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ON THE MECHANISM OF CARBON TETRACHLORIDE TOXICITY—COINCIDENCE OF LOSS OF DRUG-METABOLIZING ACTIVITY WITH PEROXIDATION OF MICROSOMAL LIPID

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Abstract—Lipoperoxidation *in vitro* of liver microsome preparations results in a coincidental loss of drug metabolism measured as aminopyrine demethylase activity. NADPH-cytochrome *c* reductase activity, on the contrary, increased as peroxidative damage to the membrane progressed. These results suggest the effect of lipoperoxidation to be remote from the flavoprotein stage of the microsomal electron transport system. The addition of carbon tetrachloride, in amounts equivalent to that found after dosage, *in vivo*, to microsomal preparations protected from lipid peroxidation did not alter the enzymic action toward aminopyrine demethylation. Carbon tetrachloride administration is known to induce microsomal lipid peroxidation as well as depress the microsomal drug-metabolizing system. The fact that aminopyrine demethylase has been shown to be very sensitive to lipoperoxidative decomposition of the microsomal membrane supports the contention that carbon tetrachloride-induced inhibition of drug metabolism is a direct result of lipoperoxidative damage to the endoplasmic reticulum.

IT IS APPARENT that phospholipids play a vital role in the functional integrity of the liver endoplasmic reticulum. Alteration of microsomal phospholipid by means of phospholipase treatment drastically curtails hepatic glucose 6-phosphatase activity,[1–3] in addition to depressing the drug-metabolizing system of the liver.[1,4] The endoplasmic reticulum can be altered *in vivo* as well. Notably, administration of carbon tetrachloride to rats depresses glucose 6-phosphatase[5–7] and various drug-metabolizing activities.[8–13]

Carbon tetrachloride induces peroxidation of microsomal lipid both *in vivo* and *in vitro*. In fact, this lipoperoxidative decomposition of the microsomal membrane is the center of the hypothesis concerning the toxic action of carbon tetrachloride.[14,15] Microsomal glucose 6-phosphatase, a lipid-requiring enzyme,[3] has been shown by Ghoshal and Recknagel[16] and Wills[17] to be very susceptible to lipoperoxidation. During the progression of lipoperoxidation, residual glucose 6-phosphatase activity steadily declined. It is believed that the depression of hepatic glucose 6-phosphatase seen after the administration of carbon tetrachloride is causally due to attendant peroxidation of the microsomal lipid.[5]

The hepatic microsomal mixed-function oxidase system is also profoundly decreased by both low doses[13] and high doses[8–12] of carbon tetrachloride. The drug-metabolizing system is dependent upon lipid for activity.[18] This communication shows that this system is likewise very sensitive to lipoperoxidation and the results provide

a rational basis for explaining the carbon tetrachloride-induced depression of microsomal drug metabolism.

METHODS

Male, Sprague–Dawley strain rats of 200–300 g were used. The animals were housed in wire bottom cages and allowed free access to standard laboratory chow (Wayne Lab-Blox) and water.

Microsomes were isolated from whole liver homogenates in saline, buffered at pH 6·5 with 0·05 M phosphate (Na–salt). The homogenate was centrifuged at 2700 *g* for 10 min to sediment the nuclear and mitochondrial fractions. The supernatant fraction was centrifuged at 100,000 *g* for 30 min and the resulting microsomal pellet was resuspended in the buffer by gentle homogenization. The suspension was diluted such that the microsome yield from each gram of liver was contained in 10 ml of buffer (100 mg Eq microsomes/ml).

Lipoperoxidation of the microsome preparation was effected by adding various amounts of ascorbic acid to microsomal suspensions of 25 mg Eq/ml (see the figures for ascorbate concentrations) followed by incubation at 38° for 30 min. Aliquots of the incubate were assayed for the extent of lipoperoxidation by measuring the amount of malonic dialdehyde produced.[16] Microsomes were recovered by recentrifugation of the incubates. Enzyme assays were determined on suspensions of these residual microsome particles.

