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Effects of Spironolactone on Carbon Tetrachloride Hepatotoxicity (37773)

GARY P. CARLSON, GEORGE C. FULLER, AND NELSON FAUSTO
(Introduced by H. W. Youngken, Jr.)

Department of Pharmacology and Toxicology, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island 02881; and Division of Biomedical Sciences, Brown University, Providence, Rhode Island 02912

A relationship between the metabolism of carbon tetrachloride (CCl_4) and its hepatotoxicity is suggested by the fact that several agents which alter microsomal enzyme activity have an effect on the hepatotoxicity of this agent. Treatment of animals with DDT (1, 2), phenobarbital (1, 3) or 3,4-benzpyrene (4), in doses known to cause enzyme induction, results in an enhanced hepatotoxicity due to CCl_4 while pretreatment with β-diethylaminoethyl diphenylpropyl acetate HCl (SKF 525A), an inhibitor of microsomal drug metabolism, prevents liver necrosis due to CCl_4 administration (5). However, in rats with microsomal enzymes induced by 3-methylcholanthrene (3 MC), there is a decrease in CCl_4 hepatotoxicity (3, 6). The contrasting findings between phenobarbital and 3 MC have been attributed to the differential effects of these two agents on NADPH cytochrome *c* reductase and cytochrome P-450 (7). In phenobarbital treated rats cytochrome *c* reductase activity was increased but P-450 content was not elevated following CCl_4 exposure. With 3 MC there was little change in cytochrome *c* reductase but a high content of cytochrome P-450 after CCl_4 exposure. Since spironolactone has been shown to stimulate microsomal drug metabolism (8), it was of interest to determine what effect spironolactone would have on CCl_4 toxicity. It differs from the agents mentioned above in that there is an increase in cytochrome *c* reductase but no increase in cytochrome P-450 (9, 10).

Materials and Methods. Male albino rats obtained from Charles River Breeding Laboratories were used. They were maintained at 21-23° with room lights on alternating light–dark cycles. Food and water were allowed *ad libitum*. The spironolactone was usually administered in corn oil twice daily at a dose of 100 mg/kg for 3 days. Controls received corn oil alone. Rats were exposed to CCl_4 in a dynamic inhalation chamber (11) 24 hr after the last dose and experiments were carried out 24 hr after exposure.

Tail vein blood samples were obtained under light ether anesthesia for serum glutamic pyruvate transaminase (SGPT) and serum glutamic oxalacetic transaminase (SGOT) according to the method of Reitman and Frankel (12). A small piece of one liver lobe was removed. One portion of this was used for measurement of glucose-6-phosphatase activity using Harper's method (13) and a second was fixed in Dietrich's solution and stained with hematoxylin and eosin for histological evaluation. The livers were then perfused with cold isotonic KCl, removed and homogenized. The homogenate was centrifuged at 9000*g* for 20 min in a Sorvall Model RC2-B refrigerated centrifuge. A portion of this was used to measure *p*-nitroanisole *O*-demethylation employing the method of Netter and Seidel (14) and the remainder was centrifuged at 105,000*g* for 1 hr in an International ultracentrifuge (Model B-60) to obtain soluble and microsomal fractions. NADPH cytochrome *c* reductase activity and cytochrome P-450 content were determined (15) in the microsomal fractions. Protein content of the microsomal fraction was determined according to the method of Lowry *et al.* (16).

Malonaldehyde formation as a measure of

TABLE I. Effect of CCl_4 Inhalation on Liver Glucose-6-Phosphatase, SGOT and SGPT in Spironolactone Treated and Control Rats.

Pretreatment	Exposure	N^c	Glucose-6-Phosphatase[d]	SGPT[e]	SGOT[e]
Corn oil	Air	10	16.3 ± 0.64	23 ± 2.5	72 ± 1.2
	CCl_4[b]	10	8.1 ± 0.30[g]	470 ± 86.7[g]	907 ± 171.6[g]
Spironolactone[a]	Air	10	16.2 ± 0.77	23 ± 2.5[f]	70 ± 2.3[f]
	CCl_4	10	10.3 ± 0.47[gh]	171 ± 27.1[gh]	374 ± 69.4[gh]

