chloride treatment, which decreases cytochrome P-450 levels without altering cytochrome *c* reductase, leads to a decrease in CCl_4-stimulated enzyme release, diene conjugation [22] and ethane expiration (Fig. 3). In addition, prior treatment with a small dose of CCl_4 also protects against the toxicity of a later larger dose of CCl_4 [23]. While a small protective dose of CCl_4 does not alter the content of cytochrome *c* reductase, cytochrome P-450 levels and drug-metabolizing activity are greatly depressed [24]. The observation that CCl_4-stimulated diene conjugation (Table 1) and ethane expiration (Fig. 2) are significantly depressed in animals treated with a prior dose of CCl_4 parallels the known decrease in cytochrome P-450 levels and strengthens the contention that cytochrome P-450 is the site of toxic metabolite formation leading to lipid peroxidation.

Administration of DEM to rats decreases the hepatic content of glutathione, an intracellular antioxidant [25]. The increase in CCl_4-stimulated diene conjugation in DEM-treated rats suggests that glutathione may be important in modulating lipid peroxidation induced by CCl_4. Furthermore, increased malondialdehyde formation was observed in hepatocytes isolated from DEM-treated rats incubated in the presence of CCl_4 [26]. Glutathione may act directly to decrease the concentration of a reactive intermediate such as the trichloromethyl free radical or may act indirectly via the glutathione peroxidase system to detoxify lipid hydroperoxides. The reason for an absence of an effect by DEM on ethane production in CCl_4-poisoned rats is not clear.

Traiger and Plaa [27] have reported that acute administration of either ethanol or isopropanol potentiates the toxicity of CCl_4 as monitored by enzyme release, hepatic triglyceride levels and plasma bilirubin content. The present study indicates that ethane production is not significantly altered by ethanol administration, while isopropanol administration produced only a transient increase in CCl_4-stimulated ethane production. These results suggest that lipid peroxidation may not be associated with the increase in CCl_4 toxicity observed after acute ethanol or isopropanol treatment.

The present investigation has demonstrated the suitability of ethane expiration as an index of lipid peroxidation *in vivo*. Furthermore, the effects of prior treatment with PB, 3-MC, CCl_4 and $CoCl_2$ on CCl_4-stimulated ethane production support the hypothesis that cytochrome P-450 rather than cytochrome *c* reductase is involved in the formation of reactive intermediate responsible for CCl_4-induced lipid peroxidation.

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