Microsomal NADPH-cytochrome *c* reductase activity was measured at 25° by observing the increase in optical density at 550 nm produced by reduction of cytochrome *c*. Volume of the final reaction was 3·0 ml and it contained 0·1 m-mole phosphate buffer, pH 7·4, 1·0 μmole KCN, 0·08 m-mole nicotinamide, 0·3 μmole NADPH (omitted in the control reaction), 0·1 μmole oxidized cytochrome *c*, and microsomal material containing approximately 0·04 mg protein. Enzyme activity was linear with respect to microsome concentration, and reaction velocity was linear for several minutes. NADPH-cytochrome *c* reductase activity was calculated as the change in absorbancy at 550 nm/minute/milligram of microsomal protein.

Drug-metabolizing activity of the microsome preparations was determined by measuring aminopyrine demethylase. Flasks containing 40 μmoles aminopyrine and microsomes containing 6 mg protein were incubated at 38° for 15 min together with a NADPH-generating system of 1·5 μmoles NADP, 20 μmoles nicotinamide, 48 μmoles isocitrate, 40 μmoles $MgCl_2$, and 50 mg isocitric dehydrogenase (Sigma, type I). Total reaction volume was 8·0 ml. The reaction was stopped by adding 1·0 ml of 15% trichloroacetic acid to 2·0-ml aliquots of the mixture. After sedimentation of the precipitated protein by centrifugation, content of formaldehyde was determined according to the procedure of Nash.[19] Production of formaldehyde was proportional to the concentration of microsomes and it was linear during the 15-min incubation period. Enzyme activity was calculated as millimicromoles of formaldehyde produced in 15 min per milligram of microsomal protein, and corrected for formaldehyde present before start of the incubation.

Protein content of the microsomes was determined by the method of Lowry *et al.*[20]

RESULTS

Incubation of liver microsomes with ascorbic acid results in peroxidation of the constituent lipids as evidenced by production of malonic dialdehyde (Fig. 1). The

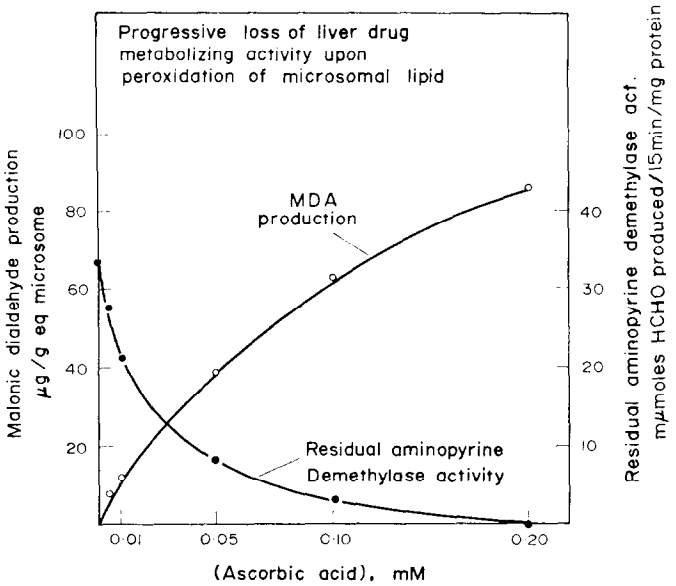

FIG. 1. Progressive loss of liver drug-metabolizing activity upon peroxidation of microsomal lipid. Microsomes were incubated with ascorbic acid for 30 min at 38° and the extent of lipid peroxidation measured by determining malonic dialdehyde production. Microsomes were recovered by centrifugation and the residual aminopyrine demethylase activity assayed.