[a]100 mg/kg twice daily for 3 days.
[b]6200 ppm for 2 hr.
[c]Number of animals.
[d]μmoles PO_4/g/min.
[e]Reitman-Frankel units.
[f]Nine animals in this group.
[g]Significantly different ($P < 0.05$) from air-exposed group receiving same pretreatment.
[h]Significantly different ($P < 0.05$) from corn oil–CCl_4 group.

lipid peroxidation was carried out by incubating microsomes from 222 mg liver in 3 ml of incubation medium containing 77.3 m*M* KCl, 34.4 m*M* Tris–HCl buffer (pH 8.0), 5.1 m*M* glucose-6-phosphate, 4.6 m*M* nicotinamide, and 1.0 m*M* methylarachidonate as substrate. One microliter of CCl_4 was suspended in a cup above the mixture and the flask was sealed. NADPH (1.2 m*M*) was added to one-half the flasks. At the end of the 15 min incubation at 37°, a 1 ml aliquot was removed and added to 2 ml of cold 10% trichloroacetic acid and centrifuged. Two milliliters of the supernatant were then reacted with 2 ml of 0.67% thiobarbituric acid by boiling for 10 min in a water bath. After cooling the samples were read at 535 nm in a Beckman DB-G spectrophotometer. The non-NADPH stimulated malonaldehyde production was subtracted to obtain a value representing NADPH stimulated malonaldehyde formation. In the *in vitro* studies, the microsomal sample was divided and assayed with and without spironolactone.

Values are expressed as mean ± standard error. Student's *t* test was used to compare means. A paired *t* test was used in the case of the *in vitro* lipid peroxidation studies.

Results. Protection against the hepatotoxicity of CCl_4 by pretreatment of the animals with spironolactone was demonstrated using two parameters (Table I). The decrease in liver glucose-6-phosphatase resulting from CCl_4 exposure was not as great in the group

TABLE II. Effect of CCl_4 Inhalation on *p*-Nitroanisole Demethylation, Cytochrome *c* Reductase and Cytochrome P-450 in Spironolactone Treated and Control Rats.

Pretreatment	Exposure	N^c	*p*-Nitroanisole Demethylation[d]	Cytochrome *c* Reductase[e]	Cytochrome P-450[f]
Corn oil	Air	5	6.2 ± 0.20	63.4 ± 3.75	126 ± 5.1
	CCl_4[b]	4	3.2 ± 0.27[g]	64.8 ± 3.27	84 ± 13.6[g]
Spironolactone[a]	Air	5	9.0 ± 0.87[h]	114.9 ± 9.01[h]	87 ± 17.3
	CCl_4	5	3.7 ± 0.26[g]	94.2 ± 4.01	63 ± 9.3

[a]100 mg/kg twice daily for 3 days.
[b]6200 ppm for 2 hr.
[c]Number of animals.
[d]μg *p*-nitrophenol formed/50 mg/hr.
[e]nmoles of cytochrome *c* reduced/min/mg protein.
[f]Difference in absorbance between 450 and 500 nm/mg protein × 10^4.
[g]Significantly different ($P < 0.05$) from air-exposed group receiving same pretreatment.
[h]Significantly different ($P < 0.05$) from corn oil–air group.

that had received spironolactone as in the control group ($P < 0.05$). Also the rises in serum transaminase levels due to CCl_4 exposure were not as great in the spironolactone pretreated rats as they were in the control animals.

Further evidence of protection was noted by histological evaluation of the liver. No cell injury was seen in the livers of animals pretreated with either corn oil or spironolactone alone. The livers of rats pretreated with corn oil and subsequently exposed to CCl_4 exhibited centrolobular necrosis and ballooning in midzonal areas. The lesion was present in virtually all lobules of all animals. In the spironolactone animals so exposed, the lesion was generally similar but less extensive than that of the corn oil group.

The combined effects of spironolactone and CCl_4 on microsomal function and components are presented in Table II. Pretreatment with spironolactone resulted in increases in *p*-nitroanisole demethylation and cytochrome *c* reductase activity. There was no change in cytochrome P-450 content. These results are in agreement with the results reported by others ;(9, 10). Spironolactone failed to protect against the decrease in *p*-nitroanisole demethylation resulting from CCl_4 exposure. Likewise there appeared to be little protection against the decrease in P-450 content following exposure to CCl_4.

TABLE III. Spironolactone Inhibition of CCl_4 Stimulated Malonaldehyde Formation.