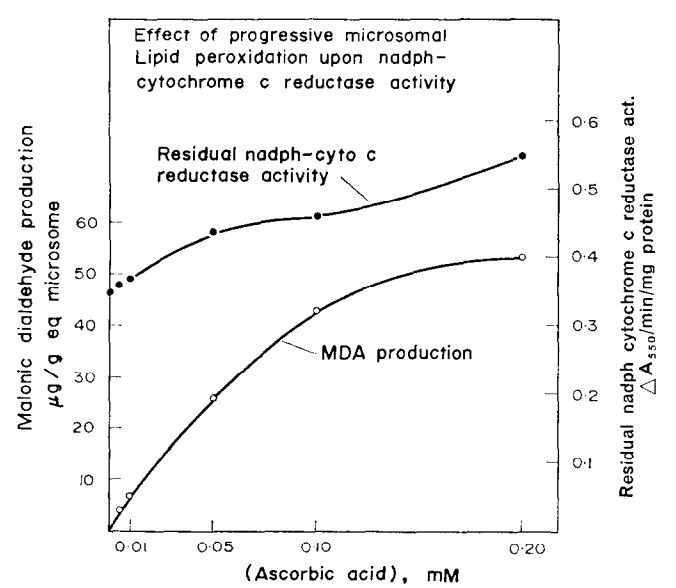

FIG. 2. Effect of progressive microsomal lipid peroxidation upon NADPH-cytochrome *c* reductase activity. Microsomes were incubated with ascorbic acid for 30 min at 38° and the extent of lipid peroxidation measured by determining malonic dialdehyde production. Microsomes were recovered by centrifugation and the residual NADPH-cytochrome *c* reductase assayed.

extent of the lipoperoxidation is proportional to the amount of ascorbate added. Microsomal drug-metabolizing activity is very sensitive to this alteration of lipid. Residual aminopyrine demethylase activity drops dramatically to zero coincident with enhanced lipid peroxidation. A relatively small amount of peroxidative damage, that produced by 10 μM ascorbate, causes a large decline in enzyme activity.

On the other hand, the activity of microsomal NADPH-cytochrome *c* reductase is not depressed after peroxidative decomposition of the membrane lipid (Fig. 2). The residual activity actually increases as malonic dialdehyde production progresses.

The toxic action of carbon tetrachloride is believed to be a consequence of its metabolism and resultant induction of peroxidative decomposition of microsomal lipid.[14,15] According to this view, if lipid peroxidation is prevented, the mere presence of carbon tetrachloride should be without effect. Such is the case for liver microsomal

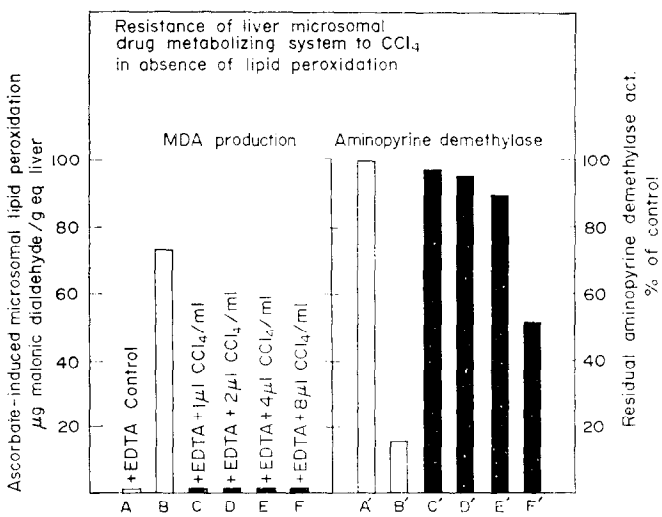

FIG. 3. Resistance of liver microsomal drug-metabolizing system to carbon tetrachloride in absence of lipid peroxidation. All of the microsomal suspensions were incubated with 0·125 mM ascorbic acid. In addition to ascorbic acid, flask A included 3·0 mM EDTA. In addition to ascorbic acid, flasks C, D, E and F included 3·0 mM EDTA and carbon tetrachloride added directly to the flask in the amount indicated. The final volume of 50 ml was incubated for 30 min at 38° and the extent of lipid peroxidation measured by determining malonic dialdehyde production. Microsomes were recovered by centrifugation and the residual aminopyrine demethylase activity assayed. The average demethylase control value (bar A') was 30 nmoles formaldehyde produced in 15 min/mg microsomal protein.

glucose 6-phosphatase.[21] The following results extend this observation to the microsomal drug-metabolizing system. If a microsome preparation is allowed to peroxidize, aminopyrine demethylase activity is markedly reduced (Fig. 1, and compare bars A and A', B and B' of Fig. 3). In the presence of EDTA, lipoperoxidation is completely inhibited (bars A, C, D, E, F). Under these conditions incubation of microsomes in the presence of up to 4 μl carbon tetrachloride/ml incubation medium was without effect on residual aminopyrine demethylase activity (bars C', D', E'). Only when the amount of carbon tetrachloride was increased to 8 μl/ml incubation medium did the residual enzyme activity significantly drop (bar F').

DISCUSSION

There is no doubt that phospholipid forms an integral part of the normal function of many microsomal enzyme activities, namely, glucose 6-phosphatase,[3] ATPase,[22] UDP-glucuronyltransferase,[23] acyl CoA: L-glycerol-3-phosphate acyltransferase,[24] and the drug-metabolizing system.[18] Treatment of microsomal preparations with lipid-altering enzymes such as the phospholipases or extraction of lipids with solvents cause significant reduction and in many instances complete inhibition of catalytic activity. The results in this communication show, in addition, that peroxidation of microsomal lipid is detrimental to the catalytic activity toward demethylation of aminopyrine (Figs. 1 and 3). The effects of ascorbic acid appear to be directly related to lipoperoxidative damage of the microsome rather than a coincidental effect of ascorbic acid not involving lipid peroxidation. Ascorbic acid added to microsomes not allowed to peroxidize (Fig. 3, bar A) did not alter the activity of the residual demethylase (Fig. 3, bar A′) when compared to the activity of microsomes incubated without ascorbic acid (Fig. 1). These results confirm and extend the observations of Wills[17,25] who showed that microsomal lipid peroxidation induced by ascorbic acid or irradiation caused decreased aniline hydroxylation and aminopyrine demethylation.

The experiment shown in Fig. 2 allows a more precise expression as to what part of the microsomal drug-metabolizing system is affected by lipid peroxidation. Hepatic demethylation of aminopyrine is catalyzed by the electron transport system associated with the microsomal membrane.[26] This system involves a flavoprotein and cytochrome P-450 and requires NADPH and oxygen for activity. It is evident that the flavoprotein part of the chain is stable under conditions in which considerable lipoperoxidative damage to the membrane has occurred. The flavoprotein activity, measured as NADPH-cytochrome *c* reductase, was not inhibited by lipid peroxidation. Therefore, the effect of membrane destruction must be remote from the flavoprotein stage of electron transport. There are at least two areas which can be considered as the center of the peroxidative damage. The first is cytochrome P-450 which is structurally closely associated with lipid[27] and second, the heat stable, lipid fraction isolated by Strobel *et al.*[18] which is necessary for drug-metabolizing activity in a reconstituted microsomal electron transport system. Which of these areas are subject to peroxidative decomposition must await further experimentation.

The central working hypothesis concerning the action of carbon tetrachloride is that through metabolism the haloalkane induces destructive peroxidative decomposition of microsomal membrane lipids.[14,15] Lipoperoxidation is the key obligatory consequence in carbon tetrachloride liver injury. It follows that in the presence of carbon tetrachloride, if lipid peroxidation is prevented, then no destructive effect of the haloalkane should be realized. The results presented in Fig. 3 bear this out. EDTA can effectively block lipid peroxidation in microsome preparations. When carbon tetrachloride is added to these incubates no effect upon aminopyrine demethylase is seen. It should be pointed out that the smallest carbon tetrachloride addition to the incubates, 1 μl/ml incubate, is about the same concentration that is seen in rat liver 1·5 hr after a rather large intragastric dose to rats.[28] A decrease in aminopyrine demethylase activity was seen only when the carbon tetrachloride addition to non-peroxidizing microsomes was equivalent to a concentration eight times that found in rat liver after dosage.