Treatment	N[b]	Malonaldehyde formed[c]	Percentage of control
In vivo			
Corn oil	4	5.4 ± 1.11	100
Spironolactone[a]	4	2.4 ± 0.38[d]	44
In vitro			
Control	6	2.0 ± 0.45	100
10^{-3} *M* Spironolactone	6	0.7 ± 0.24[d]	35
10^{-4} *M* Spironolactone	6	1.0 ± 0.26[d]	50

[a]50 mg/kg ip twice daily for 3 days.
[b]Number of animals per group.
[c]Nanomoles/mg microsomal protein/15 min; average ± SE.
[d]Significantly different ($P < 0.05$) from control group.

Lipid peroxidation, as measured by malonaldehyde formation stimulated by CCl_4 in the presence of NADPH, served as an additional measurement of CCl_4 damage. When rats were pretreated with 50 mg/kg of spironolactone twice daily for 3 days, their liver microsomes formed 56% less malonaldehyde thus indicating less lipid peroxidation due to CCl_4 (Table III). In addition, spironolactone added *in vitro* to the incubation medium inhibited malonaldehyde formation by 65% at 10^{-3} *M* and by 50% at 10^{-4} *M*.

Discussion. From the data of Suarez *et al.* (7) which suggests that the ratio of cytochrome *c* reductase activity to P-450 content is important in differentiating between the potentiating effect of phenobarbital of CCl_4 hepatotoxicity and the protecting effect of 3-methylcholanthrene, it might be predicted that spironolactone pretreatment by increasing cytochrome *c* reductase activity and not P-450 content would result in enhanced CCl_4 toxicity. However, instead of this predicted response, protection was observed.

The data substantiate the results previously reported by others (9, 10) that pretreatment with spironolactone of male rats results in increased cytochrome *c* reductase activity but no alteration in P-450 content. Furthermore, the results clearly demonstrate protection against damage to the liver by CCl_4 as evidenced by a less severe decrease in glucose-6-phosphatase activity in the liver and a decreased release of enzymes (SGOT and SGPT) from the liver into the blood. This conclusion is reinforced by histological examination of liver sections from the animals.

If the lipid peroxidation hypothesis for the mechanism of CCl_4 damage as described by Recknagel (17) is correct in that the interaction of CCl_4 with microsomal electron transport components produces free radicals that attack unsaturated fatty acids and lead to peroxidative decomposition, the agents which prevent either the free radical formation or the lipid peroxidation process itself would be expected to protect the liver. As the results demonstrated, spironolactone protected against lipid peroxidation as measured by malonaldehyde formation both when injected prior to tissue removal or when added *in*

vitro.

It is difficult to speculate how spironolactone might be interacting with the microsomal electron transport system to cause decreased toxicity since it might be expected from the increase in *p*-nitroanisole demeth ylation that spironolactone pretreatmen should lead to greater activation of CCl_4 to toxic metabolites, most likely free radicals. However, Hamrick *et al.* (10) have shown that in male rats, the metabolism of some substrates is increased and for others it is decreased possibly due to formation of abortive P-450–substrate complexes. They further suggest that there is an accelerated electron flow through the microsomal electron transport system and that there may be an uncoupling of the substrate from the electron transport system at some undetermined point leading to a decreased formation of metabolites. In view of this fast shunting of electrons to P-450 with low metabolite formation, spironolactone pretreatment should decrease lipid peroxidation and subsequent liver damage whether the free radicals responsible are formed at the cytochrome *c* reductase level as proposed by Slater and Sawyer (18) or whether they are formed at cytochrome P-450 as suggested by Garner and McLean (19).

Still another possibility is that spironolactone may act as a substrate and compete for electrons with lipid peroxidation or free radical formation as suggested for the protective effect of aminopyrine (20). It is not possible, therefore, to definitely conclude how spironolactone protects against CCl_4 hepatotoxicity until more is known about the molecular mechanism of action of CCl_4 and the peculiarities of induction with spironolactone are understood.

Summary. Pretreatment of male rats with spironolactone resulted in protection against CCl_4 hepatotoxicity. This protection was evidenced by decreased reduction of liver glucose-6-phosphatase activity, smaller rises in SGOT and SGPT, and less severe hepatic damage as observed by histological examination. Spironolactone pretreatment and spironolactone *in vitro* both decreased lipid peroxidation due to CCl_4 as measured by malonaldehyde formation.

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