The studies *in vitro* presented here indicate that depressed drug metabolism is due

to peroxidative decomposition of lipids of the endoplasmic reticulum. This conclusion does not coincide with opinions of Dingell and Heimberg,[8] Sasame *et al.*[11] and Castro *et al.*,[29] who concluded that reduction in drug metabolism as a consequence of carbon tetrachloride administration was not mediated by lipid peroxidation. The conclusion of the former[8] was based on an observation that antioxidant treatment of rats did not protect the drug-metabolizing system against carbon tetrachloride, *in vivo*. Recent work in this laboratory[30] provides a new basis for questioning the validity of the conclusion drawn by Dingell and Heimberg.[8] It has been observed that less than lethal doses of carbon tetrachloride produce maximal to near maximal changes over the first 12–24 hr with respect to three indices of liver damage, viz. the liver weight to body weight ratio, liver triglyceride content, and serum glutamic–oxaloacetic acid transaminase (SGOT) levels. In rats destined to die, liver fat and SGOT increase until death. In rats given non-lethal doses of carbon tetrachloride, the pathological changes are reversible. The critical point emerging from these observations is the realization that prior administration of an antioxidant such as vitamin E will induce an ordinary lethal dose of carbon tetrachloride to act like a large, but non-lethal dose. The rat will not die (see Hove,[31] Gallagher,[32] Cawthorne *et al.*[33]), but certain indices of liver damage observed during the first 24 hr or so will not be distinguishable from the same indices in the rat given carbon tetrachloride without antioxidant protection. Data in Table 9 of Dingell and Heimberg[8] bear on this point. They found that neither vitamin E nor *N,N'*-diphenyl-*p*-phenylenediamine (DPPD) treatment prevented a carbon tetrachloride-induced rise in the liver weight to body weight ratio. As indicated, the failure to observe an effect of antioxidant administration on this index cannot be used to conclude that there has been no antioxidant effect. To prove that carbon tetrachloride can depress liver drug metabolism without lipid peroxidation (as claimed by Dingell and Heimberg), it would have to be shown by some method which can actually detect lipid peroxidation that the former has occurred without the latter. Dingell and Heimberg simply assumed, without proof, that antioxidant pre-treatment prevented carbon tetrachloride-induced lipid peroxidation.

The conclusion drawn by Sasame *et al.*,[11] which in our opinion is erroneous, was based on comparison of effects of carbon tetrachloride and ethanol on ethylmorphine metabolism. Sasame *et al.*[11] summarize: "Carbon tetrachloride impairs the oxidative enzymes in liver microsomes by decreasing the amount of P-450. The destruction is probably not mediated by an increase in lipid peroxidation, since ethanol which promotes lipid peroxidation does not decrease cytochrome P-450 or the metabolism of ethylmorphine." The difficulty here is in the first premise, viz. that ethanol promotes (microsomal) lipid peroxidation. Hashimoto and Recknagel[34] found no evidence of liver microsomal lipid peroxidation in acute alcoholic hepatitis. Similarly DiLuzio,[35] the authority appealed to by Sasame *et al.*[11] for their first premise, found that ". . . the microsomal lipid fraction of acute ethanol-treated animals did not exhibit an increased 233 nm absorption". If the first premise in the argument of Sasame *et al.*[11] is invalid, the rest of their argument is without substance.

Castro *et al.*[29] have concluded that lipid peroxidation is not causally related to the diminished drug metabolism seen after carbon tetrachloride administration. In their experiments, prior treatment with antioxidants while suppressing necrosis had no protective effect against the decrease in ethylmorphine demethylase activity and cytochrome P-450 concentration. As previously mentioned, antioxidant treatment

serves to cause a subsequent lethal dose of carbon tetrachloride to behave as a large, but non-lethal dose.[30] Antioxidant protection against carbon tetrachloride-induced necrosis is not complete as Castro *et al.*[29] have shown. It is difficult to directly compare protection by antioxidants against necrosis to non-protection against drug metabolism and cytochrome P-450 levels, since small homeopathic doses of carbon tetrachloride will produce a severe fall in cytochrome P-450 concentration.[13] In other words, the sensitivity of cytochrome P-450 and associated drug metabolism to carbon tetrachloride administration is much greater relative to the necrosis-producing property of the toxin. The data of Castro *et al.*[29] cannot sustain their conclusion that ". . . impairment of P-450 and the ethylmorphine demethylase caused by CCl_4 is not mediated through lipid peroxidation".

In conclusion, we believe that lipoperoxidative damage to hepatic microsomes results in depressed drug metabolism. Furthermore, the arguments presented above compel us to conclude that diminished drug metabolism induced by carbon tetrachloride administration is mechanistically linked directly to attendant peroxidation of the lipids of the endoplasmic reticulum.

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